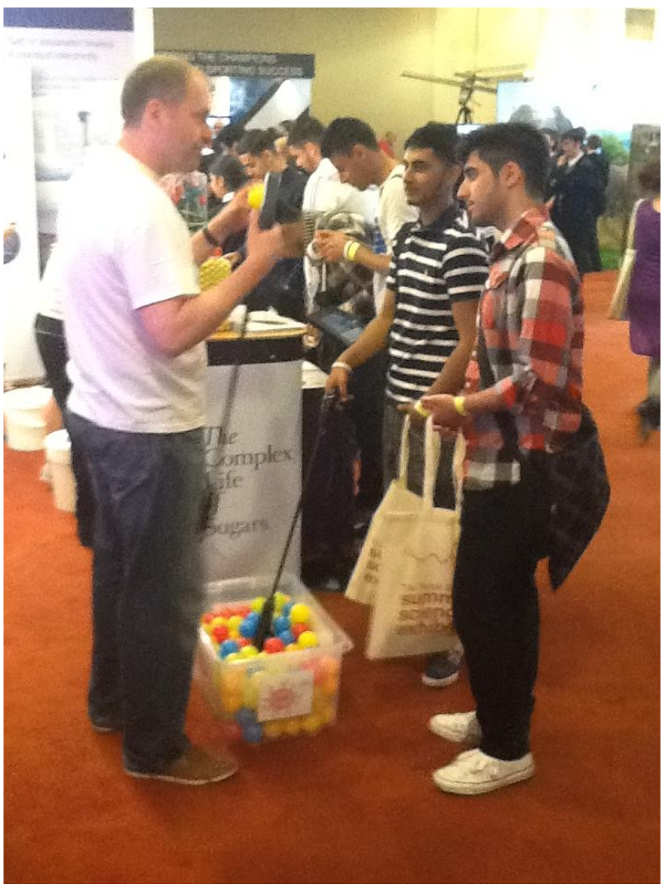
<u>**Supporting Documentation**</u>

The Royal Society Summer Science Exhibition 1-7th July 2013, London (UK)

Glycan array model making at The Royal Society Summer Science Exhibition



Race for fertilisation game at The Royal Society Summer Science Exhibition

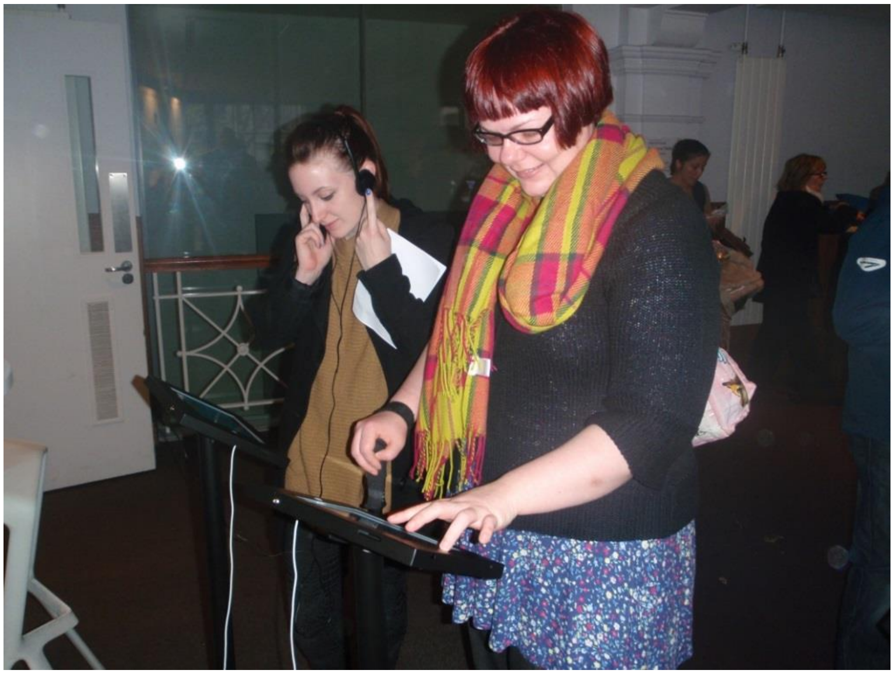
Cell Invaders game on the ipads at The Royal Society Summer Science Exhibition

Science Spectucular, 2nd November 2013 at The Manchester Museum (UK)

Glycan array model making at The Science Spectacular

Glycan array model making at The Science Spectacular

Race for fertilisation game at The Science Spectacular



The Complex Life of Sugars animation and Cell Invaders game at The Science Spectacular

Glucose testing at The Science Spectacular

BBSRC: The Great British Biosciences Festival, 14-16th November 2014, London (UK)

Stakeholder event

Public event

Sweet Complexity art exhibition, 16th July 2015, Manchester Institute of Biotechnology (UK)

Painting by artist Karen Barber 2015 – gold glycan array for diagnostic devices.

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Project full name: Tools for the Detection of Novel Glyco-biomarkers

Project acronym: GLYCOBIOM

Project website: N/A

Project reference number: 259869

Name/country of project coordinator: Sabine FLITCH, UK

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Project start/end: From 2011-01-01 to 2015-12-31

Partners from other countries:

University of Manchester (UK), Københavns Universitet (DK), Universitaetsklinikum Hamburg-Eppendorf (DE), National Institute for Bioprocessing Research and Training Ltd (IE), Genos Doo Za Vjestacenje I Analizu (HR), Galab Laboratories GMBH (DE)

Executive summary

Keeping up with the cell changes associated with cancer is no easy task. A key cancer-related cell process known as glycosylation could advance understanding significantly, leading to better diagnosis and smarter drugs. A cure for cancer has become the Holy Grail for many medic researchers, and one crucial step in this direction is understanding this elusive and complex disease through the changes that occur in cells and cell structure. Since all cell surfaces and more than half of the proteins in our bodies are linked to sugar chains, researchers have identified the need to study closely the glycosylation of biomolecules in cells – a process seen in many cancers. The EU-funded GlycoBioM project ('Tools for the Detection of Novel Glyco-biomarkers') has brought together Europe's leading scientists to study glycosylation. Hailing from Croatia, Denmark, Germany, Ireland and the UK, the team is identifying new biomarkers and tools for detection and diagnostic screening. These could be used to develop personalised treatment for cancer and related diseases.

In cancer cells, recognition between cells is disturbed, leading to invasive growth and dissemination of tumour cells. This phenomenon is reflected in the glycans of the cell coat, which is of particular interest to researchers. Recombinant glycan receptors are used to identify tumour associated changes of the glycans in the cell coat of tumour cells. The recombinant receptors are used in different ways, such as for identifying tumour associated changes to carcinoma cells in tissue sections, and recognising sub-populations of leukaemia cells. **UKE** is also using the receptors to identify soluble glycobiomarkers in tumour patients' blood samples. In parallel, project members from the **UNIMAN** have been working on a label-free analytical tool to capture and characterise glycan binding proteins event from complex mixtures. This high-throughput technology could eventually be used to pinpoint sugar biomarkers in diseases such as cancer.

The GlycoBioM project is truly a European success story, with partners from opposite ends of Europe all contributing to ground-breaking results. When **Genos** found that certain glycans can predict the speed at which colon cancer will progress (which could lead to tailored therapy – or 'smart drugs' – for individual patients), **UCPH** took up the baton, developing a new glycoprofiling method to reduce false-positive cancer diagnoses. This test kit will enable unambiguous and fast detection of ovarian/tubual cancers and is currently undergoing validation and commercialization clinical studies at SME **Galab**. The team has also made commendable progress in unravelling the complexities of breast cancer. Our results have revealed that the tumour associated glycan changes may be an independent diagnostic parameter in malignant disease such as breast cancer. We hope that our results lead to better stratification of patients regarding the choice of the most appropriate therapy. Equally noteworthy has been the project team's progress in understanding diabetes, in particular the discovery of a novel glycan biomarker related to the disease. The team expects to develop a system that will enable patients to check for maturity-onset diabetes of the young (MODY), a form of diabetes that is caused by mutations in a number of different genes.

These achievements are complemented by the award nominated online platform GlycoBase (https://glycobase.nibrt.ie) which provides tools for biomarker discovery, and has been commercialisaed by Waters Corp into the UNIFI software package for LC-MS analysis.

The project outputs will be useful for monitoring effects and safety of cancer therapies, bringing medical science a step closer to conquering some of the most complex and troublesome disease families of our time www.glycobiom.eu.

Project Context and Objectives

Keeping up with the cell changes associated with cancer is no easy task. A key cancer-related cell process known as glycosylation could advance understanding significantly, leading to better diagnosis and smarter drugs. A cure for cancer has become the Holy Grail for many medic researchers, and one crucial step in this direction is understanding this elusive and complex disease through the changes that occur in cells and cell structure. Since all cell surfaces and more than half of the proteins in our bodies are linked to sugar chains, researchers have identified the need to study closely the glycosylation of biomolecules in cells – a process seen in many cancers. The EU-funded GlycoBioM project ('Tools for the Detection of Novel Glyco-biomarkers') has brought together Europe's leading scientists to study glycosylation. Hailing from Croatia, Denmark, Germany, Ireland and the UK, the team is identifying new biomarkers and tools for detection and diagnostic screening. These could be used to develop personalised treatment for cancer and related diseases.

Glycosylation patterns on glycoproteins and glycolipids are involved in the regulation of inter- and intracellular recognition events and alteration of such glycosylation has been observed in many diseases, notably in a variety of cancers. The increasing number of reports in the scientific literature on specific alterations in glycosylation with diseases suggests that a systematic, large scale investigation in this area will provide a rich spectrum of novel and structurally diverse carbohydrate-based biomarkers.

It is becoming clear that no single biomarker is sensitive or specific enough to meet the current clinical needs for diagnosis, especially for autoimmune diseases and cancer. The availability of sensitive quantitative tools for glycan analysis is opening up the possibility of discovering useful leads by analysing total glycomes or disease associated glycans on individual glycoproteins, either isolated or excised from 2D gels. Disease markers are required for many reasons, for example to achieve an initial diagnosis, stage a disease process and determine the response to medication. Glycan processing of glycoproteins and glycolipids have been noted in many if not most diseases and combinations of glycan changes are now being tested alongside panels of protein and genomic changes. Many diseases are multi-systemic and the most effective markers will be discovered through an in-depth understanding of the disease processes. Moreover, in order to control medication and understand disease progression it will also become critical to understand how these processes relate to each other within the patient by adopting a systems biology approach.

The bottleneck for such investigation has been a lack of robust high-throughput screening techniques for carbohydrate analysis, carbohydrate-binding proteins, and glycoenzymes and a lack of diagnostic tools for carbohydrate-based biomarkers that can be used in the clinic. Recently, a number of new analytical techniques for carbohydrates have been developed by us and others and brought to proof-of-concept stage. The aim of this project will be to advance these technologies to the next stage by developing an integrated multidisciplinary approach to glycobiomarker screening and analysis based on current state-of-the-art. We will bring together a number of complementary screening techniques developed in the individual partner laboratories, notably high-throughput quantitative HPLC analysis of carbohydrates, lectins/human glycoreceptors for carbohydrate recognition and carbohydrate arrays, to develop an overall workflow for the multiplex analysis of biomarkers. Such carbohydrate-targeted methods will involve RNA/DNA and protein analysis combining high-throughput glycomics with genomics **(multimodality biomarker analysis)**. It will be important to test the workflow against serum and tissue samples of healthy volunteer/cancer patients. In addition, we envisage that the project will generate new analytical tools that can be used in routine analysis of glycobiomarkers in the clinic, including diagnostic imaging **(high throughput molecular diagnostic imaging)**. Some of the tools developed in this programme (lectins conjugated to nanoparticles, quantitative HPLC) should also be useful for finding **new quantitative imaging biomarkers for monitoring therapeutic effects and safety in cancer.** The involvement of three SME partners will ensure that the technology will be developed and validated in a format that will be suitable for commercial applications.

Progress Beyond State-of-the-Art

We propose that the complexity of glycosylation requires a holistic analytical approach in studying such glyco-biomarkers: on the one hand it requires a number of analytical techniques that allow us to determine carbohydrate structures, carbohydrate-modifying enzymes (whose specificity determines biosynthesis) and carbohydrate-binding proteins. On the other hand, carbohydrates are intrinsically linked to genome and proteome and should not be studied in isolation. Hence, it will be important to develop workflows that integrate carbohydrate analysis in multimodal schemes with DNA/RNA and protein analysis and ultimately bring glycoanalysis into the mainstream of biomarker screening and development. We believe that such an ambitious approach is timely, because a number of the key glycoanalysis technologies needed have now been developed in ours and other laboratories to proof-of-concept stage, in particular lectins/human glycoreceptors, high-throughput quantitative HPLC analysis of glycans and carbohydrate arrays for studying carbohydrate-binding proteins and glycoenzymes.

Overall strategy and general description

We have brought together nine technical workpackages (numbers 2-10; workpackage 1 concerns overall management of the project and will be discussed later; workpackage 11 concerns ethical issues and will be discussed in the ethics section) that aim to develop a workflow that can address these challenges. **Workpackage 2** is concerned with overseeing the design, planning, monitoring and development of the overall workflow of biomarker discovery to ensure that the different techniques are adapted to an integrated scheme. An important aspect will be bioinformatics. We believe that good management of clinical samples will be essential for the workflow design in order to compare and evaluate the individual analytical techniques. **Workpackage 3** will ensure that all researchers use comparable clinical samples such that any analytical results can be evaluated by the whole research network and meaningful results are generated. **Workpackes 4-9** are concerned with the individual analytical techniques that will need to be integrated into the workflow. A lot of the techniques are already set up in the partner laboratories and the main activities will be to adapt them to requirements identified in workpackage 2 and address any bottlenecks and gaps of technology. These are **Carbohydrate Arrays (workpackage 4)** for the identification and characterization of carbohydrate-binding proteins, lectins and antibodies which will either be used as tools in other work-packages or for biomarkers discovery or in the development of diagnostic tools (chip-type devices). **Glycoreceptors (workpackage 5)** and **Autoantibodies (workpackage 8)** are complementary to glycarrays in that they will identify carbohydrates as biomarkers and can ultimately be used for imaging tools and diagnostic high-throughput devices. The need for **quantitative analysis** of carbohydrate biomarkers will be addressed in **workpackage 6** by developing nano-HPLC methods. **Workpackage 7** will address the need to develop **multimodal** analysis methods and integrate glycoanalysis with genomics and proteomics. **Workpackage 9** aims to develop some of the analytical techniques, in particular glycoreceptors, autoantibodies and glyconanoparticles for ultimate application in diagnostics **Imaging tools**. Finally workpackage 10 looks at future industrial application of glycobiomarkers by validation and standardization of some of the tools developed in this programme and aims to develop prototypes by the end of the programme.

Main S&T Results/Foregrounds

WP2: Workflow Design Management. WP2 is concerned with overseeing the design, planning, monitoring and development of the overall workflow for biomarker discovery to ensure that the different techniques are adopted into an integrated scheme. **NIBRT** developed GlycoBase which is a web-based application allowing automatic assignment of peaks to glycans within existing collections and a profile browsing feature. The GlycoBioM.DB module handles data from the consortium as a whole including software tools 'GlycoProfileAssigner', 'GlycoDigest' to aid glycan structure assignment to LC peaks and GlycoMarker to help detect biomarkers from raw quantitative profiles. During the project GlycoBase was updated with UPLC/HPLC & CE data from additional intact sugars and new statistical analysis on ~2,500 colon cancer samples (CRC samples). GlycoBase has been commercialised into Waters UNIFI software.

NIBRT developed software tools GlycoBase, GlycoProfileAssigner and GlycoMarker and tested their integration into a working analytic pipeline. The main software is available at:

- GlycoBase: https://glycobase.nibrt.ie
- GlycoProfileAssigner: https://glycobase.nibrt.ie
- GlycoMarker: https://glycobase.nibrt.ie/glycomarker
- GlycoLinker: https://glycobase.nibrt.ie/glycolinker

They are also available on the GlycoBioM.DB portal.

Figure 1 shows **NIBRTs** updated, automated method for glycoprofiling which incorporates a fully integrated platform combining glycoprotein affinity purification, protein denaturation, enzymatic glycan release, fluorescent glycan labelling and sample clean-up (StoÌˆckmann, et al.; Stöckmann H.). The robotic protocol is software-controlled and is equipped with pipette tip racks, plate carriers, reagent reservoirs, a software-controlled vacuum manifold, a plate transport tool, a vacuum manifold, eight robotic pipettes with individual liquid level and pressure sensors and a temperature controlled orbital shaker. The fluorescently labelled glycans are run on HPLC/UPLC instruments equipped with hydrophilic interaction chromatography (HILIC) columns and the resulting peaks are correlated to a pre-run dextran ladder, thereby assigning a Glucose Unit (GU) value to each of the peaks. The use of standard glucose units makes these values independent of the running conditions; which allows for the direct comparison of chromatographic profile peaks and their relative glycan abundance. The platform was combined with various software tools and tested. It was concluded that *combining this automated technology with bioinformatics allows high-throughput processing several orders of magnitude higher than any protocol without bioinformatics.* In total our platform uses four such computational tools (GlycoBase, GlycoProfileAssigner, GlycoMarker and GlycoDigest in Figure 1) capable of annotating and analysing the raw glycan data in a semi-automatic manner. The glycan sample preparation platform can be easily adapted and allows glycan labelling with a variety of labels so that it can be linked to complementary analytical technologies such as mass spectrometry and capillary electrophoresis.

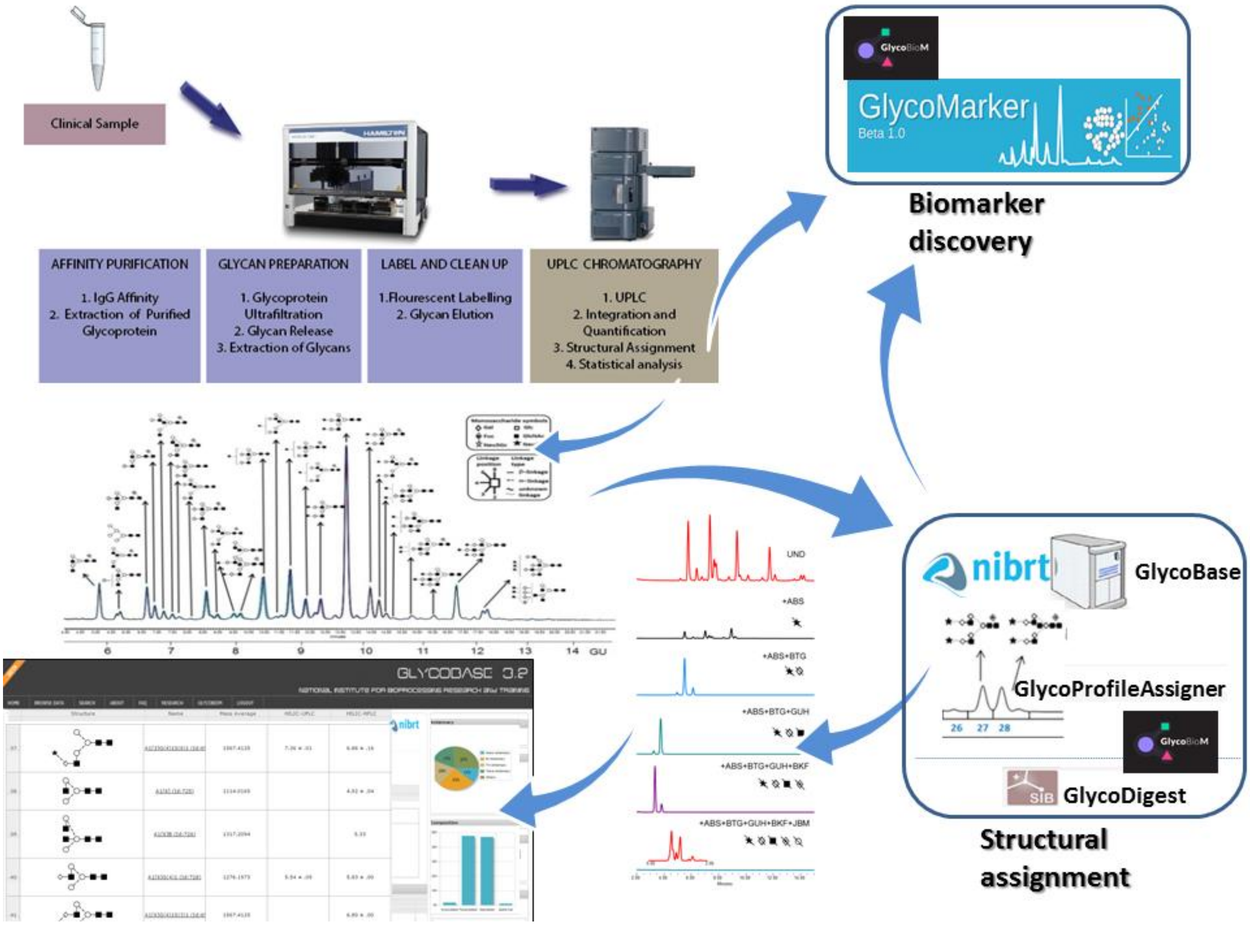


Figure 1. Glycomics workflow. Sera/plasma, individual glycoproteins, mixtures or cell culture supernatant samples are processed on a robotic workstation, resulting in fluorescently labelled glycans, which are subsequently separated into pools and quantified by ultra-high pressure liquid chromatography (UPLC). Bottom right structures are predicted using GlycoBase 3.2 and confirmed by enzyme digestion using bioinformatics software (Duffy and Rudd). Top right the high quality data produced by the platform can be analysed using new GlycoMarker bioinformatics software to find glycan biomarkers.

The platform has been successfully utilised in glycoprofiling studies in diseases such as cancer, galactosemia, rheumatoid arthritis and diabetes, (Adamczyk, et al.; Albrecht, et al.; Coss, et al.; Thanabalasingham, et al.) and current studies are ongoing in these areas for biomarker discovery. For a description of GlycoDigest see (Gotz, et al.). In the following the bioinformatics components in Figure 1, GlycoBase, GlycoProfileAssigner, GlycoMarker and GlycoLinker are described.

WP3: Sample Management

The use of a common set of clinical samples with detailed histopathological diagnoses and clinical information was proposed at the start of the multimodal workflow design. Initial evaluation of commercially available tissue and serum samples proved unsuccessful. A set of phenotyped and genotyped human tissue/serum samples from 2,000 colorectal cancer patients with the corresponding matching controls is used as the internal GlycoBioM sample set. These samples are of much greater importance for the analysis of the genetic component of glycosylation variability and health and cancer than commercially available samples. By analysing Immunoglobulin G (IgG) glycosylation in a large SOCSS cohort of patients with colorectal carcinoma (CRC), **Genos** have revealed significant associations between CRC and IgG N-glycome composition; in addition, alteration in the IgG glycome composition strongly associated with poorer all-cause and CRC mortality. During period 4, through consortium contacts we have obtained an additional cohort of CRC patients which enabled us to evaluate potential effect of surgery on the IgG N-glycome in CRC patients. To increase statistical power of our studies and possibly identify more biomarkers, we have obtained access to another cohort of more than 100 patients undergoing cardiac surgery with longitudinally collected samples; this has enabled us to perform the first large study of IgG and total plasma proteins glycosylation changes upon initiation of a major inflammation. The results are inputted into the GlycoBioM database module.

WP4: Carbohydrate Arrays

WP4 is centred on the development of carbohydrate arrays as multimodal tools for biomarker discovery and applications in diagnostics. **UKE** studied the ligand specificity of glycoreceptors/lectins in order to develop tools for the detection of new biomarkers. The carbohydrate specificity of glycoreceptors was either determined by PAA-based glycan ELISA or by glycan microarray (**UKE**) in cooperation with **UCPH**. Novel specificities of CD301 to the tumor associated structures Neu5Ac-Tn and Neu5Gc-Tn antigen were defined by PAA-based glycan ELISA. Detailed analyses of glycan-glycoreceptor interactions were carried out by surface plasmon resonance (SPR) and saturation transfer difference (STD) NMR. Using the glycan microarray we identified binding of Langerin to novel O-specific polysaccharides of several bacterial species (*Salmonella enterica, Cronobacter sakazaki, E. coli, Proteus mirabilis, Shigella boydii, Shigella flexneri*). Furthermore, substrate specificities of glycoreceptors and plant lectins were compared. Kinetic studies were performed with fucose-binding plant lectins: Lotus tetragonolobus lectin (LTL), Ulex europaeus agglutinin (UEA) and Aleuria aurantia lectin (AAL) by PAA-based glycan ELISA. In contrast to AAL and UEA, LTL detects most DC-SIGN-ligands. In additional PAA-based glycan ELISAs, we compared the carbohydrate specificity of the human glycoreceptor CD301 with the plant lectins: Vicia villosa lectin (VVL), helix pomatia agglutinin (HPA) and Jacalin. The binding domains of these proteins belong to the galactose type CRDs. Our results implicate that VVL exhibits the most similar glycan specificity to CD301. In contrast to VVL, CD301 recognizes additionally sialylated GalNAc in α2-6 linkage. Thus, it is possible to distinguish between the tumour associated Tn- and sialyl-Tn antigen for the screening of human samples by using CD301 and VVL in parallel.

A general protocol for selective capture and analysis (by MALDI-ToF MS) of pure and crude mixtures of plant lectins on a glycan array on gold-coated MALDI target plates was developed by **UNIMAN**. This protocol was further developed into a method to covalently immobilise glycans and lectins to a self-assembled monolayer on gold. This method enables label-free enrichment of glycan binding proteins (GBPs) and glycoproteins respectively, even from complex mixtures (human milk protein extract or plant extracts), which are then rapidly characterised by MALDI-ToF-MSMS on-chip. **UNIMAN** can also screen the inhibitory potential of different bacteria towards *E.coli* attachment on glycosylated surfaces as well as immobilising glycosaminoglycan (GAG) heparin to an amine terminated SAM that can also be screened against GBPs employing MALDI-ToF-MSMS. **UNIMAN** demonstrated that multivalent glycan interactions can increase protein-binding avidities in a number of commercially available samples (demonstrating new and unreported specificities), biofluid, human milk and human serum. An assay has been successfully demonstrated to capture glycan binding protein appoliprotein A-1 (APOA1) from pooled human serum and identified after tryptic digestion and database analysis. **UNIMAN** have successfully used glycoenzymes to incorporate complex glycan structure onto the glycan array and shown that Mucus binding protein from Lactobacillus reuteri (probiotic microorganism) can be characterised on the high-throughput gold glycan array. Building upon the protocols developed **UNIMAN** capitalised on this progress with a number of key results. The gold array platform is suitable for *in vitro* elucidation of the role of sugars as receptor in bacterial infections. The methodology provides a rapid screening mechanism for bacteria with unknown glycan affinity, providing insight into the complex interplay between bacteria within the human gut. *L. adenocarboxylata* and its structural polymer show excellent activity in inhibiting binding of bacteria and lectins in general, a feature much needed in probiotics. The applicability of heat killed/inactivated intact *L. adecarboxylata* and its biopolymer in advanced probiotics will be studied further. The gold array platform has been used for multimodal analysis by preparation of glycosaminoglycan (GAG) heparin to an amine terminated SAM on the same gold supported platform. The GAGs were screened against glycan binding proteins (GBPs) with a case study on stem cell growth factor (SCGF) to determine novel binding modes. The gold arrays were also used to validate the CA125 assay with **Galab**.

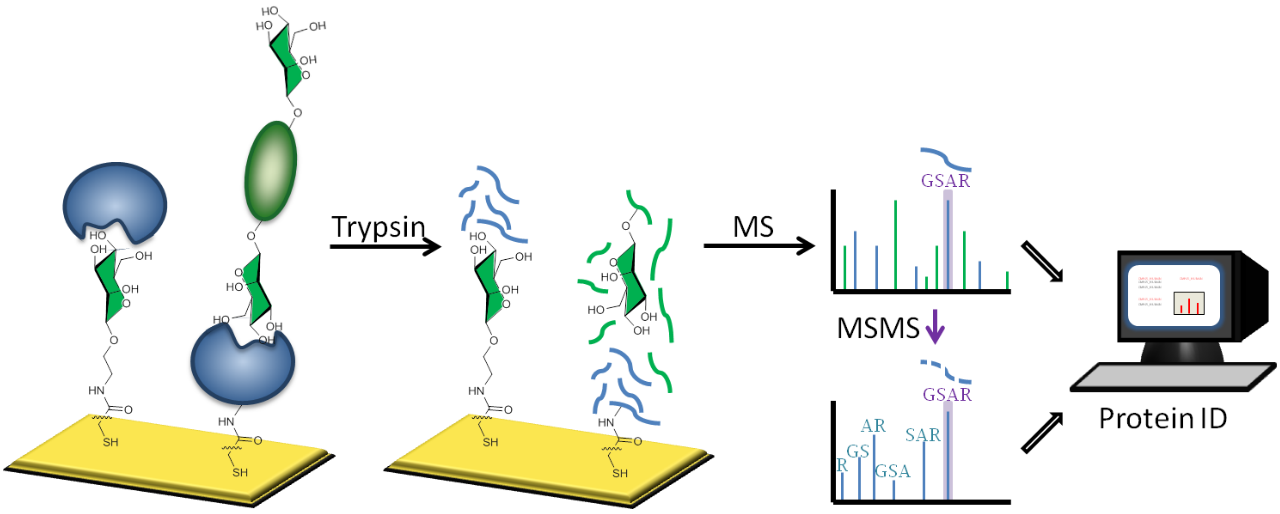


Figure 2. Discovery tool-kit for carbohydrate-binding proteins: Self-assembled monolayers (SAMs) on a gold array are functionalized with glycans (left spot) or glycan-binding proteins (right spot). When complex protein mixtures from biological samples are incubated on the array, glycan-binding proteins (left) or glycoproteins (right), respectively can be enriched, detected and identified on the array by MALDI ToF MS(/MS) with or without on-target trypsin digestion.

UCPH fabricated glass arrays to measure IgM anti-carbohydrate antibodies derived from peripheral blood of healthy donors. The results showed that a small percentage of healthy donors identified in the Western population carry antibodies in their peripheral blood which convey cytotoxic activity against certain human melanoma and neuroblastoma cell lines. Furthermore, glass arrays were used to explore binding specificities for Bacterial and Eukaryotic Chitinases. All chitinases surveyed showed increased activity towards LacdiNAc over LacNAc conjugates. These results also suggest that bacterial chitinases target either extracellular GlcNAc-containing glycans or GlcNAc-containing substrates located on the surface of host cells or intracellular vesicles.

WP5: Glycoreceptors

The major goal of work package 5 is to express, characterize and apply human glycoreceptors for the detection of glycan structures and novel biomarkers in clinical samples. **UKE** successfully expressed 8 recombinant glycoreceptors as recombinant proteins purified from eukaryotic expression systems which increased by an additional 11 glycoreceptors during the project lifetime. For 6 of 19 glycoreceptors, the binding specificity was experimentally determined by PAA-based glycan ELISA or by glycan microarray in cooperation with **UCPH**. Using glycan microarray binding, Langerin was identified to novel O-specific polysaccharides of several bacterial species (*Salmonella enterica, Cronobacter sakazaki, E. coli, Proteus mirabilis, Shigella boydii, Shigella flexneri*). The binding of LSECtin to plasma samples of colon cancer patients and patients infected by entero-aggressive strains of Escherichia coli (EAEC) and observed differential binding patterns between patients. In addition, **UKE** tested the functionality of LSECtin after affinity purification from tissue culture supernatants. Examplified for LSECtin, **UKE** successfully purified glycoreceptors without loss of binding to N-linked glycostructures allowing us to provide our cooperation partners (**UNIMAN**, **NIBRT**, **UCPH**, **Galab** and **Genos**) with purified, well characterized and functionally active glycoreceptor probes. Additionally, experimental protocols were established towards the purification of glycoproteins by pull down experiments utilizing CD301 as a bait protein, followed by identification of potential binding partners by mass spectrometry. Several cancer-associated mucins were specifically precipitated with recombinant CD301. The current data demonstrate that our glycoreceptor preparations are well suited to identify glycoproteins in tumour tissue extracts of primary origin.

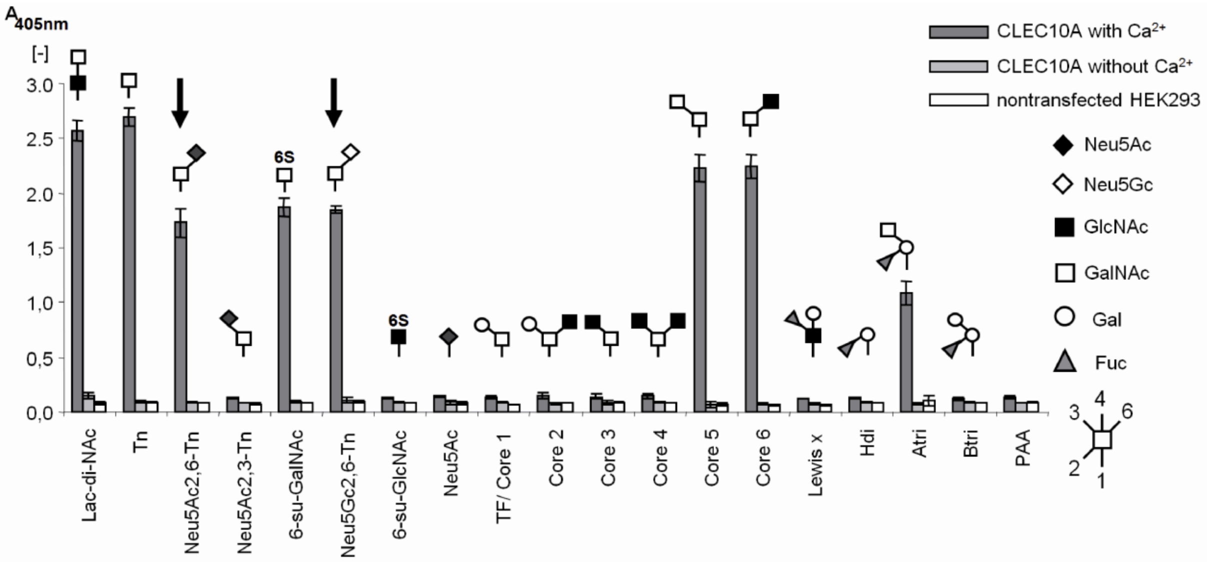
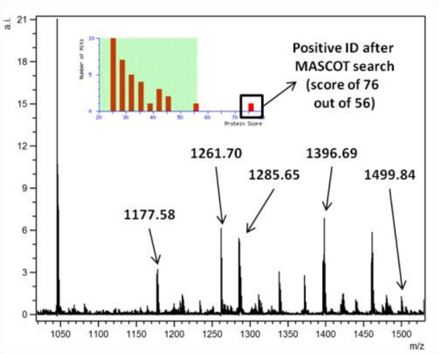


Figure 3: Glycan profiling of recombinant CD301. Glycan specificity of recombinant CD301 was measured by a Glycan-ELISA at 405 nm. Microtiter plates were coated with poly[N-(2-hydroxyethyl)acrylamide] (PAA)-based glycoconjugates. PAA served as background control. Data represent the averages of five replicates; error bars indicate standard deviations. Carbohydrate specificity of cell culture supernatant containing recombinant CD301 was analysed in the presence (dark grey column) and in the absence (grey) of Ca^{2+}. Cell culture supernatant from non-transfected HEKT293 cells served as negative control (white column). New specificities of CD301 are marked with arrows.

UKE established binding conditions for the application of labelled glycoreceptors for the detection of cellular glycan structures by flow cytometry. This has been applied to three different glycoreceptors for FACS analysis of peripheral blood cells of leukaemia patients. The percentage of positively stained cells was variable and is obviously dependent on the type of leukaemia and the glycoreceptor applied. In addition, identification of differentially glycosylated subpopulations of leukaemia cells within patient samples was possible. For the generation of CLEC10A coated nanoparticles. **UKE** successfully expressed SNAP-tagged CLEC10A fusion proteins in human cell lines and could purify the recombinant proteins for biochemical characterization.

Cell culture supernatants from HEK293 cells expressing DC-SIGN, DC-SIGNR and LSECtin, respectively, were sent to **NIBRT** for the development of a selective enrichment LC-MS platform for cancer associated glycosylation monitoring. Purified glycoreceptors CLEC11A, CLEC18A, CLEC4D and Reg3a were sent to **UNIMAN** from **Galab** for binding studies using GAG-based platform for MALDI analysis. **UNIMAN** employed a GAG-coated MALDI plate to study the metal dependency and affinity of CLEC11A towards different GAGs. A novel manganese-mediated interaction with SCGF with heparin was discovered and proved to be selective only to heparin. The results have been further verified by HPLC, competitive inhibition studies and alignment of similar protein sequences, results prepared into a manuscript. CLEC11A, CLEC4C, CLEC4D, Reg3a, CD302 and Langerin were tested on glass glycan arrays at **UCPH.** LSECtin was purified and sent to **Genos** for enriching glycoprotein markers using lectin columns. Mass spectrometry results will be verified by Western blot analysis and immunoprecipitation.



m/z value (M+H+)	Peptide sequence (position)
 1177.58 1261.70 1285.65 1396.69 1499.84	DFEAQAAAQAR (193-203) HLQEALGLPAGR (50-61) DAVQALQEAQGR (155-166) AEGLYLFENGQR (247-258) LAGLDAGLHQLHVR (115-128)

Figure 4. Peptide mass fingerprint (PMF) spectrum of SCGF obtained after on-chip tryptic digestion. A MASCOT database search yielded the sequences of the observed masses, which are depicted in the table.

UKE continued working on SNAP-tagged CLEC10A fusion proteins and coupling of nanoparticles, developing a glycoreceptor-ELISA array for detection of Tn-antigens on MUC1. In addition, **UKE** developed and validated a glycoreceptor-based array. Preliminary data indicate that glycoreceptors can be applied as capture probes on a glyco-array, however, optimization of the signal to noise ratio will be required for full applicability of the assay in the future.

WP6: Quantitative Analysis

For the purpose of biomarker detection in clinical samples, this work package is designed to enable the development of a microfluidic platform for point of care quantitative monitoring of serum glycoproteins displaying cancer associated changes. WP6 has two main objectives. The first objective is the development of an LC-MS platform for automated on-line sample preparation with subsequent quantitative glycopeptide profiling of the target glycoproteins. The second objective is the development of media for the selective enrichment of proteins carrying cancer associated glycans. **NIBRT** focussed on selecting the appropriate stationary and mobile phases to separate glycans and peptides, followed by optimisation of electrospray ionisation settings and detection of ions using a single quadrupole (SQ) mass spectrometer. For reversed phase liquid chromatography separation of glycans a porous graphitic carbon (PGC) stationary phase was used. Successful detection of (i) 2-AB labelled and unlabelled glycans, (ii) neutral and (iii) charged glycans including tri- and tetra-antennary structures was achieved using the LC-SQ both in scan and SIM modes. The stationary phase C18 was chosen for reversed phase liquid chromatography separation of the peptides. A proof of concept experiment was carried out to determine if the SQ utilised in electrospray ionisation (ESI) mode was capable of detecting a specific glycan biomarker in human serum. The *N*-glycans were run on the LC-SQ using SIM to detect ions with a m/z of 1134.9 corresponding to the *N*-glycan FA3G3 (core fucosylation) and A3FG3 (antennary fucosylation) (DG9) and ions with a m/z of 1061.9 corresponding to the non-fucosylated glycan A3G3 (DG8). Preliminary results indicated detection of the glycan biomarker using the LC-SQ in ESI mode.

An online affinity chromatography column was developed by **NIBRT** for the selective enrichment of a glycoprotein directly from a human serum sample. The platform was developed using an online enrichment column, an online albumin depletion column and an online protein A column used in sequence. Firstly, the complex serum sample was depleted of albumin and IgG prior to enrichment for haptoglobin. The affinity column contained an immobilised 13 kDa Llama antibody fragment which recognised human haptoglobin with high affinity. This column was packed in house at **NIBRT**. The advantage of this particular analytical strategy is its versatility, as it can be readily applied for purification of any glycoprotein by targeting the affinity column towards a particular glycoprotein.

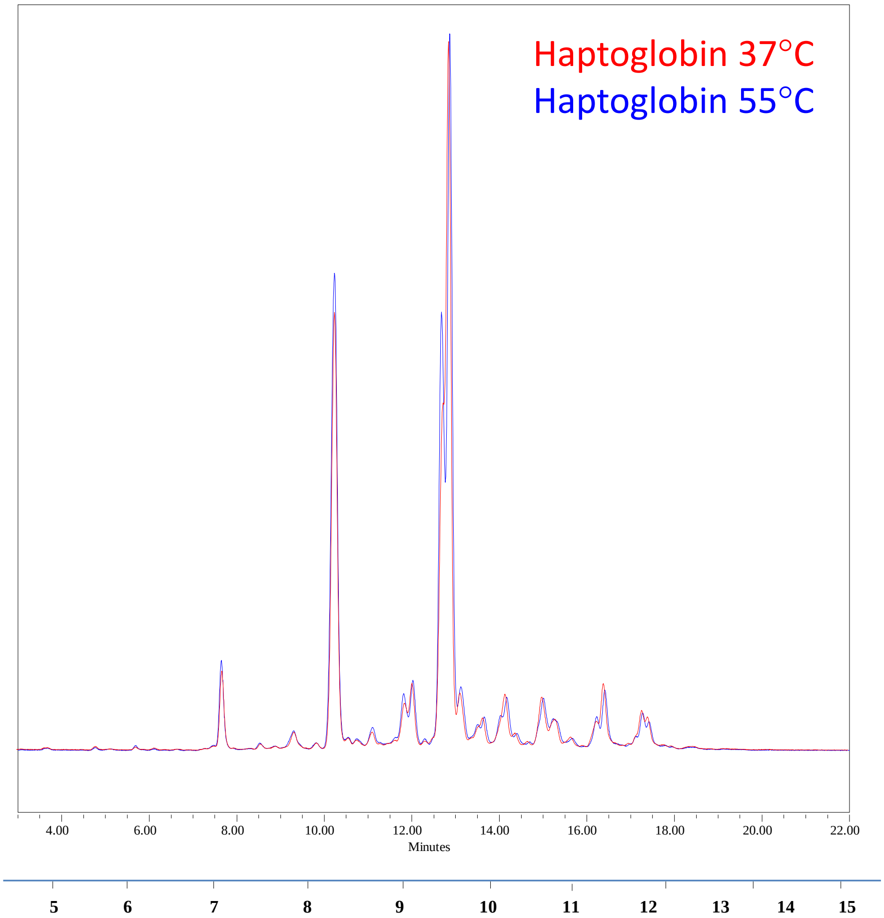


Figure 5: Overlay of haptoglobin glycan release profile at 37°C and 55°C

The immobilisation of enzymes has been demonstrated to significantly improve the reaction kinetics, reducing the sample preparation time. The use of immobilised enzymes also has the advantage of a reduction in reagent consumption. Following in-line digestion of target glycoproteins, the next task in this workpackage is the separation of the enriched glycans and peptides using high efficiency chromatographic media with subsequent mass selective detection. The performance of currently available stationary phases is quite poor, thus limiting the ability to separate a number of sample components in liquid chromatography based glycomics with fluorescence detection. Multiple reaction monitoring mass spectrometry (MRM-MS) with the use of a triple quadrupole mass analyser was employed for absolute quantitation of sample components. This was used at the nanoscale level due to such advantages as reduced sample consumption and increased ionisation efficiency of glycans. Anti-albumin antibody resin tips were employed prior to the capture of other individual glycoproteins i.e. Transferrin(Trf), IgG, IgM, IgA, Haptoglobin(Hpt) and Alpha 1 antitrypsin (A1AT) and exoglycosidase digest conditions for the UPLC-FLD glycoprotein glyco analyses were optimised for AQC labelled glycans.

WP7: Glycoproteomics

The main objective of this work package is to address the complexity of glycobiomarkers by analysing the total glycome and glycosylation of individual proteins in plasma and tissue samples of cancer patients together with the expression of relevant glyco-genes. New integrated methods and procedures developed in other work packages, as well as their results are integrated and used in this work package. By analysing associations between plasma glycans and different elements of the metabolic syndrome, potential new biomarkers were identified by **Genos**. To enable analysis of glycosylation of individual proteins a high-throughput method of isolation and glycosylation analysis of IgG was developed. A pilot study on 225 individuals indicated a number of promising candidates for prognostic and diagnostic markers. Analysis of IgG glycosylation from four European populations revealed a complex pattern of changes in IgG glycosylation with age. Several IgG glycans change considerably and the combination of these glycans can explain up to 58% of variance in chronological age. The remaining variance in these glycans strongly correlates with physiological parameters associated with biological age; thus, IgG glycosylation appears to be closely linked with both chronological and biological ages. The ability to measure human biological aging using molecular profiling has practical applications for diverse fields such as disease prevention and treatment. **Genos** and **NIBRT** worked together to analyse both serum and IgG from the CRC sample cohort (2,000 samples) and **NIBRT** utilized a newly developed high-throughput robotic quantitative UPLC platform for the analysis of glycan profiles.

This first genome-wide association study (GWAS) of the glycome of an individual protein, Imunoglobulin G was performed by **Genos**. Nine genetic loci were found to associate with glycans with genome-wide significance. This study showed that it is possible to identify new loci that control glycosylation of a single plasma protein using GWAS and generated guidelines. **Genos** compared four different analytical approaches (UPLC-FLR, xCGE-LIF, MALDI-TOF-MS and LC-ESI-MS) for analysis of IgG glycosylation and have shown that all the methods generate glycan data of sufficiently high quality to be used to detect associations with genetic polymorphisms.

Genos has also performed the first large scale study of intra-individual changes of total plasma and IgG glycans during the early course of systemic inflammation caused by cardiac surgery. Individual variation in IgG glycosylation changes during acute systemic inflammation associated with increased mortality risk which indicates new avenues for the development of personalized diagnostic and therapeutic approaches. Finally, association analysis of measured IgG glycans with clinical data available in the SOCCS database revealed significant alteration of IgG glycome in CRC patients which is strongly associated with poorer all-cause and CRC mortality. Specific elements of IgG glycosylation, which are linked to CRC prognosis, showed potential to act as novel prognostic biomarkers of CRC mainly in late stage (systemic) disease. **Genos** built a model for predicting disease status for CRC patients and healthy controls based on age, sex and IgG glycan traits (AUC = 0.755). Furthermore, by examining the expression of all genes previously associated with the IgG glycome in CRC and matching tissue, a significantly increased expression of *FUT8* was found, implying that the same features of genetic makeup influence glyco-gene expression in both the tumour tissue and in B cells.

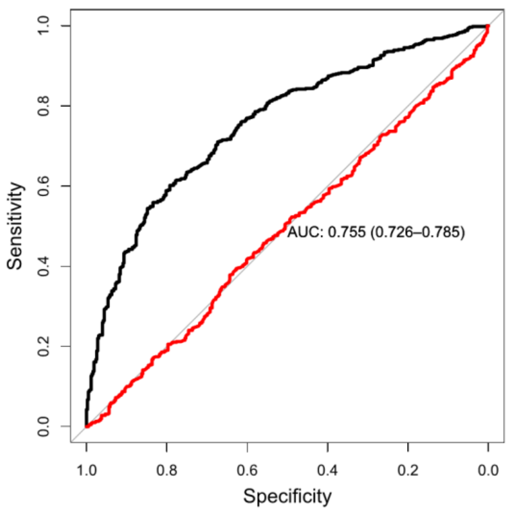


Figure 6: Classification of CRC patients using IgG glycans. ROC curve illustrating the performance of regularized logistic regression model in predicting disease status for CRC patients and healthy controls. While models based only on age and gender did not show predictive power (red line), addition of glycan traits increased predictive power of model (black line).

WP8: Autoantibodies

A sensitive antibody capture microarray platform was developed by **UCPH**. The platform was first evaluated with tumor cell line model system designed to express and secrete CA-125 and CA-15.3 with STn and Tn glycoforms. In a pilot study, **UCPH** demonstrated that aberrant glycosylations of CA-125 and CA-15.3 tumor proteins are present in serum and can be detected with glycan specific antibodies and lectins. Validation studies were performed with a unique and unprecedented sample cohort (UKCTOCS and UKOPS biobanks), and **UCPH** demonstrated for the first time that current CA125 biomarker assay of ovarian cancer can be significantly improved by measuring tumor-specific glycoforms of secreted and circulating CA125 (MUC16) glycoprotein. This work was published recently in the Journal of Proteome Research.

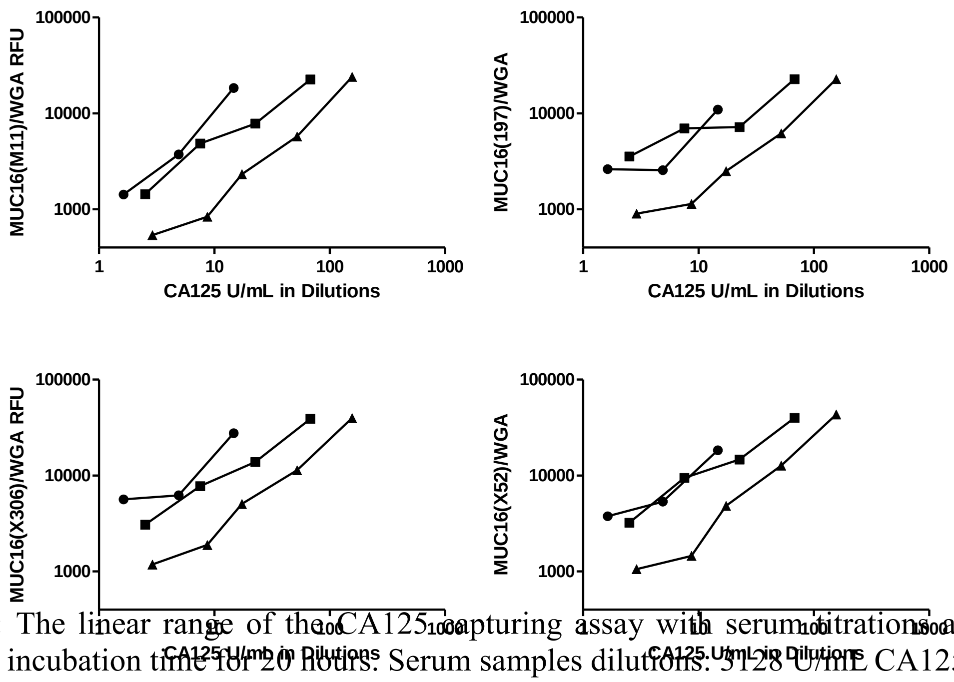


Figure 7: The linear range of the CA125 capturing assay with serum titrations and WGA lectin detection, incubation time for 20 hours. Serum samples dilutions: 5128 U/mL CA125 in PLIP buffer

with 1/20; 1/60; 1/180; 1/360 dilution factors (solid triangle); 340 U/mL CA125 in PLIP buffer with 1/5; 1/15; 1/45 dilution factors (solid square); 29.4 U/mL CA125 in PLIP buffer with 1/2; 1/6; 1/18 dilution factors (solid circle).

Successful production of recombinant glycoform standards by **UKE** resulted in a unique resource of baits for validation of the glycoform assay but also to explore presence of autoantibodies to these antigens. Unfortunately, **UCPH** could not see any significant elevation of circulating autoantibodies which might be hidden on various immune complexes. However, capitalising on the developed glycopeptide and glycoprotein microarray technologies, **UCPH** investigated other disease samples such as viral infections: Herpes, Varizella, Epstein barr, Tick-borne encefilitis, and Crimean-Congo virus. Preliminary conclusions (published in Journal of Virology, 2012) showed the potentials of detecting novel GlycoBioMarkers such as type-specific serum antibodies in such samples.

UCPH has established a high-throughput SPPS tip-synthesis protocol and microarray display of peptides and glycopeptides for display and screening serum antibodies. Furthermore, **UCPH** has successfully screened large cohorts from Herpes, Varizella, Epstein barr, Tick-borne encefilitis and Crimean-Congo virus infected individuals and identified several novel epitopes with biomarker values. The development of a lateral-flow device (LFD) assay for simple diagnostic tests will also undergo commercialisation. A lateral flow device assay (LFD assay) is a quick and easy test to run in the field. The development of such combines principles from chemistry, biology, physics, and engineering. The test is built up by a nitrocellulose membrane where you immobilize the antigen and human IgG. The LFD assay is a method widely used by professionals and patients since its invention in the 1980s. The advantages of this type of assay are the ease of manufacture, stable shelf life, high sensitivity and specificity, relatively low cost and short timeline for development.

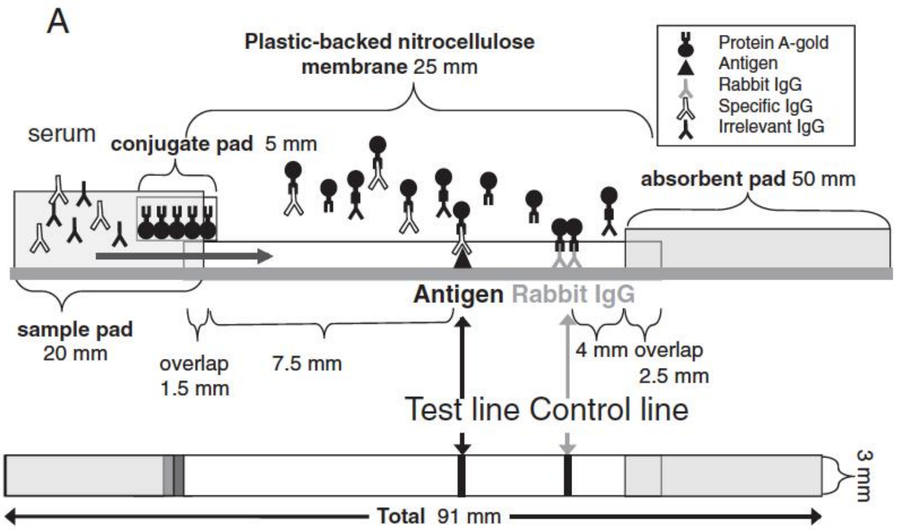


Figure 8: The antigen and human IgG were immobilized on a nitrocellulose membrane, which was assembled with the conjugate pad containing the protein A conjugated gold particles, a sample pad, and an absorbent pad. The membrane cards were cut into thin strips and tested in different concentrations of human infected sera (scheme in Figure 8A). Tests with CCHFV epitope 84 (AVCKRMCFRATIEASRRALL) showed positive results for both the test line and the control line.

WP9: Imaging Tools

The aim of work package 9 is to establish new imaging strategies for detection of glycan structures by recombinant glycoreceptors. Glycoreceptors are suited to image cells, which express the respective glycans. The human glycoreceptor SRCL binds to HEKT293 cell line expressing CEACAM1 and fucosyltransferase IX creating Lewis x structure on CEACAM1, whereas DC-SIGN binds to CEACAM1 expressed from the same cell line through Lewis a/b/x/y or high mannose. In far western blot analysis of tumor lysates, the binding patterns of eight glycoreceptors (DC-SIGN, SRCL, LSECtin, CLEC11A, Langerin, DDB27, CD301 and KLRF1) were tested. Comparison of the binding patterns showed significant binding of DC-SIGN, SRCL and Langerin to tumor samples. Glycoreceptors are also suited to delineate subpopulations of cells by flow cytometry. We tested the binding of several glycoreceptors to the colon carcinoma cell line HT29 in the absence or presence of Ca^{2+}. These results fit very well to far western blot analysis. HT29 cell extracts were probed positively with Langerin, whereas no specific band pattern was observable with LSECtin. In other flow cytometry experiments we probed CLEC4C to four different cell lines indicating subpopulations of differentially glycosylated cells. For the detection of ligands in sections from formalin-fixed, paraffin-embedded normal and cancerous mammary tissues, we tested wild-type CD301 and mutant CD301. In comparison with normal mammary glands, a pronounced staining of tumor tissues was observed with wild-type CD301. Because the mutant construct did not show any tissue staining, the binding of wild-type CD301 can be attributed to its carbohydrate recognition domain. Taken together, glycoreceptors of inflammatory and endothelial cells are important recognition molecules, which could interact with distinct glycan structures of normal and malignant cells. Thus, glycoreceptors are suited to identify glycan structures on defined glycoproteins and on cells and tissues.

STORM microscopy provides substantially enhanced resolution over standard fluorescence microscopy allowing the investigation of direct molecular interactions in cells and cell-cell interactions in tissues and cell lines on a nano-meter scale (Fig. 9). As STORM microscopy is well suitable for the analysis of glycostructures on glycoproteins using recombinant glycoreceptors as probes UKE will adopt this technique for co-localisation studies in primary tumour tissues.

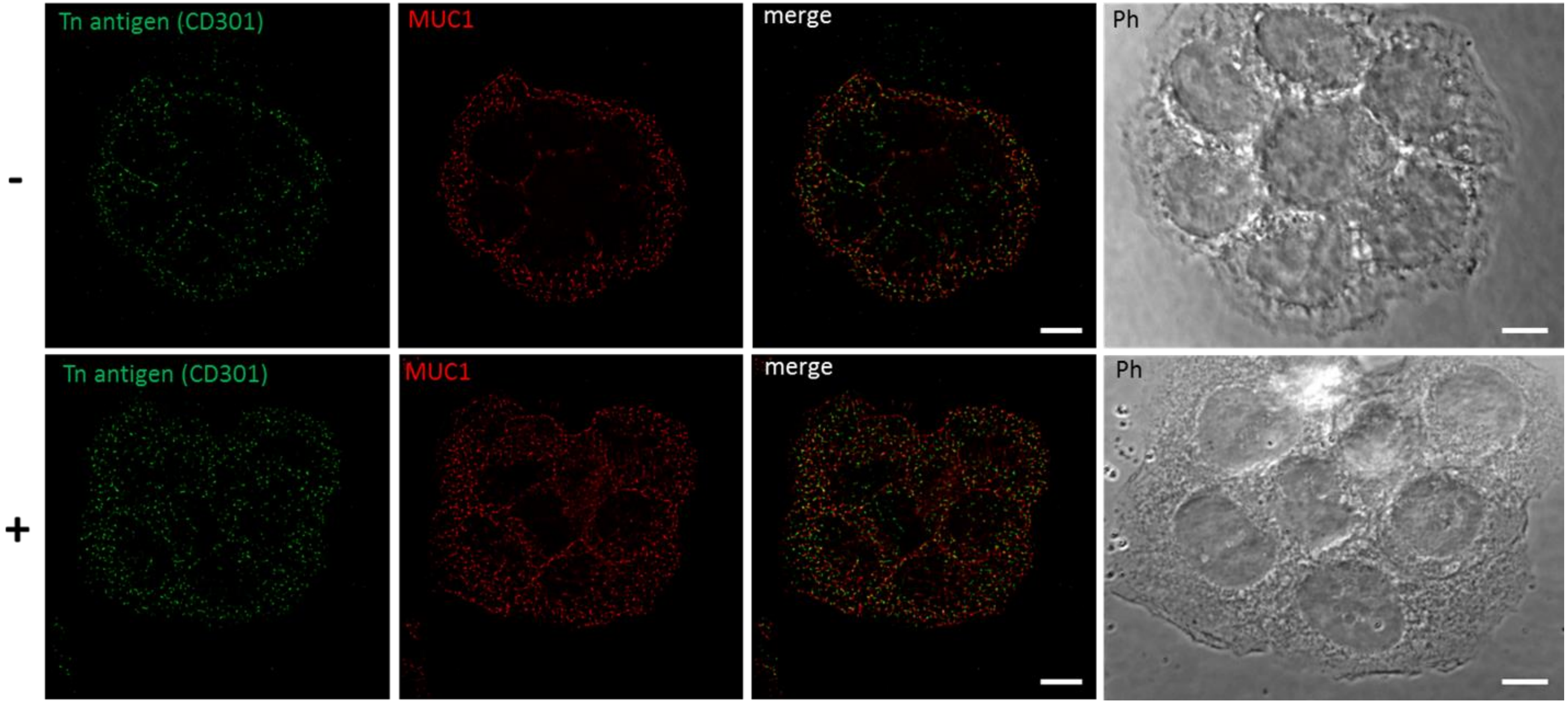


Fig.9. STORM microscopy of T47D breast cancer cells stained with recombinant CD301 (green) and anti MUC1 antibody (red). Cells were fixed with 3 % paraformaldehyde, incubated with 0.1% Triton X-100 (+) or not (-) and stained with CD301 complexed with anti-myc-antibody and detected by an anti-rabbit-antibody coupled with Alexa 568. MUC1 was detected with a murine anti-MUC1 antibody and an anti-mouse-Alexa 647 secondary antibody. Right most panel shows corresponding phase contrast (Ph) images (scale bar indicates 10 µm).

UKE reported on the successful generation of a *Cosmc* ko mouse with specific alterations of O-glycosylation in the pancreas and confirmed by histochemical staining and mass spectrometry. Based on this mouse model **UKE** primarily addressed two questions. First, is the enhanced expression of the Tn-antigen tumorigenic? **UKE** observed no tumor specific alterations in the pancreas of the *Cosmc* ko mice suggesting that additional genetic lesions are required for tumor development. For this reason, we will intercross our *Cosmc* ko mice with a *Kras* mutant mouse strain, an oncogenic mutation frequently observed in human pancreas carcinomas to recapitulate carcinoma development and to provide a mouse model system affected in O-glycosylation for a multitude of future applications (Fig. 10). The second aim was to provide a model with altered O-glycosylation for the development of imaging strategies based on recombinant glycoreceptors coupled to nanoparticles. **UKE** successfully expressed CLEC10A-SNAP-tag fusion proteins in human cell lines and could further purify the recombinant proteins. Direct binding assays showed maintenance of glycan-specificity of recombinant CLEC10A and functionality of the SNAP-domain. However, Click-chemistry coupling of the CLEC10A fusion proteins to functionalized quantum dots was so far not successful. To establish and test **UKE'**s imaging strategy future work focussing on nanoparticle coatings and the optimisation of the coupling chemistry will be required (Fig. 10)

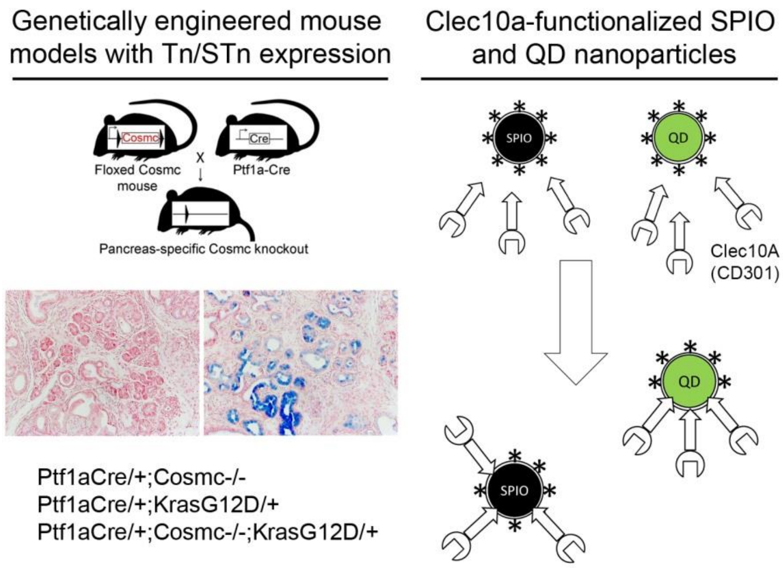


Fig. 10. Future perspectives utilizing the *Cosmc* ko, Tn-positive mouse model in pancreas carcinogenesis and serving as a versatile tool for imaging studies e.g. by MRT or PET.

WP10: Validation and Standardisation Tools

Work package 10 is centred on the validation and standardisation of tools which have been developed in the previous work packages. CA125 was selected as the assay for validation and test kit development. The CA125 cancer biomarker assay plays an important role in the diagnosis and management of primary invasive epithelial ovarian/tubal cancer (iEOC). However, a fundamental problem with CA125 is that it is not cancer-specific and may be elevated in benign gynaecological conditions such as benign ovarian neoplasms and endometriosis. Aberrant O-glycosylation is an inherent and specific property of cancer cells and could potentially aid in differentiating cancer from these benign conditions, thereby improving specificity of the assay. In WP8, **UCPH** reported on the development of a novel microarray-based platform for profiling specific aberrant glycoforms, such as Neu5Aca2,6GalNAc (STn) and GalNAc (Tn), present on CA125 (MUC16) and CA15-3 (MUC1).

In a blinded cohort study of patients with an elevated CA125 concentration (30-500kU/L) and a pelvic mass from the UK Ovarian Cancer Population Study (UKOPS), **UCPH** measured STn-CA125, ST-CA125 and STn-CA15-3. The combined glycoform profile was able to distinguish benign ovarian neoplasms (BE) from invasive epithelial ovarian/tubule cancer (iEOCs) with a specificity of 61.1% at 90% sensitivity. The findings suggest that microarray glycoprofiling could improve differential diagnosis and significantly reduce the number of patients elected for further testing. The approach warrants further investigation in other cancers which will continue post-project. **Galab** developed the MTP assay for CA125 with neuraminidase treatment which increases sensitivity of the assay by at least a factor of 5 compared with non-neuraminidase treatment. However, unspecific binding of VVL to anti-CA125 required further investigation. **Galab** have put the test through validation and test kit stages to produce a robust production protocol with sensitivity within the limits for detection and differentiation. The CA125 assay is now at TRL6 and ready for large clinical validation studies. This test can improve the speed at which ovarian/tubual cancers are diagnosed in a less invasive manner with positive impact on patients' lives. After clinical validation the test will be delivered to clinics and hospitals and **Galab** will also market the assay on their internet platform and via external suppliers for research purposes.

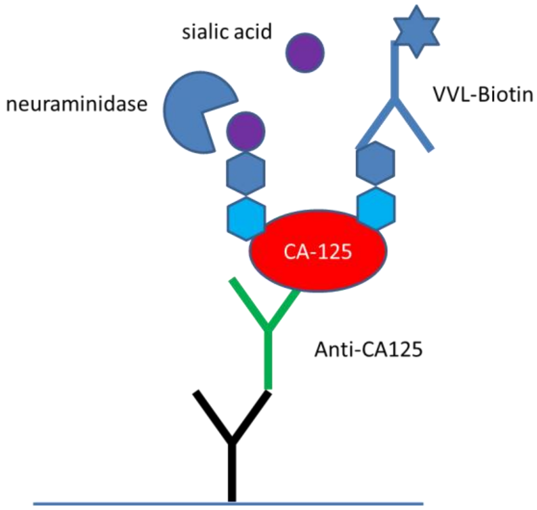


Figure 11: MTP- CA-125 GlycoBiomarker assay

WP11: Ethical Reviews

The table below outlines all sample sets used within the consortium.

Deliverable	Sample Set Name	Reference Number	Beneficiaries Involved
D3.1	CRC Samples	11-SS-0109	**All**
D4.3	Glycol-Medical Study	08/G/103	**UNIMAN**
D7.3	Croatian-Vis Study	2181-198-03-04/10-11-008	**Genos**
D7.3	Croatian-Korčula	2181-198-03-04/10-11-008	**Genos**
D7.3	ORCADES	27-02-2004-ORCADES	**Genos**
D7.3	EHEC	PV4447	**Genos**
D8.2/8.3	UKCTOCS	05/Q0505/57	**UCPH**
D8.2/8.3	UKOPS	05/Q0505/58	**UCPH**

Impact, Dissemination Activities and Exploitation of Results (not exceeding 10 pages).

Impact and Exploitation of Results

There has been considerable impact and exploitation during the course of the GlycoBioM project and looking ahead for the next 7 years.

Non-confidential

Spin-out company Bio-Shape Ltd. www.bio-shape.com co-founded in November 2015 by Dr Hannah Roberts (**UNIMAN**), Professor Sabine Flitsch (**UNIMAN**), Professor Perdita Barran (**UNIMAN**) and Professor Claire Eyers (**UNIMAN/UoL**). Bio-Shape Ltd. is a contract structural characterisation and method/instrument development facility for biomolecules operating from The BioHub in Cheshire (UK). Bio-Shape Ltd. provides state-of-the-art native mass spectrometry services utilising ion mobility mass spectrometry (IM-MS). Using this technique, the molecular non-covalent interactions are retained during the ionisation process and subsequent mass analysis. Detailed post-translational modification (PTM) analyses can be performed at the intact protein level or following the enzymatic release of glycans. A more in-depth glycan analysis service is also available for quantitative glycan monosaccharide composition analysis and structural characterisation of glycans including separation of isobaric species. Bio-Shape Ltd. was successful in their application to Pitch at the Palace http://pitchatpalacenews.com/ and will attend Pitch in the Palace 5.0 on 7th March 2016. These types of analyses will be used by SME Pharma, Big Pharma, SME Biotech and Big Biotech companies initially throughout the UK and Europe. These analyses can significantly reduce research and development timelines to bring safer, more efficient products to people faster thus also improving patent lifetimes. As an example, a third of all drugs approved by the FDA in 2015 were biological. By using Bio-Shape's analyses we can bring safer products to market faster to ensure the best possible drugs are available to the population.

Commercialisation of GlycoBase into UNIFI Software NIBRT and its Glycosceince group have a multi-level partnership with Waters Corp. designed to support advanced concepts and deliver the promise of more effective biotherapeutic innovation. All of this was made possible by the scientific contributions of GlycoBioM to Glycobase development. GlycoBase has been extended with experimentally with glycan information from a diverse set of therapeutically relevant glycoproteins. GlycoBase is a web-enabled proprietary resource that contains normalised retention data, expressed as glucose Units, GU values, for more than 600 2AB labelled N-linked glycan structures. These values were obtained by systematic analysis of released glycans from a diverse set of glycoproteins using Waters HPLC and UPLC technologies and the NIBRT glycan analysis platform. GlycoBase was commercialised into Waters UNIFI software in 2013 aimed at biopharmaceutical organisations challenged to deploy high-resolution analytics across the innovative biotherapeutic or biosimilar development process. The impact of this database and workflow is to significantly reduce drug research and development timelines, extending patent life and lowering costs to being more efficacious drugs to market faster which will have a positive impact on patients' lives across Europe. **NIBRT** were shortlisted for the US-Ireland Research Innovation Awards 2015 for the commercialisation of GlycoBase into UNIFI software.

CA125 MTP Assay CA125 was selected as the assay for validation and test kit development during the GlycoBioM project. The CA125 cancer biomarker assay plays an important role in the diagnosis and management of primary invasive epithelial ovarian/tubal cancer (iEOC). However, a fundamental problem with CA125 is that it is not cancer-specific and may be elevated in benign gynaecological conditions such as benign ovarian neoplasms and endometriosis. Aberrant O-glycosylation is an inherent and specific property of cancer cells and could potentially aid in differentiating cancer from these benign conditions, thereby improving specificity of the assay. The combined glycoform profile was able to distinguish benign ovarian neoplasms (BE) from invasive epithelial ovarian/tubule cancer (iEOCs) with a specificity of 61.1% at 90% sensitivity. The findings suggest that microarray glycoprofiling could improve differential diagnosis and significantly reduce the number of patients elected for further testing. The approach warrants further investigation in other cancers which will continue post-project. **Galab** developed the MTP assay for CA125 with neuraminidase treatment which increases sensitivity of the assay by at least a factor of 5 compared with non-neuraminidase treatment. **Galab** have put the test through validation and test kit stages to produce a robust production protocol with sensitivity within the limits for detection and differentiation. The CA125 assay is now at TRL6 and ready for large clinical validation studies with the support of further H2020 SME Instrument funding. This test can improve the speed at which ovarian/tubual cancers are diagnosed in a less invasive manner with positive impact on patients' lives globally. After clinical validation the test will be delivered to clinics and hospitals and **Galab** will also market the assay on their internet platform and via external suppliers for research purposes.

Global Health: 50 Year Road Map Glycoscience under the GlycoBioM remit has been propelled onto the worldwide stage. Professor Pauline Rudd (**NIBRT**) gave a invited lecture at European Parliament "Global Health" 50 year road map to the future of medicine 2013. This lecture addressed global health challenges, their related socio-economic implications and the future of medicine. Professor Rudd addressed what the upcoming global health challenges are from a glycoscience perspective and the economic implications of those challenges. Healthcare systems have no proper solutions in place to cope with these challenges and countries have no strategy how to finance healthcare under these expected developments. There is a need for new solutions. The 50 year roadmap for the future of medicine outlines that these solutions may only be delivered by top level research and development. However, several paradigm shifts in R&D and healthcare are required to occur: to change from a fragmented R&D landscape to a multidisciplinary and international collaborative community and to take advantage of the 'massive data' century to change research focus from individual molecules to biological systems. This has several implications ranging from restructuring medicine, new strategies in drug development and prescriptions, new organization and healthcare providers to developing new curricula for education and training. In order to allow such a shift common technical standards, harmonized ethical and legal frameworks are essential. The R&D landscape will also affect the academia-industry relationship. There is a need for increased collaboration between academia and industry particularly in the pre-competitive field. This will also impact on the role of patients and open access information.

Centre of Innovation Award from Waters Corporation to Professor Pauline Rudd 27/03/12 (**NIBRT**)

Confidential

Antennary fucosylation as a biomarker for MODY subtype of diabetes. 1-2% of people with diabetes have MODY-subtype. This equates to approximately 7.2 million affected people. It is important to know if you have got MODY to make sure you get the right treatment and advice for your type of diabetes (e.g. stopping insulin), there is a 50% chance of a parent passing on MODY to their child and genetic testing can be offered to other family members. A non-genetic biomarker specific for a monogenic subtype, which could be used as a high-throughput screening test would allow both identification of patients at highest risk (who could then undergo genetic sequencing) and functional assessment of novel mutations. **Genos's** GWAS of the plasma glycome identified HNF1A as a master regulator of antennary fucosylation of plasma proteins. Mutations in HNF1A cause HNF1A-MODY subtype of diabetes and **Genos** confirmed that antennary fucose is an excellent biomarker for HNF1A function (AUC up to 95%). **Genos** holds 1/3 IPR for the respective (WO2012042020-A2) and has the freedom to operate in the field. This biomarker is currently also in clinical trial in UK and Croatia (funded by EFSD).

The exploitation plans for HNF1A-MODY is completion of clinical trials to enable test-kit production by **Genos** and preparation of roadmap to market into clinics and hospitals globally. The alternative is franchising technology to limited number of large clinical laboratories and production and sales of diagnostic kits that can be used by routine laboratories. Clinicians, including both diabetes specialists as well as non-specialists will gain from the new screening technologies – particularly from improvements in their ability to correctly diagnose and treat patients with less common forms of the disease. Such patients will benefit from receiving proper treatment for their disease and therefore lowers risk of developing serious complications of long-term diabetes as they age. Through **Genos's** initial studies they learned that individuals diagnosed with diabetes early in their lives are highly motivated to undergo this test (and pay for it if needed), even while it is still in the research phase. This test will ensure the rapid diagnosis of MODY globally which will have a positive impact patients' lives. Since healthcare costs are growing faster than the GDP for decades, global health systems need to be transformed to ensure long-term sustainability and access to high quality healthcare. For example, the total annual economic impact of only RA in Western Europe has been reported to be €45 billion. Moreover, approximately 40% of expensive RA biological therapies (€10,000 per patient per year) annual budget is wasted because it is given to people who will not respond to the drug. Also, although the prevalence of MODY is relatively low the cost savings per patients are significant. Switching a patient from insulin to low dose glicazide would save around €950 per year per patient. We estimate that there are ~400,000 cases of HNF1A-MODY in Europe resulting in cost savings on medication of more than €300M per year, not to mention the decrease in incidence of diabetic complications and consequential decrease in direct and indirect costs.

IgG galactosylation as a prognostic marker in acute and chronic imflammation and as a glycan-age index and biomarker for sub-optimal health. IgG is an important regulator of inflammation and results of **Genos's** GWAS study of the IgG glycome suggest IgG galactosylation has an important role in different inflammatory diseases. By performing studies of people undergoing major heart surgery as part of the GlycoBioM project, **Genos** identified the potential of IgG glycans to predict mortality after surgery. **Genos** have filed a patent (application #P20140082A) and expect to secure IPR for this inventions. Large population studies performed as part of GlycoBioM also identified strong association of IgG glycans with biological age, most probably through the process of inflammaging. A separate patent for this invention has been filed (#P20130568A) and **Genos** expect to secure IRP. **Genos** believe that IgG could be a general indicator of suboptimal health that could benefit diagnostics of various diseases (PCT patent application #PCT/HR2014/000022) and in collaboration with several large clinical centres **Genos** are currently planning several clinical trials that could confirm this hypothesis. Significant gender specific differences have been identified in past studies, thus future studies will be planned to have sufficient power to identify gender-specific differences.

IgG glycan analysis is not being performed as routine diagnostics so patients are not benefitting from this knowledge. Powerful biologics are available for treatment of many inflammatory diseases but are effective in just a subset of patients. Furthermore, the clinical utility is hampered by the absence of adequate biomarkers. This means that patients are given biologic drugs sequentially on a 'trial and error' basis. This approach has significant detrimental consequences on health and is a waste of healthcare resources since many patients are exposed to expensive drugs (with significant side effects) to which they are unlikely to respond. Recent results indicate that characteristics of individual IgG glycosylation are important for the efficacy of treatment with immunoglobulins and the availability of effective affordable glycomics tests for IgG glycosylation resulting from future projects would allow (a) stratification of patients with inflammatory diseases before treatment and (b) monitoring of the biomolecular response of a patient to their treatment regime. This system could be extended to other diseases for which glycosylation pattern biomarkers have been found – so expanding the impact on patient healthcare and financial savings through more considered use of expensive biological drugs.

Genos's studies on over 10,000 individuals indicated that glycan-age index is a promising biomarker with prognostic power in numerous different diseases. Changes of IgG galactosylation do not seem to be specific for a given disease but apparently reflect that state of suboptimal health that is about to develop into a disease, or predict more severe disease course. Therefore this simple test could potentially be included in routine screening as a part of preventative medicine check-ups. At the moment this is not fully validated but such a prognostic marker could have a very significant impact because it could be an early indicator of disease development and could enable lifestyle changes that may even prevent the disease.

In principle, there are three options to introduce glycan biomarkers into routine use: (1) Analysis of glycan biomarkers as a service provided by **Genos**; (2) Franchising technology limited to a number of large clinical laboratories with mass spectrometry capabilities and (3) production and sales of diagnostic kits that can be used in routine laboratories. There are successful examples of market positioning using all three approaches and as part of **Genos's** feasibility study they will evaluate all three of them using market studies and customer surveys. **Genos** is already providing analytical service and selling kits in the field of molecular genetics, thus they have the necessary experience for all approaches within the company. IgG in these contexts is currently positioned at technology readiness level (TRL 4). Labelling and derivatisation (currently TRL3) will still need to be optimised and tailored software for data analysis will have to be developed (currently TRL2). The individual steps will have to be integrated into one workflow, the sample preparation will be transformed into kits and transferred onto robotic platforms for automation. Sample preparation, measurement and data analysis will be integrated, validated and demonstrated in the relevant environments of a diagnostic service and in the second stage in clinical centres as end users. TRL9 will be reached by demonstrating the successful application of the technology for the analysis of diagnostically valuable markers in clinical sample cohorts. All of this will create conditions for positioning of **Genos** as a global leader in clinical application of glycan biomarkers. **Genos** will transform from an academic start-up into a professionally managed company.

Crimean-Congo hemorrhagic fever virus (CCHFV) is an acute, highly contagious viral zoonosis transmitted mainly by ticks of the genus Hyalomma, with a fatality rate of 30-50% in humans. CCHFV is widely distributed throughout large areas of the world, including Africa and Asia. The disease has the potential to cause epidemics in Southeastern and Southern Europe. Recent detection of the CCHFV vector in Portugal, Spain and Germany is alarming and observations point towards the possibility of future outbreaks taking place in Western Europe. Diagnostics for CCHFV is restricted both by limited diagnostic tools available in several endemic areas as well as difficulties in the international transfer of samples for logistic and economic reasons. An inactivated suckling mice vaccine has been in use in Bulgaria since 1970's for high-risk groups living in CCHFV endemic regions. There is no vaccine against CCHFV licensed in any EU member state and it is therefore necessary to identify relevant lacunas and build up capacity for virus prevention, virus control and distribution of new diagnostic tools. A CCHFV-LFD assay test strip has been developed and transfer of technology for commercialisation with Coris BioConcepts (Belgium) initiated by **UCPH**. Preliminary studies on the preparation of anti-CCHFV scFv monoclonal antibodies anticipate commercialisation with Coris BioConcepts (Belgium) initiated by **UCPH**. This will have huge impact on the mortality rates of those in affected areas during outbreaks.

Herpes Simplex Virus. Invention disclosure (**UCPH**): HSV serosorting. Identification of new immunogenic epitopes for type-specifc serodiagnosis of oral and genital Herpes Simplex Virus

(HSV) infection. University of Copenhagen, 2013, SAG:21-0210/13-7000. Detection of type-specific antibodies is an important and essential part of accurate diagnosis, even in silent carriers of HSV-1 (oral) and HSV-2 (genital) infections. Serologic assays that identify HSV-1 and HSV-2 type-specific antibodies have been commercially available for more than a decade but is generally an expensive and labor-intensive test procedure whose results may be difficult to interpret due to crude antigen preparations. Attempts to identify type specific peptide epitopes for use in serology for both HSV-1 and HSV-2 have been limited. Identification of new immunogenic epitopes for type-specifc serodiagnosis of oral and genital Herpes Simplex Virus (HSV) infection by **UCPH** can be capitalised upon. The explotation plan for this result is to use the new biomarker and make it into a commercial prototype test kit. This will be used by clinics and hospitals globally within the next 3-5 years and have significant impact on the rapid diagnosis of HSV so patients can receive treatment and the right care faster.

Patent filed for on-site test for early detection of pregnancy in cattle. Accurate detection of non-pregnancy thus informing farmers if re-insemination required considerable costs savings to farmers globally **(NIBRT**). The glycomarker must now be validated in further studies during which time a roadmap to market is established.

Dissemination activities

A wide variety of dissemination activities have been achieved. Notably, During Period 2, **UNIMAN** were successful in their bid to host "The Complex Life of Sugars" at The Royal Society Summer Science Exhibition, 1-7th July 2013, London. The exhibition was visited by over 12,500 people including the following categories: students, teachers, public, scientists, media, potential donors/key decision makers and celebrities. The complex life of sugars webpage http://sse.royalsociety.org/2013/exhibits/sweet-complexity/ proved a great success, including one of the most popular 'how it works' videos. The webpages also include scientist profile videos for four members of the GlycoBioM **UNIMAN** team http://sse.royalsociety.org/2013/exhibits/sweet-complexity/scientists/. The videos range from a PhD student just beginning their career all the way up to an internationally established Professor leading their field. We hope that these videos show the range of job opportunities available within an academic career (STEM subject) and also the person behind the scientist. As part of the exhibition we also commissioned a three minute animation which provides an introduction and overview of the whole area of carbohydrate science. This video has had over 700 views http://www.youtube.com/watch?v=9uT9dQ-s1yo in three different places (available on other websites). We were also successful in the Royal Society Games Jam competition. Our game, 'Cell Invaders' was voted the best game at the exhibition and won £2,000 worth of development. It is now available to download on PC http://coolcherrytrees.com/cellinvaders/web/web-new.html and ipad https://itunes.apple.com/gb/app/cell-invader/id664340722?mt=8. One of the main exhibition activities was focused on cell surface sugars and visitors were encouraged to build a cell surface sugar and explore its interaction with cell invaders both on a cell surface and also a gold glycan array. This activity was designed to highlight and directly promote the GlycoBioM work (WP4; D4.3, D4.4: Tasks 4 & 5) at **UNIMAN** and proved very popular (see photographs below). For more contents please visit our blog http://sugar-complexity.tumblr.com/ and twitter feed @sugarcomplexity.

The Royal Society Summer Science Exhibition proved so popular that we were asked to be part of The Science Spectacular, 2nd November 2013 at The Manchester Museum http://www.manchestersciencefestival.com/whatson/science-spectacular and also the MIB Open Day for A-Level Students, 8th November 2013. The Science Spectacular was aimed primarily at young children and families; whereas the MIB Open Day was specifically for A-Level students wanting to find out more about the applications of biotechnology and potential STEM careers.

- GlycoBioM chosen as an FP7 success story for the European Commission website (**UNIMAN**, **NIBRT**, **Genos**, **UKE**, **UCPH** and **Galab**) http://ec.europa.eu/research/infocentre/success_stories_en.cfm?item=All&subitem=&start=1 (story awaiting publication)
- Beilstein TV (**NIBRT**) http://www.beilstein.tv/tvpost/bioprocessing-and-protein-glycosylation-analysis/
- BIONEXGEN dissemination meeting, Brussels 2-4th December 2013 (**UNIMAN** & **GALAB**)
- Waters & **NIBRT** platform press release http://www.bizjournals.com/prnewswire/press_releases/2013/01/28/NE49082
- **NIBRT** makes front page of BioPharm International, Sept 2013 http://www.biopharminternational.com/biopharm/Feature+Articles/Elucidating-Biosimilars-Characterization/ArticleStandard/Article/detail/822761?contextCategoryId=50125
- GlycoBase the LC database developed by **NIBRT**, freely available http://glycobase.nibrt.ie/
- **Genos** on facebook https://www.facebook.com/Genos.DNA.Laboratorij
- **Genos** among finalists for the prestigious "European Universal Biotech Innovation Prize" 2013
- **Genos** TV/radio/newspaper coverage
    - ***http://www.hrt.hr/index.php?id=enz&tx_ttnews[cat]=540&cHash=370ecd75be***
    - ***http://www.hrt.hr/index.php?id=enz&tx_ttnews[cat]=597&cHash=638064648a***
    - ***http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1003225***
    - **http://www.index.hr/vijesti/clanak/uspjeh-znanstvenika-iz-tvrtke-genos-novo-otkrice-omogucit-ce-razumijevanje-razlike-izmedju-bioloske-i-kronoloske-dobi-pojedinca-/716973.aspx**
- Individual partner websites: http://www.galab.de/; http://genos.hr/en/; http://www.nibrt.ie/; http://www.flitschlab.chemistry.manchester.ac.uk/; http://icmm.ku.dk/ansat/curis/beskrivelse/?id=335329; http://www.uke.de/index_ENG.php

During period 3, a wide variety of dissemination activities have been achieved. Notably, **UNIMAN** were successful in their bid to host "The Complex Life of Sugars" at BBSRC The Great British Biosciences Festival, 14-16th November 2014, London. The exhibition was visited by over 6,500 visitors including people from the following categories: students, teachers, public, scientists, media, potential donors/key decision makers and celebrities. One of the main exhibition activities was focused on cell surface sugars and visitors were encouraged to build a cell surface sugar and explore its interaction with cell invaders both on a cell surface and also a gold glycan array. This activity was designed to highlight and directly promote the GlycoBioM work (WP4; D4.3, D4.4: Tasks 4 & 5) at **UNIMAN** and proved very popular. For more contents please visit our blog http://sugar-complexity.tumblr.com/ and twitter feed @sugarcomplexity.

The Great British Biosciences Festival proved so popular that we were asked to be part of The Science Spectacular, 2nd November 2014 at The Manchester Museum http://www.manchestersciencefestival.com/whatson/science-spectacular and also the MIB Open Day for A-Level Students, 8th November 2015. The Science Spectacular was aimed primarily at young children and families; whereas the MIB Open Day was specifically for A-Level students wanting to find out more about the applications of biotechnology and potential STEM careers. These events provided excellent training opportunities for PhD and PDRA/early stage researchers.

IBCarb http://ibcarb.com/ is a network for Glycoscience Tools for Biotechnology and Bioenergy (BBSRC NIBB). On 16th July 2015 at The Manchester Institute of Biotechnology**, UNIMAN** supported the launch of a vibrant, colourful collection of abstract paintings by artist Karen Barber that promote the incredibly diverse and vital roles of sugars and their applications. A number of paintings displayed gold glycan arrays for diagnostic devices and GlycoBioM staff at **UNIMAN** were on-hand to discuss their projects and future outlook for this technology. The paintings will now tour the UK and Europe and showcase further the application of glycans to non-science specialists and strengthen the links between the arts and science.

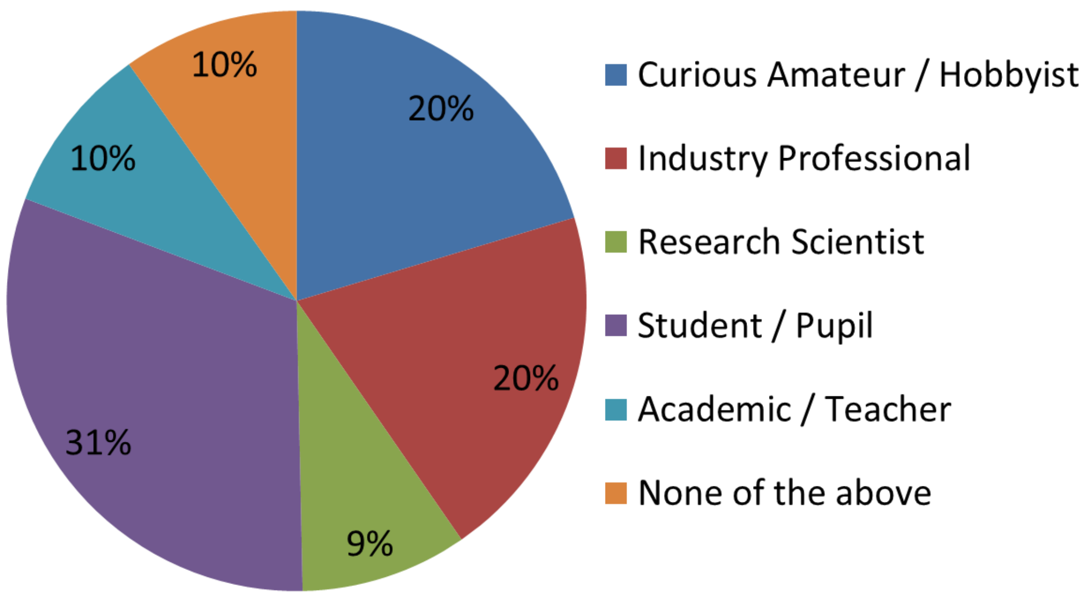
- The President of the Republic of Croatia, Ivo Josipovic, conferred the Charter of the Republic of Croatia to **Genos**: http://radio.hrt.hr/clanak/nagraena-znanost/61628/

- Prof. Gordan Lauc (**Genos**) was awarded with the Croatian State Award for 2013 in the field of biomedical sciences: http://www.vecernji.hr/znanost/gordan-lauc-dobitnik-je-godisnje-drzavne-nagrade-za-znanost-951774
- **Genos** TV and radio coverage
    - ***http://www.hrt.hr/index.php?id=enz&tx_ttnews[cat]=540&cHash=370ecd75b***
    - ***http://www.hrt.hr/index.php?id=enz&tx_ttnews[cat]=597&cHash=638064648***
    - **http://www.index.hr/vijesti/clanak/uspjeh-znanstvenika-iz-tvrtke-genos-novo-otkrice-omogucit-ce-razumijevanje-razlike-izmedju-bioloske-i-kronoloske-dobi-pojedinca-/716973.aspx**

- **NIBRT** shortlisted in the US-Ireland Research Innovation Awards 2015 for their research collaboration with Waters Corporation. **NIBRT** shortlisted in 4 categories in the Irish Pharma Industry Awards 2015 (www.pharmaawards.ie): Pharma Research Centre of the Year, Pharma Education and Training Award, Partnership Alliance of the Year, Leader of the Year

- During period 4, filming of opening lectures at Glyco23 Conference http://www.glyco23.org/ 15-20th September 2015 made available as an online resource on www.glycobiom.eu and www.ibcarb.com (**Genos**, **UNIMAN**).

During period 4, **UNIMAN** were asked to design, integrate and deliver 3 modules on Glycoscience as part of a MOOC (massive open online course) in Industrial Biotechnology with particular emphasis on the tools developed within GlycoBioM. A MOOC provides: open education, global reach, improves recruitment in emerging areas (for example glycoscience), widening participation. The MOOC contents level is aimed at students aged above 16 studying A-Levels in Science or adults with an interest in Science. However, the demographic split of students is typically presented in Figure 1.



The MOOC will be made available on Coursera https://www.mooc-list.com/initiative/coursera?static=true for free; however, if students require a verified certificate then they must complete the quizzes and pay a small fee. The course takes typically 6-8 weeks to complete and comprises of interactive video lectures, a narrative, supplementary links, self-test quizzes, forums and assignments. The glycoscience module is based on 'A Roadmap for Glycoscience in Europe' produced by IBCarb and the EGSF. It covers an introduction to glycoscience, key results and challenges in the areas of Pharmaceuticals and Personalised Medicines (addressing challenges in Health), Food (food security and wellbeing) and biomaterials (resource efficiency and raw materials). Underpinning these opportunities are a number of emerging Glycoscience Tools, particularly in synthesis, analysis, bioinformatics and modelling which open up glycoscience to a much wider scientific and industrial community and overcome barriers to entry for commercial applications. Importantly, full exploitation will only be possible, if the broader community is educated in the opportunities brought by the emerging field of glycoscience, through cooperation with the media and policy makers, as well as Education and Training for students and scientists at all levels. The course will be available online from May 2016 on Coursera.

During period 4, **Genos** had a press release on Croatian TV, newspapers and web news portal "Croatian scientists have discovered potential biomarker of several diseases" directed at civil society in Croatia and globally via web-news.

The GlycoBioM website (see screen shot below) http://www.glycobiom.eu and twitter feed @glycobiom has been regularly updated by **UNIMAN** and will remain active post-project.

Publications detailing results obtained during the GlycoBioM project are listed below and within the EU participants portal and totals 121 which includes 8 internal GlycoBioM collaborations. The number of conferences/invited lectures for GlycoBioM staff totals >250 since the start of the project. GlycoBioM members have successfully disseminated their scientific results on a worldwide stage.

Dr Hannah Roberts

The University of Manchester

Tel: +44(0)1613065174

E-mail: hannah.roberts@manchester.ac.uk

Project website address: http://glycobiom.eu/