Methods for high-throughput (HTP) analysis of protein glycosylation

Final Report Summary - HIGHGLYCAN (Methods for high-throughput (HTP) analysis of protein glycosylation)

Executive Summary:
The HighGlycan project developed technology for the analysis of protein glycosylation in a high-throughput (HTP) manner. Protein glycosylation plays an important role in determining the stability of proteins. Glycosylation targets proteins to specific subcellular compartments or – in the case of secreted proteins – to specific sites in the organism. Changes in glycan structure modulate the activity of proteins, by influencing for example receptor-ligand interactions. Protein glycosylation is highly regulated, and cellular development steps consistently coincide with glycosylation changes. Glycosylation patterns may mark various pathological and physiological situations. Diseases associated with glycosylation changes include cancer and diabetes, as well as congenital, cardiovascular, immunological and infectious disorders. Various glycoproteins have been found to be useful clinical biomarkers in diagnostics as well as targets for
There is an increasing need in both biomedical research and biotechnology for robust high-throughput methods for the characterization of protein glycosylation. The HighGlycan consortium addressed this need by developing analytical methods which can reliably quantify a multitude of glycan structures in complex biological samples. For this purpose, it brings together leading European companies as well as innovative academic groups in the field of high-throughput glycosylation analysis.

On the basis of analytical potential as well as acceptance in the field, the HighGlycan participants have chosen the following three technologies for further development of advanced, mature glycosylation profiling methods:
1. Ultrahigh-performance liquid chromatography (UPLC)
2. Capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF)
3. Mass spectrometry (MS)

The HighGlycan consortium worked on method development including robotisation of sample preparation methods. Another focus is on data processing and analysis methods. In addition, method comparison is an important aspect of the activities, in order to evaluate the particular strengths and limitations of the different analytical approaches. The developed methods show a considerable overlap in terms of the analytical information they provide, but also exhibit divergent properties with regard to throughput as well as required expertise and investments.

Strong participation of SMEs in the project and close contacts with large industrial partners made that research accomplishments achieved by collaboration between academic and industrial scientists were swiftly transformed into innovative products and services for the benefit of European industry. Moreover, close association with clinical research initiatives ensured the efficient transfer of the developed technology into biomarker discovery programs.

Project Context and Objectives:
Glycans play critical roles in both natural cells and biopharmaceuticals. Oligosaccharides (glycans) are key components in major biological processes from the fertilization of eggs by sperm to cell death. Cell surface oligosaccharides undergo complex changes during cellular development, differentiation and activation and precise regulation of the dynamic changes in glycosylation patterns is essential for normal occurrence of these events. When aberrant glycosylation is found on cells it is invariably associated with biological dysfunction such as autoimmune reactions or a fatal disease such as various types of cancer.

For most biopharmaceuticals correct glycosylation is vital for maintaining consistent safety and efficacy profiles. Even small changes in glycosylation can lead to serious issues such as anaphylaxis in patients and the destruction of therapeutic activity. These effects have been found in drugs such as Cetuximab (a mAb for treatment of metastatic colorectal cancer), Herceptin (a mAb for treatment of breast cancer) and erythropoietin (EPO - a drug for increasing red blood cells in patients with renal failure and cancer patients undergoing chemotherapy).

Glycosylation can play a crucial role in modifying the efficacy of glycoprotein vaccines. This phenomenon
is not well studied at present but the evidence for a real and significant effect is growing. For example, changes in a single N-linked glycan in Human Immunodeficiency Virus (HIV) type 1 gp120 (which is heavily glycosylated) results in an enhanced ability to induce neutralizing antibody responses [Li Y, Cleveland B, Klots I, Travis B, Richardson BA, et al. (2008) J Virol 82: 638-651.].

Our understanding of the relationship between the glycosylation and clinical efficacy of glycoprotein vaccines is at early stage but indicates that glycoengineering could be exploited for the design of more potent vaccines. For biopharmaceuticals our knowledge of the sensitivity of therapeutic performance to changes in glycosylation patterns is more advanced but raises anxiety. It is of particular concern for drug regulators and manufacturers because (a) oligosaccharides greatly increase the heterogeneity and structural complexity of recombinant therapeutics and (b) glycans can be very difficult to measure and control during biomanufacturing. To compound matters, for many biopharmaceuticals we have a limited understanding of how the glycan structures influence the safety and efficacy of the drug in patients. As a result, glycosylation is currently the major source of batch to batch variation for biopharmaceuticals and the acceptance criteria for these variations for drug lot release are often arbitrary. This leads to wastage due to failed batches with out-of-specification glycosylation patterns and, consequently, higher costs for drugs that are already exceedingly expensive for patients and healthcare providers.

Current Glycoprofiling Technology is Too Slow, Expensive and Low-Throughput

The evidence for the critical nature of protein glycosylation has relied on both experimental biology and profiling of the glycan structures. The glycoprofiling tools available to analysts have had severe limitations but many advances have been made over the years. For example, in the mid-1980’s a detailed glycoprofiling study was performed to map changes in the glycosylation of immunoglobulin G (IgG) from patients with rheumatoid arthritis and osteoarthritis. This was done in the laboratory that later became the Glycobiology Institute at the University of Oxford. Over 1,400 oligosaccharides from 46 IgG samples were analysed by a team of specialists [Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, et al. (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. Nature 316: 452-457.]. The analytical work took over two years to perform. With today’s glycoprofiling techniques, that study would take one glycoanalyst around three weeks.

The glycoprofiling platforms available to today’s glycoanalysts include a wide range of orthogonal analytical HPLC methods, capillary elektrokinetic separation techniques, various types of mass spectrometry, hyphenated techniques thereof (such as LC-MS), and microarray systems. However, even this impressive array of glycoprofiling methods still fails to deliver the power needed to solve the challenges now faced by life scientists. Those scientists need to analyse not tens of samples but thousands – and with glycosylation patterns orders of magnitude more complex than that found on IgG. For modern life sciences, today’s glycoprofiling methods are too slow, too expensive, and too low throughput.

Aim of the HighGlycan Project – Development of High Throughput (HTP) Glycoprofiling Technology

The HighGlycan project aims to address these deficiencies by establishing robust, efficient and fast high-throughput (HTP) glycoprofiling technologies. These will be optimised for two main applications:
The technologies are aiming at commercial-grade glycoanalysis modules which will be developed to the prototype level in HighGlycan. These will be developed to the prototype level in the HighGlycan project. Each module will typically consist of a kit with materials and reagents, a protocol and troubleshooting guide, data analysis tools (including informatics software), and documentation for regulatory compliance (including health and safety legislation). These modules will be used in conjunction with specific analytical instruments, sample handling robots and other laboratory equipment. Together, the modules will form a flexible toolkit, the components of which can be combined in various ways to tackle key glycoanalysis problems in systems biology and the realization of therapeutics.

The objective of this project is to establish comprehensive technologies for glycoprotein isolation and high-throughput (HTP) measurement of glycosylation for glycomics studies in medicine and systems biology for biomarker discovery and early-stage clinical diagnostics and to support development and production of biopharmaceuticals and vaccines. To succeed we will need to overcome the major challenges that have hampered the efforts of other world-class consortia from the US and Japan. To this end, the partners of the proposed HighGlycan programme have followed the progress of those glycomics groups and studied what has worked well and what has not. We believe we understand the core elements needed to overcome the problems and have designed a programme to combine these elements into a coherent and practical framework.

These core elements are as follows:

a. Focus on orthogonal methods for reliable quantitation of glycans first and HTP analysis second. The three analytical platforms in this programme - HPLC, MS and CE - work well as an analytical toolkit for reliable identification and relative quantitation of biopharmaceutical oligosaccharides. These form the basis of the HTP tools for quantitative glycomics work.

b. Glycan and glycan receptor microarrays will not take centre stage in the HighGlycan programme. Glycan microarrays have captured the imagination of many glycomics researchers. They have been chosen as the main analytical platform for the US Consortium for Functional Glycomics (CFG) in part because they offer easy scale up sample throughput and a model for studying the interaction of glycans with receptors (the main focus of the CFG). However, current implementations of glycan microarrays struggle to deliver reliable quantitation data.

c. Focus on tools to study effects of glycan modification of protein function. In this programme we are interested mainly in the effect that glycans have on modifying the behaviour and functions of the proteins that bear them. The complex topic of glycan interactions with protein receptors (the main focus of the CFG) is of secondary importance - so we will not be hindered by the special challenges faced by researchers in that field.

d. Industry focus. The industry focus of the HighGlycan programme operates in three areas. Firstly, the participants are predominantly SME’s who make their living by developing, manufacturing and selling...
useful technology. This creates urgency for the programme to yield practical, affordable, commercial grade technology. Secondly, the overall objectives of the programme are shaped by our close collaboration with the Industrial Impact Board - who represent an important group of clients for that technology. Thirdly, there is a natural and efficient way for the results of the HighGlycan programme to be disseminated, shared and built upon in the long term. This differs from most other glycomics consortia which have a predominantly academic focus without the urgency, emphasis and longevity that a commercial perspective brings.

e. Integration of the four key stages for HTP glycoprofiling. The HighGlycan programme brings together partners with the expertise and vested interest to develop methods for the four main stages of HTP glycoprofiling - sample preparation, analytical separation, informatics and the applications (glycomics for systems biology and glycoprotein therapeutics).

f. Systems Biology – Approach. Acquired glycomic data will be integrated with genomic, lipidomic, physiological and health data about individuals in large population cohorts generated within the recently completed FP6 EuroSpan project. This approach would capitalize on the global leadership of European scientists in the large population studies and enable development of databases and tools which would enable systems biology approach to protein glycosylation. Genome Wide Association Studies (GWAS) of quantified glycosylation traits will enable discovery of complex genetic networks which govern protein glycosylation. In addition, this approach will enable integrated genomic/glycomic approach to biomarker discovery and reveal otherwise cryptic disease biomarkers.

The focus of the HighGlycan project will be on developing HTP glycoprofiling methods based on three analytical platforms - ultra-high-pressure liquid chromatography (UPLC), mass spectrometry (MS) and multiplexing capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF). The approach for each of these platforms will be to formulate practical systems for HTP sample preparation, analysis on the instrument, and analysis of the data in a way that provides useful information for each of the two application areas. Key themes in this work will be simplification of processes, preservation of relative quantitation information on the component glycans, reduction of running times and use of parallel processing.

Project Results:
In the following, the science and technology results of the HighGlycan project will be presented per work package, for the 8 experimental work packages WP2 to WP9.

WP2: High throughput isolation and of individual glycoproteins
WP leader: BIA Separations
Results related to deliverable D2.1: High-Throughput Immunoglobulin G Analysis

The main task of Deliverable D2.1 was to develop 96-well monolithic plates for high throughput isolation of IgG from human plasma and analysis of the glycan structures of IgGs isolated using a HTP protocol. 96-well plates are a powerful tool for the analysis of a large number of samples and the used chromatographic monolithic support enables fast separation and purification of target biomolecules from a matrix and can be involved in an automated process. BIA Separations had already experience in developing 96-well CIM monolithic plate with other chemistries. Monoliths with immobilized protein G were developed, and transfer
Immobilization of Protein G

Monoliths were polymerized in a special polymerization housing in which 100 monolithic units with the volume of 200 µL can be polymerized simultaneously. Subsequently, monoliths were washed several times with 96% ethanol. Monoliths were rinsed with deionized water before hydrolysis of epoxy groups with 0.5 M sulfuric acid. After hydrolysis water was removed from the pores by using the organic solvents acetonitrile and carbonyldiimidazol (CDI) was applied on the surface of monoliths by flushing monoliths with a solution of CDI in water free media. The immobilization of protein G on the monoliths in a 96-well plate was later performed by flushing the monoliths with a protein G solution prepared in an aqueous buffer. Afterwards the monoliths were flushed with deionized water and with 0.1 M sodium hydroxide for 10 min, followed by washing with water to reach the neutral pH of eluted solution. Plates were stored in 20% ethanol prepared with 20 mM TRIS + 0.1 M sodium chloride.

Testing the Protein G plates for DBC on IgG

After immobilization the dynamic binding capacity (DBC) of 96-well Protein G monolithic plates for human IgG was measured. Commercial IgG solution from Octapharma was prepared in 20 mM TRIS buffer pH 7.4 and as an elution buffer 0.5 M acetic acid was used. IgG solution was overloaded onto the 96-well plate CIM Protein G and then washed with the binding buffer (20 mM TRIS, pH 7.4) to remove unbounded proteins. Afterwards an elution with 1.0 ml (5 CV) of acetic acid was done. Absorbance of the elution fraction was measured (Epoch, BioTek) at wavelength of 280 nm and concentration of IgG in collected elution fractions was measured by using dilution curve with known concentrations of IgG in acetic acid. For this purpose a simple platform for calculating the capacity of each well of 96-well plate was developed. The needed input for the calculation is only the information on the absorbance of a known concentration of IgG and absorbance of diluted elution fractions of each well. DBC was measured each time after the new immobilization. The coefficient of variation was below 10 %, which is in line with our expectations.

Due to the high price of Protein G from the first producer, a cheaper Protein G with the same or even better specifications has been searched. We prepared a plate with immobilized Protein G from producer 2. After the optimization of the immobilization procedure for the second protein G (changing buffer composition, time of immobilization) on single monolithic units, a transfer of the immobilization to a 96-well plate was successfully applied. The DBC values for IgG were tested and the results were comparable with those obtained with protein G from the first producer.

The Protein G 96-well plate prepared with protein G from the producer 2 was sent to Genos to compare its performance with the plate prepared with protein G from the producer 1. Additionally, a comparison between protein G ligands from other producers immobilized on CIM monolith analytical columns was performed with the intention to improve the product and lower the immobilization costs. These Protein G samples were tested at an analytical scale (single monolithic units), and the resulting monoliths were used to evaluate the Cleaning in place (CIP) stability with 0.1 M NaOH of different ligands as well.

It was found that the CIP procedure with 0.1 M NaOH decreases the capacity for IgG. We performed also consecutive 13 CIP procedures on 96-well plate with immobilized Protein G from producer 1 and results were matched with one obtained on analytical column. After 13 CIP cycles on the plate the capacity was still above 5 mg of IgG per 1 mL of monolithic support. In case of loading real samples of human plasma a
DBC value above 4 mg/ml is sufficient for experiments, where 100 µl of human plasma per 0.2 ml monolith is loaded.

**HTP isolation of IgG from human plasma using 96-well CIM® Protein G monolithic plates**

The method for HTP isolation of IgG from human plasma was successfully developed in collaboration between Bia Separations d.o.o. Ajdovščina, and Genos Ltd., Zagreb. IgG has been isolated from 1800 plasma samples from adult human residents of Croatian islands of Vis and Korčula. Aliquots of IgG have been sent to three different laboratories (Genos, LUMC and MPG) within the HighGlycan project for subsequent glycosylation analysis (UPLC, MALDI-TOF-MS, LC-MS and CGE-LIF). In addition, IgG has been isolated and its glycosylation analysed in over 5000 samples. Very complex patterns of changes in IgG glycosylation with age were observed.

**IgG glycan analyses**

Aliquots of the same IgG samples were used for comparative analysis of IgG glycosylation by four different methods: UPLC analysis was performed in Genos, MS analysis was performed in LUMC, and CGE-LIF analysis was performed in MPG. To evaluate the accuracy of the four methods, analysis of association with genetic polymorphisms and age was performed. Chromatographic methods with either fluorescent or MS-detection yielded slightly stronger associations than MS-only and multiplexed capillary gel electrophoresis, but at the expense of lower levels of throughput. Advantages and disadvantages of each method were identified, which should inform the selection of the most appropriate method in future glycosylation studies.

**Results related to deliverable D2.2: High-Throughput Plasma Protein Analysis**

The main task of deliverable D2.2 was to develop 96-well monolithic plates for high throughput isolation of glycosylated proteins from human plasma, enabling glycan-analysis of said proteins with a HTP protocol. 96-well plates are a powerful tool for the analysis of a large number of samples and the used chromatographic monolithic support enables fast separation and purification of target biomolecules from a matrix. As monoliths offer a quick and reliable platform for antibody immobilization and affinity chromatography, the usage of 96-well CIM® monolithic plates could be extended to immunoaffinity applications as well. Immobilization of monoclonal antibodies specific for different glycosylated proteins from human plasma on such format would become a unique and efficient high throughput tool for fast analysis of glycosylation pattern of different plasma proteins. Immunoaffinity monolithic 96-well plates were successfully developed and representative examples were sent to Genos, where isolation of proteins from plasma samples was performed using the new plates followed by the release and analysis of glycans.

**WP3: HTP Sample Preparation for Glycomics**
**WP leader: Daniel Spencer and Daryl Fernandes, Ludger**

**Results related to deliverable D3.1: Glycan Release and Labelling**

We established a protocol for the release and labelling of glycans that is included in a Standard Operating
Procedure (SOP) and was automated on a Hamilton STARlet liquid handling robot. The protocol can easily be modified and transferred for use on a wide range of liquid handling robots.

- All kits and consumables used in the sample preparation protocol are commercially available through Ludger

- The sample preparation method takes just over two days to perform and subsequent analysis of 96 samples takes upwards of two days. Multiple plates can be analysed on multiple UHPLC systems

- The sample preparation method has been automated which means the method user only has to start the program on the robot and can walk away and perform another task

- The process is repeatable and accurate as a Hamilton robot is being used to perform all the pipetting steps removing potential sources of human error in the process

- The protocol can easily be modified and transferred for use on a wide range of liquid handling robots

As part of the protocol for the rapid release and labelling of glycans, we have implemented a high throughput labelling kit that can label up to 100 samples at a time.

- A key part of the optimisation of the labelling chemistry was the replacement of sodium cyanoborohydride with 2PB (2-picoline borane), lowering toxicity and improving stability.

- Comparable results achieved using the labelling kit containing 2PB and incubating for one hour at 65°C, resulting in the shortening of the sample preparation method by two hours.

Automated high throughput sample preparation method for post labelling clean-up of samples using LudgerClean T1 cartridges.

- Cartridges used in a 96 well plate format that is compatible with Hamilton vacuum manifold along with other automated, robot and manual vacuum manifolds.

- Scalable use so that anywhere from one to ninety-six samples can be cleaned up in parallel (empty holes in the plate being blocked with strips of plugs)

- The method user only has to start the program on the robot and can walk away and perform another task

- The process is repeatable and accurate as a Hamilton robot is being used to perform all the pipetting steps removing potential sources of human error in the process

The sample preparation protocol for N-glycan analysis was shown to be repeatable (intra-assay precision) with CVs of <0.5% on GU values and CVs of <5% on peak areas for glycan peaks with an average relative % area >1.0% (48 samples of human IgG and 48 samples of bovine fetuin).

The sample preparation protocol for N-glycan analysis was shown to have intermediate precision (inter-assay precision) with CVs of <0.5% on GU values and CVs of <10% on peak areas for glycan peaks with an average relative % area >0.6% (48 samples of human plasma analysed on two different days).

Results related to deliverable D3.2: Membrane and Tissue N-Glycome

The objective was to establish a protocol for high-throughput release of cell membrane and tissue glycome. The vast majority of glycoproteins are associated with cell membranes, however, due to technological limitations, membrane N-glycome has not be evaluated as a biomarker. The major obstacle is homogenization of cells and tissues which inherently introduces large variability and blurs the results. To alleviate this problem we have developed and optimized several approaches for the release of membrane N-glycome from different types of cells:

Release of plasma membrane N-glycome from intact cells
Homogenization of cells disrupts the cellular integrity and results in a mixture of plasma membrane N-glycans and intracellular N-glycans. Intracellular N-glycome originates mainly from Golgi and ER and contains both complete and party synthesized glycans. Since our preliminary experiments indicated that the analysis of N-glycome from embedded cells results in improved analytical precision due to elimination of the homogenization step, we have decided to further exploit the method of glycan analysis from embedded cells. We were able to show that the integrity of the cell membrane is preserved during the embedding process, however, we could not exclude the possibility of leakage during the subsequent steps. To test for possible contamination from intracellular N-glycans, we have analysed glycans from embedded cells that were not treated with N-glycanase F (PNGase F) and observed presence of high-mannose N-glycans (validated by mannosidase treatment) due to an existing efflux of high-mannose N-glycans.

Release of plasma and internal membranes N-glycome using Triton X-114 solution

To enable isolation of membrane proteins and soluble proteins as separate phases, we have tested a cloud-point extraction (CPE) method for isolation of all cell membranes’ proteins. CPE is a detergent based enrichment method for hydrophobic analytes and the idea behind CPE is that non-ionic detergent forms clear micellar solutions in water at concentrations above the critical micellar concentration. Upon an increase in the temperature, the solution becomes turbid (reaches the “cloud point”) followed by the separation into two distinct phases (aqueous phase with soluble proteins and detergent phase with membrane proteins). We have used a non-ionic detergent, Triton X-114, to solubilize cell membranes after which the detergent was removed by filtration and proteins were extracted by methanol/chloroform extraction. Extracted membrane proteins were deglycosylated, labelled and analysed by HILIC. Use of Triton X-114 enabled isolation of membrane proteins and soluble proteins as separate phases which was tested on THP cells (human monocytes), leukocytes and lysosomes. By analysing membrane and soluble proteins as separate fraction, their differences in glycosylation were observed.

Release of plasma membrane N-glycome using polylysine beads

To isolate plasma membrane alone from the cells we adjusted a method using polylysine beads (. The idea behind this method is that cells with a net-negative surface charge tenaciously bind to the beads which have a positive surface charge. This is followed by cell lysis, protein extraction, deglycosylation, labelling and HILIC analysis. This method enabled analysis of glycoproteins from plasma membrane while contamination from other intracellular membranes was avoided. We used it to isolate exclusively plasma membrane from leukocytes and platelets.

High-throughput analysis of plasma membrane N-glycans from the cell surface

To enable high-throughput analysis of N-glycans from the cell surface we have developed and optimized a protocol for cell surface N-glycan release, derivatization, purification (all on the 96 well plate format) and MALDI-TOF-MS analysis. This approach enabled analysis of both cell surface N-glycans and N-glycans released from the residual pellet revealing significant differences in their abundances (especially prominent in high-mannose content). The developed protocol was successfully used for N-glycome characterization
of 25 colorectal cancer cell lines.

To analyze tissue N-glycome we have developed a protocol for murine brain tissue homogenization, glycoprotein extraction, deglycosylation, labelling and HILIC-UPLC. For this purpose brain tissue was first homogenized by FastPrep system developed by MPBio (participant 8) which enables simultaneous fast and reproducible homogenization of up to 24 samples. This was followed by methanol/chloroform glycoprotein extraction, denaturation and reduction with SDS and 2-mercaptoethanol and overnight deglycosylation with PNGase F. Released glycans are then filtered through 30kDa filters, labelled with 2-aminobenzamide and analyzed by HILIC-UPLC. The developed method enables processing of up to 48 samples per week. Using this method we analysed four different parts of murine brain (cerebral cortex, hippocampus, striatum and cerebellum) and showed significant differences in their N-glycomes.

Conclusions

We have successfully developed several approaches for the analysis of the cell N-glycome on different analytical platforms (UPLC-FLR and MALDI-TOF-MS). Cloud-point extraction method using Triton X-114 enables analysis of the total cell membranes’ N-glycome while method with polylysine beads enables analysis of exclusively plasma membrane N-glycome. In addition, to enable cell surface N-glycome analysis in a high-throughput fashion, we have optimized a protocol for sample preparation based on a 96-well format plates with MALDI-TOF-MS analysis. To alleviate problem of laborious and time-consuming analysis of tissue N-glycome, we have developed a robust and reproducible sample preparation protocol for the analysis of up to 48 murine brain tissues’ N-glycomes per week.

References

next generation of liquid chromatography, the upcoming UPLC systems (Bones et al, Anal Chem 82:10208, 2010). Improvement with respect to accuracy and precision could be achieved by upgrading the established external to an internal retention-time normalization, utilizing differentially labelled samples and standards. The number of different glycan structures in complex biological samples is very large requiring high-resolution LC runs for successful analysis. The use of internal standards is compromised as glycans of complex samples elute over a broad range. An alternative approach could be the introduction of standards labelled with a structurally similar fluorescent dye which emits at different wavelength and can therefore be detected without interference. Multicolour fluorescent labelling is routinely used for DNA analysis by gel electrophoresis for decades, but to the best of our knowledge this approach has not been applied for HPLC analysis. To be successful, this kind of application needs to meet the following demands: (i) fluorophores need to have non-overlapping emission and excitation spectra; (ii) they should not compete or interact in any way with each other during HPLC separation; and (iii) the chemical properties of the fluorophores (size, structure and polarity) should be similar enough to enable simultaneous separation without changes in gradient conditions. Preliminary experiments indicated that 2-aminobenzamide (2-AB), 2-aminoacridone (2-AmAc) and aniline could be used for simultaneous multiplexed analysis of glycans, but extensive testing of intra and inter-assay reproducibility and sample interference needs to be performed before the final conclusion of the feasibility of this approach can be made. Both individual glycans and reference samples will be evaluated as internal standards for quality control. We believe that this effort is worth doing since introduction of non-interfering internal standards over the whole chromatographic range is expected to allow determining relative retention positions with much greater accuracy, therefore resulting in higher confidence structural assignment on the basis of retention-time matching.

In our work (High throughput plasma N-glycome profiling using multiplexed labelling and UPLC with fluorescence detection. Knežević A, Bones J, Kračun SK, Gornik O, Rudd PM, Lauc G. Analyst. 2011 Nov 21;136(22):4670-3. doi: 10.1039/c1an15684e) 2-Aminobenzamide (2-AB), 2-aminoacridone (2-AmAc) and aniline were selected as the fluorophores of choice for the development of multicolour glycan labeling protocol. Maximal excitation and emission wavelengths for aniline, 2-AB and 2-AmAc were experimentally determined as ex250/em340 for aniline, ex330/em440 for 2-AB and ex428/em580 for 2-AmAc using a standard solution of maltoheptaose labelled with each fluorophore via reductive amination with sodium cyanoborohydride in 70:30 DMSO : acetic acid at 65°C for two hours. The emission wavelength chosen was not necessarily \( \lambda_{\text{max}} \) but the wavelength at which there was minimal interference from the emission of the other fluorophores in the other detection channels.

A standard glycan mixture was next prepared by mixing 3.47 mM maltotetraose, 1.8 mM maltopentaose, 0.95 mM maltohexaose and 3.37 mM maltoheptaose. Equal quantities of this glycan mixture were labelled with aniline, 2-AB and 2-AmAc and subsequently separated using UPLC on the 1.7 mm HILIC stationary phase (Waters BEH Glycan, 50 x 2.1 mm i.d.) with multi-wavelength fluorescence detection. The UPLC instrument used was a Waters Acquity UPLC consisting of a binary solvent manager, a sample manager and a FLR fluorescence detector (Waters Corporation, Milford, MA, USA). Oligosaccharides labelled with aniline, 2-AB and 2-AmAc demonstrated <1% spectral interference in the monitored fluorescence channels of the other labels using the experimentally determined emission wavelengths. All chosen fluorophores are spectrally separated and essentially optically transparent to each other with negligible cross-detection of samples in different detection channels of the multi-wavelength fluorescence detector. Limits of detection were also experimentally determined as 3.6 2.0 and 6.1 fmol for maltotetraose labelled with aniline, 2-AB and 2-AmAc, respectively. The slightly higher limit of detection for the 2-AmAc labelled
oligosaccharide is due to monitoring 2-AmAc fluorescence not at \( \lambda_{\text{max}} \) but under conditions of minimal spectral interference in the other detection channels.

Retention time reproducibility was evaluated by five consecutive injections of the same mixture of the standard malto-oligosaccharides labelled with three fluorophores. Experimentally determined variations in retention time were less than 1% RSD. Reproducibility for quantitation was also evaluated using repeated injections of the malto-oligosaccharides. Deviations of less than 1% RSD were again noted based upon relative peak area quantitation. The high levels of reproducibility for both retention time and relative peak area observed in both cases demonstrate that glycans labelled with the three fluorophores did not interfere with each other when co-injected on column. Furthermore no loss of separation efficiency or resolution was recorded in any of the chromatograms indicating no sample mass loading effects on the chromatographic process. The selected fluorophores differ moderately in both size and polarity. Despite these differences no alterations in selectivity for the labelled oligosaccharides were recorded in any of the resulting standard or sample chromatograms. This suggests that retention on the HILIC phase is governed predominantly via interaction of the glycan with the amide stationary phase with minimal contribution to retention from the attached neutral labels. Moreover, the fluorophores appear to govern the degree of retention based upon their interaction with the high solvent environment in the mobile phase. It was observed that glycans labelled with aniline were less retained than glycans labelled with 2-AmAc which were in turn less retained than glycans labelled with 2-AB, respectively.

To investigate the practicality of the multiplexed glycomic profiling method using complex samples, the three dye on-column approach was applied to the simultaneous analysis of healthy human plasma N-glycans using standard HILIC-fluorescence conditions. The plasma N-glycome represents one of the most complicated and analytically challenging oligosaccharide mixtures and consists of bi-, tri- and tetra-antennary glycans with variable sialylation as well as core and outer arm fucosylation. Run time and resolution were maintained; thus the data obtained after integration are comparable to that obtained during our previous large scale plasma N-glycan studies wherein a single fluorophore was used, however, now with the advantage of a three-fold increase in analytical speed.

To further increase sample throughput the three dye on-column method for plasma N-glycome profiling was transferred from HPLC to UPLC using the recently introduced 1.7 mm HILIC stationary phase using conditions of maintained HPLC performance but at maximised analytical speed. These criteria were specifically chosen to guarantee comparability with data generated in our previous studies. It was possible to separate a mixture of three differently labelled plasma N-glycome samples in a gradient time of 3.9 minutes whilst maintaining the desired performance for use in subsequent profiling studies. Faster analytical runtimes afforded by UPLC and co-injection of the three differently labelled glycan pools enables a significant increase in sample throughput.

Conclusions

The three dye on-column approach offers a significant increase in high throughput glycan analysis. The method described is specifically tailored for large scale population profiling studies or targeted glycomics rather than studies focused on discovery of altered glycosylation.

Results related to deliverable D4.2: GlycoBase Update

Structure elucidation of glycan peaks requires reliable techniques and glycan reference data. Challenges
in structural analysis include the large number of glycan classes and the efficient exploitation of analytical and bioinformatics tools that are available for structural interrogation. Data sources for glycan analytics encompass several orthogonal methodologies such as ultra-high pressure liquid chromatography (UPLC), capillary electrophoresis (CE) and mass spectrometry (MS), all of which have inherent difficulties in data interpretation. Assignment and characterization of glycan structures in biotherapeutic products or high-throughput data from clinical profiling is a difficult and time-consuming process and is often a bottleneck in this type of research. It therefore requires automated data-integration, data-mining and statistical analysis tools coupled with software engineering and database technology to advance this field of research. This would bring glycomic analysis in line with both the proteomic and genomic fields.

NIBRT’s GlycoBase (www.glycobase.nibrt.ie Campbell et al., 2008) is an integrated solution for rapid and reproducible characterisation of glycan samples. Originally developed from the database EurocarbDB, GlycoBase is a resource for the storage, classification and reporting of glycan structures as well as their associated experimental values obtained using various chromatographic techniques such as HPLC, UPLC and CE. GlycoBase is a web-enabled, open-access resource that contains glycan data as normalized chromatographic retention time data, expressed as GU values, for hundreds of 2-AB labelled N-linked glycan structures. These values were experimentally obtained by systematic analysis of released N-glycans from a diverse set of glycoproteins on the NIBRT glycan analytical platform utilising both Waters HPLC and UPLC analytical instruments. The database was built using data from many samples over the course of one decade. The UPLC data were obtained from many analytes including human serum. For instance, the “Waters Collection” is a list of GU values pertaining to the analyses of a number of therapeutically important glycoproteins including Erythropoietin and Herceptin, haptoglobin, RNAse B and transferrin and is continually being expanded. Hydrophilic interaction liquid chromatography combined with fluorescence detection (HILIC-fluorescence), supplemented by exoglycosidase sequencing and mass spectrometric confirmation, was used to generate this high confidence glycan library. The resulting database has been made accessible through a customized web-application containing a simple and intuitive interface for assignment and confirmation of glycan structures.

GlycoBase has undergone a number of important updates, which enable users to search for specific glycans using a variety of powerful search tools. These include searching by the regular expression name or by antennary composition. Alternatively searches can be carried out according to a GU value (± 0.3) or the user can search for a particular glycan feature, for example the presence or absence of sialic acid or core-fucose. The user also has the ability to carry out a stoichiometric search and thus search by, for example the number of Hexoses or Xyloses. All of these searches can be performed on a global basis, thus searching the entire collection, on a selected collection or a particular sample within a collection. GlycoBase also provides users with access to a “summary report” which collates all the available data for a selected glycan. This includes information on general glycan properties such as the monoisotopic mass and the monosaccharide composition. Individual experimental records containing for example all the UPLC derived GU values recorded in the database are also shown on this summary page. Similarly the user can view links to literature records, profile information as well as the instrument running conditions. Reliable glycan peak assignments and structure elucidation are achieved through GU data from GlycoBase combined with glycan sequencing. Glycan sequencing is performed by exoglycosidase glycan digestion and is an ideal method for rapid oligosaccharide characterisation including monosaccharide sequence and linkage information. Exoglycosidases remove carbohydrate residues from the non-reducing end of a glycan in a linkage-specific manner. For example, almond meal fucosidase (AMF) removes terminal α-fucose residues attached with a (1→3) or (1→4) linkage but not residues attached with a (1→6)
linkage. In glycan sequencing, the glycan pool is analysed before and after sequential digestion with arrays of linkage-specific exoglycosidases. Glycan digestion results in peak shifts, the extent of which depends on the nature and the number of monosaccharides removed. The entire pool of glycans can be digested without separating individual peaks and aliquots of the pool can be digested simultaneously with panels of enzyme arrays.

A new module, termed GlycanProfileAssigner, has been developed and will soon be part of GlycoBase. The software uses exoglycosidase glycan digest panels as the input. GlycanProfileAssigner assigns each peak in a profile a set of potential glycans using preliminary assignments based on GU-values. Any glycan structures that would have been digested by any exoglycosidases applied to the profile are excluded. Next, profiles are ordered from most to least digested, and simulated digestions are performed on the different combinations of potential glycans. Simulated digestions that match observed digestions are used to identify the correct set of glycans. Currently, the assignment algorithm has been written and tested on a representative IgG digestion panel. A basic GUI interface for the software has been created, and the software will shortly be distributed to testers, for bug fixing and improvement.

GlycoBase also enables the user to carry out in-silico “GlycoBaseDigests”. This predictive tool enables the generation of in-silico exoglycosidase digests using various enzymes that are frequently used in the full characterisation of glycans. The user can select a particular glycan and perform a “virtual” digestion. GlycoBase returns the predicted digest product, and if experimental GU values associated with the queried and digested exist in the database, then these are also reported. GlycoBaseDigest currently provides in-silico digestion for the following exoglycosidases: JBM, GUH, ABS, BKF, NAN1, AMF and SPG.

If information regarding the exoglycosidase digest panels is available, GlycoBase users can navigate from parent to child profiles. Thus this allows the user to reconstruct the logic that was followed during the full characterisation of an undigested profile and enables the user to fully comprehend how each and every structure was identified.

Moreover, GlycoBase allows users to conduct in-silico “Extrapolated Profiling”. GlycoBase stores glycan profiles using both the GU values as well as the area under the peak. Using this information and data, GlycoBase re-constructs the original profile computing the Gaussian kernel density estimation. The relative percentage areas are used as a numeric vector of non-negative observation weights, while the GU values are the data from which the estimate is to be computed. The result is an in-silico approximate profile. The computation is performed on the server side using the “R” statistical and graphic package.

Conclusion

A number of important updates to GlycoBase have been implemented, so that GlycoBase has matured into a powerful platform to enable both the research community and the biopharmaceutical sector to perform released glycan analysis with greater confidence and speed than previously possible with the existing analysis workflows. A dedicated biotherapeutics sub-collection is available, which contains GU values for oligosaccharides released from therapeutically relevant glycoproteins such as Erythropoietin, Herceptin, and Infliximab. Hydrophilic interaction liquid chromatography combined with fluorescence detection, exoglycosidase sequencing and mass spectrometry were used to generate this high confidence glycan library. The resulting database has been made accessible through a customized web-application containing a simple graphical user interface for assignment and confirmation of glycan structures.
WP5: CGE-LIF HTP glycosylation analysis
WP leader: MPG
Results related to deliverable D5.1: CGE-LIF Data Analysis

Objectives
• To develop a user-friendly rapid and robust HTP technology (system and method) for analysing fluorescently labelled oligosaccharides / glycans by multiplexed CGE-LIF
• To develop data processing and analysis software for automated fingerprint comparison, quantification and structural assignment
• To extend the method with respect to absolute quantification, maximum long-term reproducibility, and high-confidence structural assignment
• To extend and diversify the database used for automated structural annotation

Work performed

Development of a CGE-LIF specific glycan structure/migration time database and data analysis software

This report summarizes the work and output leading to the software “glyXtool” and the integrated database “glyXbase” for (multiplexed) CGE-LIF based high performance glycoanalysis. Which both are meanwhile commercialized by glyXera. Our work comprised the development of glyXtool that provides automated raw data smoothing, baseline correction and our patented orthogonal double normalization to internal standards, thus enabling structural elucidation and annotation by referring to an oligosaccharide database. Furthermore, functions for peak comparison and overlay as well as several implemented statistical tools facilitate HT-screening for biomarkers in large sample sets. The resulting combined high-performance system (method, software & database) enables fully automated, highly sensitive instrument-, lab- and operator-independent glycoanalysis in high-throughput, even when operated by non-experts.

Development of data processing and analysis software

To tackle the lack of processing and analyzing experimental CGE-LIF glycodata, we developed the software glyXtool for data preprocessing and for in-depth data analysis. glyXtool is a standalone platform-independent application written in Java. The software provides an easy to use graphical user interface (GUI) for processing and interpreting of CGE-LIF based data. The basic input format for glyXtool is XML. The XML files are generated by converting raw files using the “Data File Converter”. The converted input files contain raw data information about sample measurement, i.e. electropherogram data of the recorded dye channels and separation parameters.

To facilitate peak detection, the raw data are smoothed by using the Savitzky-Golay algorithm and applying baseline correction. Both are implemented to run fully automated. To make processed samples comparable and to account for migration time shifts, the software features our patented orthogonal double-normalization: The first normalization is applied to an internal standard ladder. The second normalization is optional and uses an internal quantitative oligosaccharide standard mix. After finishing the pre-processing the actual peak picking begins: The peak picking criterion is to calculate the local maxima of pre-processed data points. An intensity threshold is set to filter out small peaks in the noise region. Additionally, a parameter can be set to filter for the most abundant peaks. The peak picking range must be
provided as well before the peak picking process can be started. After finishing the peak picking, the identification process compares peaks found in the oligosaccharide channel to known peaks at specific migration time points provided in a separate XML database. A glycan structure is claimed to be identified if the difference between observed and theoretical peak lies within a certain migration time tolerance window.

Enlargement, diversification and integration of the database

During the project the multiplexed CGE-LIF oligosaccharide/glycan-database “glyXbase” reached now a mature state for N-glycans and a large number of entries for human milk oligosaccharides (HMOS) built a second sub-library. This XML database can be loaded, fully configured and saved by the user. Database entries may contain structure annotation, major and minor migration times. Thus, our software allows for the creation of tailor-made databases for particular glycan structures.

Conclusion

Due to the lack of convenient and user-friendly software tools analysis and interpretation of large datasets remains the major bottleneck in high-throughput glycomics. Until now, no software has been available for processing and analysis of CGE-LIF based data. We assessed this shortcoming developing the glycoanalysis software glyXtool. Our software comes with an intuitive GUI making it easy for the non-experienced user to work with. Another advantage is the speed of the application, as our tool is able to process input data files in parallel processes. Our application is robust against processing errors by warning the user when providing bad-quality data. Additionally, it contains the integrated and fully configurable database system glyXbase for N-glycan identification. glyXtool presents a user-friendly, self-contained, platform-independent tool for CGE-LIF based N-glycan data analysis which should help researchers to facilitate the high-throughput derived data processing and analysis. Furthermore, glyXbase was enlarged and diversified e.g. with respect to HMOS and that all this was done for two different gel matrices.

Results related to deliverable D5.2: CGE LIF Kits and Standards

The work comprised of isolating glycans for glycan standard production that were of relevance mainly in IgG/blood serum workflows, bulk labelling of these glycan standards with APTS dye and bulk cleanup, developing the relevant QC and finally commercialisation of the product. In addition to the glycan standards which would be used as system suitability controls in CGE-LIF workflows, we also created an APTS glycan labelling kit based on picoline borane reduction for high throughput automated sample preparation.

APTS labelled Glycan Standards

Ludger has isolated the glycan standards from natural sources using a combination of hydrazinolysis, PNGase release techniques, bulk glycan purification procedures followed by bulk APTS labelling and cleanup to produce 11 APTS labelled glycan standards. These standards are commercially available on the Ludger Ltd website:
QC of the products involves comparison to a quantitative APTS labelled chitotriose standard, followed by glycan purity assessment using UHPLC-FD. Ludger has now produced several batches of these glycans which are sold internationally to biopharmaceutical and academic institutions.

High throughput compatible APTS labelling kits

In order to support high throughput workflows Ludger developed a high throughput compatible APTS labelling kit that can be used on automated liquid handling robot platforms. APTS is an expensive chemical to purchase and so Ludger has successfully synthesised APTS in-house for use in a 96 sample labelling kit. In-house APTS has been extensively tested and compared with the commercial one. The in-house APTS and commercial APTS gave comparable results when used concurrently to label human IgG glycan standards. The in-house APTS has been incorporated into a 96 sample, robot compatible, APTS labelling kit utilising the reductant picoline borane whose use was tested extensively in WP3 as a non-toxic alternative to sodium cyanoborohydride.

The Ludger 96 sample compatible APTS labelling kit has been extensively tested using UHPLC-FD as the analysis method. From this we have determined that the current prototype kit gives comparable results to our gold standard glycan labelling and analysis method utilising 2-AB as the reductive amination label. The labelling method is performed in only two hours, at 37oC. Which allows a quick sample throughput workflow and reduces any potential desialylation that can occur under the mild acidic conditions utilised. The labelling method is suitable for HT analysis.

Ludger has also developed a robot compatible, 96 well clean up kit post APTS labelling. The technology most suitable for this was determined to be vacuum manifold solid phase extraction (SPE) technology developed also for high throughput 2-AB cleanup in WP3, plus an ammonium formate buffer for good, non-skewing APTS labelled glycan recovery.

Conclusion

Ludger Ltd has developed a range of CGE-LIF compatible products that have been available commercially since 2013 and will continue to be distributed and supported by Ludger Ltd.

WP6: Robust mass spectrometric glyco-analysis methods

WP leader: LUMC

Results related to deliverable D6.1: Mass spectrometry of derivatized glycans

In work package 6 we aimed for the development of robust and high throughput (HTP) methods for mass spectroscopic glycan analysis. Main points of interest are the development of a HTP matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS) method for analysis of native glycans, and glycans labelled at the reducing end. Furthermore, to stabilise sialylated species for reflectron mode MALDI-MS, it was thought to develop methods for the analysis of permethylated glycans. In addition to the permethylation, we also focus on the sialic acid specific derivatization of glycans. Finally, in order to enable absolute quantification and to enhance the robustness of the methods, the development of internal standards in addressed.
Development of standards and automation for permethylation of released glycans

Ludger LTD has optimised and automated glycoprotein release and enrichment, automated HTP permethylation of released glycans, automated data acquisition of permethylated glycans using MALDI-MS. The HTP permethylation method can be used to process 1-96 samples using either a manual method or an automated method that has been adapted to a Hamilton liquid handling robot. The derivatization leads to enhanced stability and sensitivity of glycan MALDI-time-of-flight (TOF)-MS measurements, as well as increasing the structural information for MS/MS experiments. This HTP microplate based permethylation method is fast, reliable and gives comparable results to the standard Ultra High Performance Liquid Chromatography (UHPLC) method. This analytical technique delivers a cost effective and HTP method for biopharmaceutical realization and biomarker studies. The kit has already successfully been applied for the analysis of human IgG, sialylated glycan standards from Ludger LTD, human plasma, inflammatory bowel disease serum samples, and biopharmaceutical samples such as IgG4 monoclonal antibodies (for monitoring levels of galactosylation), as well as glycosylation profiles of N- and O- glycans from recombinant human erythropoietin (rhEPO). These data will be submitted for publication in the near future.

Ludger LTD has developed 12C and 13C permethylated human IgG N-glycan standards, which are now commercially available; these present an example of system-suitability standards used for relative quantitation of permethylated glycan samples (Shubhakar et al., Chromatographia, 2015).

2-Picoline borane to facilitate reducing end labelling by reductive amination

For mass spectrometry and liquid chromatography with fluorescence detection, glycans are commonly labelled via reductive amination by a fluorophore or ionising label. For example, 2-aminobenzoic acid (2AA) can be used to enhance negative ionization by MALDI. After the reaction of the amine group of the label with the reducing-end aldehyde group of the oligosaccharide, the resulting Schiff base should be reduced to a secondary amine. For this reducing step, the toxic sodium cyanoborohydride is often used. However, it was shown that the non-toxic 2-picoline borane (2-PB) is a good alternative, with less toxicity (Ruhaak, L.R. et al., Proteomics, 2010). This labelling, in combination with LC-fluorescence, was used for the analysis of human plasma N-glycome in samples from the Leiden Longevity Study (LLS), showing that plasma protein N-glycan profiles are associated with calendar age, familial longevity and health (Ruhaak, L.R. et al., Journal of Proteome Research, 2011). Furthermore, the labelling can be used in combination with MALDI-Fourier transform ion cyclotron resonance (FTICR)-MS to analyse released N-glycans. The labelling of glycans at the reducing end using 2-PB as the reductant was successfully transferred to Ludger LTD, which now produces commercially available labelling kits (2-AB, 2-AA, procainamide kits) that can be used for HTP labelling. The 2-AB labelling kit was utilised for analysis of serum samples in determining “Changes to Serum Sample Tube and Processing Methodology Does Not Cause Inter-Individual Variation in Automated Whole Serum N-Glycan Profiling in Health and Disease”. (Ventham et al., PLOS One, 2015).

Ethyl esterification

To allow reflectron positive mode MALDI-TOF-MS measurements, stabilisation of the sialylated species is required. The development of the recently published ethyl esterification protocol has led to a robust, facile,
inexpensive, one-pot derivatisation method (Reiding, K.R. et al., Analytical Chemistry, 2014): during derivatisation, α2,6-linked sialic acids are ethyl esterified (leading to stabilisation and a mass increase of 46.042 Da), while α2,3-linked sialic acids are lactonised (leading to stabilisation and a mass loss of 18.011 Da). After the reaction, efficient detection of sialylated and non-sialylated glycans can be done in positive ion mode. In addition, the induced mass difference between α2,3- and α2,6-linked sialylation isomers allows their discrimination in MS (without derivatisation the masses of these isomers are the same), and adds an additional clinical parameter when studying glycosylation by MALDI-MS. The method was proved to be suitable for profiling of highly complex samples, like the total human plasma N-glycome.

Automated platform for high-throughput ethyl esterification

The aforementioned ethyl esterification method has been transferred to an automated sample preparation system containing an 8-channel Hamilton MicrolabSTAR, a Hamilton MicrolabSWAP (with plate storage, and automated vacuum centrifuge), and a Hamilton MicrolabSTARplus (Bladergroen M.R. Reiding K.R. et al., J. Proteome Res., 2015). The system has been utilised to perform derivatisation, purification and MALDI target spotting of released N-glycans without manual intervention. Current throughput times are 3 h for the complete workup of one 96-well sample plate, but efficient use of the robot resources means that each additional 96-well plate beyond the first one takes only one additional hour. As such, 384 samples can be prepared and measured by MALDI-TOF-MS during a single working day, while currently performed upgrades are expected to result in a throughput of 768 samples per day. The method is highly reproducible, showing an average main peak CV of 4.8% when analysing a standard-containing 96-well plate nine times across three days. MALDI-TOF-MS spectra resulting from the method are informative for the analysis of N-glycan compositions from a complex biofluids like plasma, and provide relative quantification of the sialic acid linkage.

Conclusion

High-throughput protein glycosylation analysis is often performed by (tandem) mass spectrometric analysis of released glycans. This provides a broad overview of the glycome of any given sample, from a single isolated protein to complex samples like plasma or tissue. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) is a fast and convenient method for the analysis of released glycans. Using this method, however, information on monosaccharide linkages is often difficult to obtain and the analysis of sialylated glycoconjugates is biased by metastable decay and variations in ionization and salt adduction. The use of labelling and derivatisation protocols can help to overcome these problems. To enhance negative MALDI ionization, N-glycans were labelled with 2-AA using 2-PB. This protocol is now incorporated in a commercially available kit. To overcome the instability of the sialic acids and enable measurements in reflectron mode, chemical derivatisation was applied to neutralize the carboxylic acids on the structures. The derivatisation methods include permethylation, which can be combined with the incorporation of C13 atoms creating the possibility to use the products as internal standards. Another way to stabilize the sialic acids is to perform sialic acid specific derivatisation. Ethyl esterification causes the linkage-specific modification of sialic acids. Both permethylation and ethyl esterification are at the moment usable in a HTP, robotized set-ups. Finally, automated data analysis can be performed with the in-house developed program, MassyTools.
In addition to the work performed for deliverable 6.1 on the high-throughput analysis of released glycans, we focussed in 6.2 on the development of mass spectrometric profiling methods for glycosylation analysis at the glycopeptide level. This approach has several advantages when compared to the profiling of released glycans: 1) Protein- and site-specific information on glycosylation is obtained. 2) Information on site occupancy may be obtained. 3) N-glycosylation and O-glycosylation may be analysed in parallel.

While the first part of this report focuses on the specific analysis of immunoglobulin G (IgG) glycopeptides, a substantial amount of work was performed on the development of universally applicable methods for N- and O-glycopeptide analysis as described later in the report.

Linkage-specific sialic acid derivatization for MALDI-TOF-MS profiling of IgG Fc-glycopeptides

As described in the comparison of mass spectrometric methods for the analysis of therapeutic IgG Fc-glycosylation and reported before for released glycans, the analysis of sialylated glycopeptides by MALDI methods is biased by metastable decay and variations in ionization and salt adduction. In addition, information on monosaccharide linkages is often difficult to obtain. Therefore, we developed the recently published dimethylamidation protocol for the robust analysis of glycopeptides derived from the Fc-portion of IgG (Linkage-specific sialic acid derivatization for MALDI-TOF-MS profiling of IgG glycopeptides. de Haan N, Reiding KR, Haberger M, Reusch D, Falck D, Wuhrer M. Anal Chem. 2015 Aug 18;87(16):8284-91. doi: 10.1021/acs.analchem.5b02426). Similar to the ethyl esterification reported previously, the method makes use of the carboxylic acid activator 1-ethyl-3-(3-dimethylamino)propyl)carbodiimide (EDC) and the catalyst 1-hydroxybenzotriazole (HOBt). However, we found that the alcohols used for the previously reported esterification, while linkage-specific for the sialic acid, showed variable reactions on the peptide portion of the glycoconjugates. Testing a range of reagents and conditions yielded a protocol employing dimethylamidation of the glycopeptides, which shows selectivity for the sialic acid linkage as well as for the carboxylic acids on the peptide portion. The protocol proves highly repeatable for the subclass-specific glycosylation analysis of tryptic IgG glycopeptides and provides, next to the sialic acid linkage information directly in MS, the possibility to generate informative MS/MS spectra.

The method described above was applied on a human plasma sample cohort consisting of 108 plasma samples of healthy subjects between 0.1 and 40 years of age and 22 samples derived from umbilical cords of newborns (Changes in Healthy Human IgG Fc-Glycosylation after Birth and during Early Childhood. de Haan N, Reiding KR, Driessen G, van der Burg M, Wuhrer M. J Proteome Res. 2016 Jun 3;15(6):1853-61. doi: 10.1021/acs.jproteome.6b00038). While subclass-specific characterization of IgG Fc-glycosylation was performed previously for healthy adults, pregnant women and newborns, the subclass specific description of IgG Fc-glycosylation was still lacking for young children. With this study we were able to fill this gap in our knowledge about healthy human IgG Fc-glycosylation.

Software for automated integration and quality control of LC-MS data (LaCyTools)

As the high-throughput analysis of glycopeptides often includes the separation of a mixture prior to MS detection, the high-throughput processing of this kind of complex data is required. We addressed the issue of efficient and robust data processing before for MALDI-MS data (MassyTools: A High-Throughput Targeted Data Processing Tool for Relative Quantitation and Quality Control Developed for Glycomic and
Glycoproteomic MALDI-MS. Jansen BC, Reiding KR, Bondt A, Hipgrave Ederveen AL, Palmblad M, Falck D, Wuhrer M. J Proteome Res. 2015 Dec 4;14(12):5088-98. doi: 10.1021/acs.jproteome.5b00658). We developed a modular software package designed to tackle the individual aspects of an LC−MS experiment (LaCyTools: A Targeted Liquid Chromatography-Mass Spectrometry Data Processing Package for Relative Quantitation of Glycopeptides. Jansen BC, Falck D, de Haan N, Hipgrave Ederveen AL, Razdorov G, Lauc G, Wuhrer M. J Proteome Res. 2016 Jul 1;15(7):2198-210. doi: 10.1021/acs.jproteome.6b00171). With this package, targeted alignment is performed using user defined m/z and retention time (tr) combinations. Subsequently, sum spectra are created for each user defined analyte group. Quantitation is performed on the sum spectra, where each user defined analyte can have its own tr, minimum, and maximum charge states. Consequently, LaCyTools deals with multiple charge states, which gives an output per charge state if desired, and offers various analyte and spectra quality criteria. These quality criteria include an isotopic patter comparison, calculation of the mass accuracy and signal-to-noise value per analyte. We compared throughput and performance of LaCyTools to combinations of available tools that deal with individual processing steps. LaCyTools yielded relative quantitation of equal precision (relative standard deviation <0.5%) and higher trueness due to the use of MS peak area instead of MS peak intensity. In conclusion, LaCyTools is an accurate automated data processing tool for high-throughput analysis of LC−MS glycoproteomics data. Released under the Apache 2.0 license, it is freely available on GitHub (https://github.com/Tarskin/LaCyTools).

Conclusions

For deliverable 6.2 state of the art MS based glycopeptide methods were compared to find strengths and shortcomings of current methodologies. Also, time was invested in the better understanding of biases in- and the optimization of- the enzymatic digestion of glycoproteins. The development of MS based methods for glycopeptide profiling resulted in a toolbox of different methodologies, including MALDI-TOF-MS, MALDI-FTICR-MS, CE-ESI-QTOF-MS and C18-PGC-LC-ESI-QTOF-MS. All these methods have their own applicability, ranging from glycoprotein-specific glycopeptide analysis, to targeting a broad range of glycoproteins in either a high-throughput, very sensitive or in-depth way. In addition to the profiling methods developed, software to handle the often complex data was established in the form of LaCyTools.

WP7: Systems biology of protein glycosylation
WP leader: UEDIN
Results related to deliverable D7.1: Analysis of Glycome Data

The main objective of this project was to expand list of genes associated with protein glycosylation by performing genome-wide association studies of datasets collected in glycome database for systems biology.

Genome-wide association studies of Ultra-Performance Liquid Chromatography measured IgG glycans

We performed genome-wide association analysis of seventy-seven UPLC IgG glycans and 2.5 million HapMap2 imputed genotypes in seven cohorts of European ancestry, with 8875 individuals comprising a discovery study and 2479 individuals included in replication study. Following extensive quality controlling of cohort-based GWAS results performed by two independent researches, we performed inverse-variance
weighted fixed effect meta-analysis, resulting in a final discovery sample size of more than eight thousand individuals. We identified 1541 significantly associated SNPs (genome-wide significance level Bonferroni corrected for 21 independent glycans, \( p \leq 2.4 \times 10^{-9} \)) with additional 283 suggestively significant SNPs (\( p \leq 5 \times 10^{-8} \)) which cluster in 36 loci. Loci were defined as regions of SNPs in LD \( r^2 > 0.5 \) with the most strongly associated SNP in the region.

Twenty seven out of 36 loci reached genome-wide significance, tripling the number of loci associated with IgG glycosylation compared to our previous study\(^2\) (Table 2). Ten out of sixteen loci from Lauc et al\(^2\) replicate in this study, with two non-replicated loci (IL6ST-ANKRD55 and SUV420H1) were genome-wide significant and four (BACH2, LAMB1, RECK, PEX5) were strongly suggestive in the previous study. Both significant and suggestive SNPs have been functionally annotated using Ensembl’s Variant Effect Predictor (release 82)\(^4\). As expected, majority of SNPs mapped to non-coding regions.

Given that majority of significantly associated SNPs map into the non-coding regions of the genome, it is unclear what genes these SNPs are having influence on. Rather than reporting a gene that is physically the closest to the variant with the strongest association in the region, we employed the following gene prioritisation strategy – for each locus we checked for evidence of non-synonymous variants, variants associated with expression (eQTLs) and evidence of co-expression with genes from similar pathways (Data-driven Expression-Prioritized Integration for Complex Traits - DEPICT).

Unlike the common practice of comparing that relies on overlap of a single SNP trait and expression association studies, we applied summary level Mendelian Randomization (SMR) with heterogeneity in dependent instruments (HEIDI) that expands the test to the whole region in proximity of a shared SNP. For regions with SNPs with significant SMR \( p \) value, test is expanded to surrounding region by testing for heterogeneity in ratio of z-scores from two association studies. Given that immunoglobulins are primarily synthesised in B-lymphocytes, we applied this method on publicly available peripheral blood expression data, and cell-type specific expression data from CEDAR dataset, provided by prof. Michele Georges from University of Liege, Belgium. Among others, this dataset contains expression of B and T lymphocytes. Nine loci exhibited overlap with expression in various tissues, out of which three are known glycosyltransferase genes.

In the case of discordant gene prioritisation in a locus based on different tools, we consider that stronger evidence lies in genes with overlap with expression in appropriate tissue or genes with probably damaging non-synonymous variants. After merging results from all three annotation sources, the list was manually curated to its final form. While in majority of loci single gene was prioritised, in the HLA region on chromosome 6 multiple hits have been prioritised with different annotation algorithms.

To explore potential pleiotropy with other traits and diseases, all SNPs in LD \( > 0.8 \) with significant or suggestive glycan SNPs were queried against published GWA studies using Ensembl's BiomaRt\(^9\) to search for other phenotypes associated with same SNPs. In total, 58 other phenotypes shared at least one SNP with IgG glycosylation SNPs. In general, IgG glycan SNPs exhibit potential pleiotropy with autoimmunity related diseases and various cancers and complex traits.

Conclusions

We performed genome-wide association studies of seventy seven ultra-performance liquid chromatography measured immunoglobulin G glycans and discovered 26 novel loci associated with IgG glycosylation and replicated 10 loci from our previous work. For nine of these loci we have evidence of potential regulation through expression. Novel glycan loci show potential pleiotropy with a number of genes...
autoimmune diseases, cancers and complex traits.

WP8: Glycosylation of plasma proteins in diabetes – validation of the integrated glycome / genome approach
WP leader: Genos
Results related to deliverable D8.1: Report on glycosylation changes in MODY-3 patients

Objectives

A large number of genetic polymorphisms have been associated with different diseases using GWAS technology in the past few years. GWAS of the plasma glycome identified HNF1 as the major regulator of plasma protein fucosylation. Mutations in HNF1 are known to be causative of MODY3 subtype of diabetes and this knowledge enabled us to screen a subgroup of diabetic patients for a selected specific structural change in glycans and identify aberrant plasma protein fucosylation as the most promising biomarker for MODY3. This finding would not be possible without the prior knowledge of association between fucosylation and HNF1 since fucose is not a significant marker of diabetes in general (due to low incidence of MODY3)

Although changes in antennary fucosylation appear to be promising biomarker for MODY-3, it is unclear whether these significant aberrations in glycans have any functional implications. Glycans are known to be important in regulation of activity of many membrane receptors (including GLUT2 and GLUT4 glucose transporters), but compensation capacity in the glycosylation pathway is very large and many of these aberrations do not have a visible phenotype in a compensated state. To address whether under challenge conditions these aberrations become functionally important we analyzed leukocyte membrane glycome in MODY-3 patients and matching controls in both steady state and under immune challenge.

The finding that antennary fucosylation is a good biomarker for MODY3 is potentially very important because therapeutic approach for these patients is different than for other subtypes of diabetes, therefore early diagnosis is essential for good prognosis. In our research group we analysed a large number of diabetic patients, both selected and unselected which displayed a great potential of antennary fucosylation as biomarker for diabetic patient stratification.

However, despite the large number of different glyco-biomarkers which were discovered in the past none of them have been successfully transferred into clinical use. One of the main problems is the complexity of demanding analytical methods which are difficult to apply in routine clinical laboratories. Development of a simple assay for reliable quantification of complex glycans would be an important step towards the successful implementation of fucosylation analysis in the clinical laboratories. In the scope of the project we developed quantification of antennary fucosylation as an ELISA-like assay.

Aberrant glycosylation in MODY-3 patients.

Given the role of HNF1A in regulating important components of the acute inflammatory response, we investigated the changes in plasma N-glycans and WBC (white blood cell) membrane glycans in HNF1A-MODY during an acute inflammatory response. The study included 24 patients in total, encompassing 9 HNF1A-MODY patients, 7 type 2 diabetes patients and 10 healthy controls. Endotoxin (purified lipopolysaccharide prepared from Escherichia coli, 0:113 - U.S. Standard Reference Endotoxin) was used for inducing an acute inflammatory response. Experimental human endotoxemia is a well-tested model of
inflammation, and has been used in a large number of studies involving over 2,000 subjects. Sampling was performed on day 0 (baseline sample, before the initiation of acute inflammatory response), day 1 (several samples), day 2 and day 8.

Plasma N-glycome and WBC membrane N-glycome in HNF1A-MODY, type 2 diabetes patients and healthy controls before and after acute inflammatory response induction were analysed by HILIC-UPLC. Plasma N-glycome and WBC N-glycome chromatograms were separated into 39 and 44 peaks, respectively. The profile of membrane N-glycans obtained from isolated white blood cells was successfully established and compared to plasma N-glycome profile. Baseline levels of plasma N-glycans for three of the observed glycan peaks were found to be significantly different in HNF1A-MODY compared to controls. The results were consistent with the previous work, showing that patients with HNF1A-MODY have lower baseline levels of antennary fucosylated glycans and higher levels of highly branched tetraantennary glycans. No significant difference in plasma N-glycans over time was observed in subjects with diabetes and healthy controls.

WP9: Biotechnological application of HTP Glycoanalytics

WP leader: Ludger

Results related to deliverable D9.1: Glycoprofiling of Biopharmaceuticals

Objectives

- To analyse the glycosylation of glycoprotein drugs using the methods of WP2-8
- To assess and validate the HTP glycoprofiling methods for process monitoring and product release tests of therapeutic glycoproteins

Ludger has realised the potential from the High Glycan project for the analysis of glycoprotein glycosylation by implementing and optimising work flows for the sample preparation and processing of biopharmaceutical samples using an automated liquid handling robot for fast, reliable and robust N-glycan analysis. Ludger has integrated the multiple, orthogonal glycoanalysis methods developed in WP2 to WP6 into its LongBow glycomics system. LongBow represents the practical realisation of the HighGlycan concept of an integrated suite of automated HTP glycoanalysis modules that connect glycoprotein samples and biopharmaceutical glycoprotein samples with the three main glycoanalytical platforms: UHPLC, MS (including MALDI-MS and LC-MS) and CE.

Ludger have used the automated HTP sample preparation methods set up in WP3 along with the analytical techniques relating to WP4 and WP5, UHPLC and CE analysis of fluorescent labelled glycans, and WP6, MALDI and ESI-MS analysis of derivatised glycans, fluorescent labelled glycans and glycopeptides, to enable Ludger, laboratories, clients and companies around the world to be able to assess the glycosylation of their biopharmaceuticals. These techniques have been investigated using a number of standard glycoproteins and monoclonal antibody glycoproteins that are good models for glycoprotein drugs and a biopharmaceutical glycoprotein. The simplicity, accuracy and repeatability of the above automated analytical techniques has been determined.

One practical application of the Longbow glycomics system is the provision of a commercial monoclonal antibody (mAbs) glycosylation service (GX-mAb) for the HTP glycoanalysis of therapeutic mAbs (http://www.ludger.com/glycoprofiling-services-GX-mAb). Ludger launched a commercial mAbs glycosylation service (GX-mAb) for the developers of mAbs in the US and China incorporating several of
the automated HTP glycoanalytical methods developed in WP3, WP4 and WP6. This includes the glycoanalysis modules LC-MS analysis of procainamide labelled N-glycans, UHPLC analysis of 2AB labelled N-glycans, MALDI analysis of permethylated N-glycans and UHPLC and MALDIMS analysis of VTAG labelled glycopeptides. The service can be used for mAb development and analysis at all stages of the product lifecycle including early stage development, PAT (Process Analytical Technologies) and lot release of monoclonal antibodies produced in mammalian expression systems.

WP10: Dissemination and Exploitation
WP leader: Ludger

The results of WP10 are covered in the next section of the HighGlycan final report addressing potential impact, main dissemination activities, and the exploitation of the results.

Potential Impact:

Science Activities 1: Glycomics to support Biopharmaceutical Realisation

Glycosylation is a major source of problems in the development and production of therapeutic glycoproteins. From monoclonal antibodies (mAbs) and fusion proteins to hormones and erythropoietin (EPO), glycosylation increases structural complexity, and the resulting heterogeneity can significantly impact safety and efficacy. Glycosylation is typically the biggest contributor to batch variations in a biological drug’s clinical performance. There is an increasing need to perform effective ‘glycoshaping’ or glycosylation design / glycoengineering to produce drugs with consistent and optimized structures. Glycoshaping is a complex process involving the optimisation (molecular design, cell line engineering and clone selection), specification, measurement and manufacturing control of the glycosylation patterns of a biologic drug. Current approaches are not robust and the resulting changes in glycosylation between batches and throughout the product’s life cycle can cause significant variations to the clinical safety and efficacy profiles. Poorly executed glycoshaping has caused: (a) financial losses of $1 to $2 billion globally from out-of-specification batch failures (average 7% failure rate) and delays in getting drugs to market, (b) denial of regulatory approval, (c) higher than necessary cost of drug substance and protein load per therapeutic dose, (d) loss of market share to competitors and (e) patient suffering due to adverse reactions and delays in accessing drugs. These problems could be reduced through improved QbD-based drug glycoshaping. The HighGlycan consortium has developed glycoanalytical technologies that is highly useful in glycoshaping and found its way into commercialization via Ludger, Genos and glyXera, three SMEs that are HighGlycan beneficiaries.

GlyXera was founded during the HighGlycan project and joined the consortium via an amendment. The company joined for the development and commercial exploitation on high-throughput glycomic technology and is in the meanwhile a well-established commercial player for the characterization of the glycosylation of biopharmaceuticals as well as human samples via robust high-throughput technology.

HighGlycan: Development of high throughput (HT) glycomics technologies for medicine

HighGlycan addresses a major barrier to advancement of the field of medical glycobiology – i.e. that glycan populations as found on drugs and cells of the human immune system are immensely complex and
very difficult and expensive to analyse. This hampers both modern drug design by QbD (which typically requires thousands of glycoanalyses) and measurement of potential glycosylation pattern biomarkers of disease.

The idea of the HighGlycan project was to connect various types of biological, medical and pharmaceutical samples with a variety of analytical platforms for reliable quantitative measurement of glycosylation profiles. This would be done through an integrated set of validated technology modules. This makes it easier for scientists to tune glycoanalysis methods from expensive, low throughput analyses giving high detail to affordable and streamlined but reliable high throughput methods.

For Ludger, the practical outcomes of HighGlycan include Ludger’s LongBow glycomic engine which is now being used in several programmes to support realisation of glycoprotein therapeutics and development of glycomics based clinical diagnostics (see below).

LongBowTM: Ludger’s High Throughput (HT) glycomics engine

Ludger’s business depends on our ability to provide advanced, reliable and affordable glycoanalysis technologies for healthcare. To maintain our commercial edge we have developed LongBowTM – the glycomics system that will power most of our business growth over the next decade. LongBow has been six years in the making. Much of the innovation was done within the FP7 funded HighGlycan (technology development) and IBD BIOM (application) programmes and future development will be supported from our commercial revenue. LongBow is an integrated set of orthogonal quantitative glycoanalysis workflows that are robust, reliable and automatable. The system is very flexible and can be tuned in terms of analysis detail and sample throughput. Of glycan biomarkers in complex biological samples including blood, saliva, mucous and gut tissue. 2016 marks the transition from the development phase of LongBow to its commercial exploitation.

At Ludger we have focussed on the transition from development phase to commercial exploitation / promotion of LongBow. The technology developed during HighGlycan was a result of the support, feedback and guidance from the members of the Executive Committee, the Industrial Impact Board and other key biopharma representatives who provided relevant insights for developing and optimising advanced technology that is constantly required for drug developers and biomanufacturers. By utilising this knowledge Ludger has coordinated and met the demands of both research and industry partners by developing and promoting LongBow technology.

Science Activities 2: Glycomics to support Precision Diagnostics

IBD-BIOM: Discovery and validation of glycan biomarkers of inflammatory bowel diseases

IBD-BIOM was a 4 year collaborative FP7Health R&D project to discover glycomics (and other omics) biomarkers for inflammatory bowel disease (IBD). The IBDM-BIOM website (http://www.ibdbiom.eu) gives an overview of the scientific aims. Several HighGlycan partners contributed via glycomics analysis relying on technology developed in HighGlycan, contributing to technology dissemination and exploitation. The programme was completed in September 2016, and a range of scientific findings of the IBD-BIOM
The inflammatory bowel diseases (IBD), Crohn’s disease and ulcerative colitis, affect around 2.5 million people across Europe. These diseases are associated with high morbidity, definite mortality and an increasing economic burden. Typical symptoms include uncontrolled diarrhoea with intestinal bleeding. Current diagnostic tools and therapeutic alternatives are unsatisfactory for many patients for IBD are unsatisfactory. With current clinical practice and tools IBD patients often remain undiagnosed for several years. This means they get inappropriate clinical treatment and exacerbating the impact of the disease on morbidity and mortality. Currently, there is no recognised cure for IBD but the disease is managed using anti-inflammatory drugs (including anti-TNF-alpha therapeutics) and surgical removal of inflamed bowel sections.

Recent genome-wide association studies (performed in a large part by IBD-BIOM partners) have identified nearly 100 genes associated with IBD, but clinical application of these discoveries is so far limited to date. IBD-BIOM complements the existing clinical, genetic, biochemical and immunological data through a study of over 6000 patients and matching controls with four novel types of analytical data: epigenetic, glycomic, glycoproteomic and activomic. These were chosen because the glycosylation patterns of plasma proteins and epigenetic and activomic profiles of individuals are known to change with molecular disturbances to the immune system. This has allowed us to find patterns and correlations between the different types of clinical and biomolecular data relevant to IBD. In effect, we can filter out the confusing background noise in the data to uncover the (currently hidden) molecular patterns relating to initiation and progression of inflammatory bowel disease. IBD-Biom partners have already proven the utility of this large-scale combination approach. By combining genomic and glycomic profiles of several hundred patients Prof Lauc’s group discovered a clinically useful glycosylation biomarker for a subgroup of diabetes patients (this is being exploited in the GlycoDx-MODY programme). The IBD-BIOM programme has built on this systems biology approach, extending both the scale, type and complexity of profiles analysed to identify novel early diagnostic and prognostic biomarkers of inflammatory bowel diseases, as well as the new potential targets for therapeutic intervention.

The practical outcome of the IBD-BIOM programme is the development of a method of combining glycomics and other omics biomarkers for stratification of patients with IBD. The early evidence is that we can distinguish Crohn’s Disease, Ulcerative Colitis and Irritable Bowel Syndrome by glycomics analysis of a drop of blood and that combination with other omics data further improves reliability and sensitivity of the clinical diagnostics.

GlySign: Discovery of glycan biomarkers for inflammatory diseases and cancers

This is a four year EU-funded research training network for translational aspects of glycomic clinical biomarkers for Precision Medicine (PM). These are specific, complex changes that occur in the glycomics profiles or Glycan Signatures of serum and tissue glycoproteins during progression of diseases such as cancers and inflammatory conditions. The three members of the GlySign Consortium – LUMC, Ludger and Genos – have furthered knowledge in this field through development and use of advanced glycomics technologies for discovery of novel, clinically important glycan biomarkers. Ludger’s focus will be development of:
• Reliable and affordable workflows for glycomics based clinical diagnostics and
• Methods to incorporate glycomics based diagnostics (GlycanDxTM) into precision medicine diagnostic pathways.
The work will be carried out by six PhD students, two in each partner lab of the consortium, working in collaboration.

GlycoCan: Discovery of glycan biomarkers for colorectal cancer

This is a four year EU funded Marie Curie European Training Network: Exploiting Glycosylation of Colorectal Cancer for the development of improved diagnostics and therapeutics. We are using LongBow methods to explore glycomic biomarkers in colorectal tissue and blood glycoproteins. The aim is to develop a GlycanDx precision diagnostics test to monitor patients with genetic and lifestyle pre-disposition to colorectal cancer.

Application of developed methods for the benefit of European Industry

Our work for this objective has focussed on two areas:
1. Exploitation of HighGlycan technologies by members of the HighGlycan Consortium.
   This includes use of HighGlycan technologies:
   a. to enhance R&D conducted by partner organisations,
   b. solo commercial ventures by the industrial partners and
   c. joint commercial ventures led by industrial partners in collaboration with other Consortium members.
   These are at various levels of progress – from early stage discussions for joint ventures (e.g. discussions between Ludger and Genos) to pre-launch of commercial methods (e.g. for the Ludger VTag glycopeptide labelling kit) and fully launched (e.g. the Ludger VP/T1 system for 2AB and 2AA glycan labelling systems incorporating 2PB reductant technology licensed from LUMC).
2. Use of HighGlycan methods by European biotech companies. These fall into two categories:
   a. Application of HighGlycan technologies in glycoprofiling services for biotech companies (e.g. commercial glycoprofiling services now offered by GlyXera and Ludger) and
   b. Use of kits implementing HighGlycan technologies (this includes Ludger’s Velocity glycan, glycopeptide and sialic acid analysis kits designed for the LongBow glycomics system).

Relevant activities at BIASep: The platform technology of preparing (immuno) affinity chromatographic columns and 96-well plates, which was successfully developed and optimized within WP2, was implemented in its commercialization. BIASep now offers a service of antibody immobilization on the chromatographic monoliths, enabling European and global biotech companies’ usage of HighGlycan methods. The examples of dissemination of immunoaffinity chromatography for glycan analysis are gathered in BIASep Application notes.

The Highglycan project, its technologies and methods were additionally disseminated on different scientific and industrial conferences, such as Vaccipharma (Cube, June 2015) and Affinity (September 2015).

Relevant activities at NIBRT: Project results generated at NIBRT have been disseminated to policy makers, the scientific community, the general public and key stakeholders in drugs and food industry. The
importance of automation and bioinformatics in glycomics, exemplified by High Glycan results, has been showcased directly to policy makers including experts in the glycomics pharmaceutical industry. Members of the HighGlycan consortium have participated in global Waters Workshops to highlight the importance of UPLC and mass spectrometry in glycomics to the scientific community in Taiwan, Tokyo, Singapore, China, Malaysia, CCRC (Athens, Georgia) in this timeframe. Involvement in glycan notation standardization meetings, with ramifications for glycoscientists from both academic and industrial have been undertaken with the results published (Borman, Chemical and Engineering News, 94, 1, 28, 2016). Dissemination to the general public include co-chair at Ordinary Genius symposium 2015, with a goal of the initiative to bring about the discovery of new spiritual information by furthering high-quality scientific research and an article presented to a general engineering audience (High-throughput robotic platform for disease diagnosis, Engineers Journal, 2015, http://www.engineersjournal.ie/2015/04/21/high-glycan-nibrt/).

Evergreening of the HighGlycan initiative

In 2012 a very strong policy paper published by the US National Academies declared Glycoscience as one of their research priorities. Subsequently NIH opened several dedicated calls for the development of technologies for glycoscience. At that point HighGlycan project was already active for several years, which gave us significant head start in this direction. Consequently EU researchers managed to preserve their globally leading position in the field of high-throughput glycomics and glycoproteomics. Technologies developed in HighGlycan enabled glycomics to be included in a number of large clinical and epidemiological studies, including some funded by EC: IBD-BIOM and Pain-Omics within FP7, and GlycoCan and GlySign within H2020. Through these projects HighGlycan initiative is spreading outside of glycoscience and enables researchers from other disciplines to benefit from the results of the HighGlycan project.

Through HighGlycan our SME partners (Ludger, Genos and MPI) are developing into global leaders for contract analysis and contract research in the field of glycoscience. They are also partners in several new FP7 and H2020 projects, but are also launching new commercial products in the field, all of which is contributing to evergreening of the HighGlycan initiative.

Dissemination – Website

The official HighGlycan consortium website is http://www.highglycan.eu/. The website was kept up-to-date with a list of details shown below and the list was not exhaustive to:

- About
- Project progress
- Participants
- Publications
- Contact
- Partner pages

The partners had access to details regarding objectives, collaborative activities, scheduled meetings, list of dissemination activities, links to resources and results for the project work that was performed.
Dissemination - Spoken word

During the programme a vast number of presentations were made by consortium members about the science involved in the HighGlycan programme. These were mainly presentations to peers in scientific meetings but also included some outreach and press interviews. It is anticipated that the number of presentations made will continue to increase significantly as the data published from the other glycomics programmes which is the result of the application and implementation of HighGlycan technology.

Dissemination – Written Word – journals / news articles and posters

Up until November 2016, more than 150 peer reviewed scientific articles were published by consortium members about HighGlycan research. Whereas much of the written dissemination was to the scientific community during this phase of the HighGlycan consortium’s work it is anticipated that the commercial and clinical outcomes of the project will create more dissemination towards industry and clinicians in the glycomics sector.

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