



# Clinical glycomics in the diagnostic laboratory

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## The potential of glycosylation analysis for the diagnostic laboratory

Glycosylation is the most diverse type of protein modifications. Glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus. The presence of multiple competing glycosyltransferases and glycosidases results in a highly heterogeneous population of glycan structures on each protein. Every cell and tissue contain different factors that influence the process of glycosylation, thus further contributing to the rich biological information as hold in a protein's glycan repertoire. Glycans can have a strong influence on important biological processes such as brain development, immunity and growth. Abnormal glycosylation has been found in a broad range of human diseases including rheumatoid arthritis, cancer and liver disease. This offers an interesting potential to use glycans as diagnostic biomarkers with improved specificity. Thus far, a first example of a clinically validated assay that is based on glycosylation profiling in human blood is the Glyco Liver Profile test from Helena Biosciences. This test is used as a non-invasive risk stratification tool for chronic liver disease. Ratios of four total protein released N-glycan biomarkers from plasma provide an index that correlates with liver disease status (1). Mass spectrometry based analysis of total plasma released N-glycans, i.e., glycomics, potentially offers additional specificity, however, further improvement is needed for broad clinical implementation.

## Lessons from monogenetic glycosylation disorders

Monogenetic inherited diseases, congenital disorders of glycosylation (CDG), form a good starting point to study the causes of abnormal glycosylation profiles in blood. CDG is a group of rare genetic diseases consisting of more than 130 gene defects, affecting different glycosylation types. CDG's with abnormal N-glycosylation include ~45 types and are divided into two main types. CDG-I includes CDGs with a defect in the assembly of the oligosaccharide or the transfer of the oligosaccharide chain on the protein in the ER. These defects result in reduced occupancy of glycosylation sites. CDG-II includes defects in the processing of glycan chains already bound to the protein, resulting in altered glycan structures. Some CDGs are directly involved in glycosylation pathways such as MAN1B1-CDG and MGAT2-CDG, while others secondarily affect the glycosylation such as TMEM199-CDG, and SLC10A7-CDG which influence Golgi homeostasis in general. To diagnose CDGs with abnormal glycan structures (CDG-II), mass spectrometric analysis of transferrin has been introduced in a couple of clinical diagnostic laboratories (2-4). For CDG-I, mass spectrometric analysis of transferrin allows very sensitive detection of a defect in site occupancy. One of the main advantages of the use of transferrin as diagnostic marker for genetic diseases is that it is not influenced too much by

non-genetic factors. Glycomics profiling of total plasma proteins has thus far mainly been applied as a discovery tool in CDG research to identify glycan structures that are specific for CDG subtypes and to identify novel disease genes. In the last years, combination of whole exome sequencing and glycomics profiling has resulted in a large increase in the number of novel disease genes (5). Studies on the combined use of transferrin and total plasma protein analysis have even revealed treatment possibilities, as shown for PGM1-CDG. Additionally, these studies indicated that the glycosylation of transferrin and other, as yet undefined, plasma proteins is differentially affected (6). In addition, it is known that transferrin glycosylation is not affected in several CDGs such as MOGS-CDG and SLC35C1-CDG, while plasma glycomics profiling provides information on subtype specific glycan abnormalities. This illustrates the need to translate glycomics profiling into a standardized clinical assay, which is however hampered by the current lack of available internal standards, as commonly used for other mass spectrometry based clinical chemistry methods.

### Use of internal standards

There are several approaches that can be taken to tackle this issue. One approach is the addition of isotope labeling of N-glycans of a monoclonal antibody as internal standard (7). Another approach is the use of isotope labels during glycan derivatization. 2-aminobenzoic acid (2-AA) and 2-aminopyridine (PA) are two examples of labels that have been used as  $^{13}\text{C}$  labelled variants for relative quantitation (8). In a current issue of *Clinical Chemistry*, Chen *et al.* tried to tackle this problem with the use of a  $^{13}\text{C}$ -labeled custom-synthesized glycopeptide. This glycopeptide consisted of a N-glycan with two NeuAc residues labeled with  $^{13}\text{C}$ , linked to the asparagine of the hexa-peptide NH<sub>2</sub>-Lys-Val-Ala-Asn-Lys-Thr-COOH. Semi-quantitative flow injection-electrospray ionization-quadrupole time-of-flight (ESI-QTOF) mass spectrometry was applied to measure N-glycans. N-glycans were released from total plasma proteins with PNGase F. The resulting N-glycans have a transient amine-group that was coupled to a quinolone via an N-hydroxysuccinimide carbamate tag. The derivatized N-glycans were isolated with hydrophilic interaction chromatography (HILIC) and analyzed by mass spectrometry. Using the above mentioned internal standard allowed to identify and quantify low abundant, clinically relevant glycan biomarkers for several CDG subtypes which had normal transferrin glycosylation. Several high

mannose glycan structures were indicated as biomarker for a couple of CDG-I defects that were analyzed. The presence of high mannose glycans was suggested to be the result of their more stable presence in the bloodstream due to slower clearance as compared to transferrin (9). However, this ignores the known presence of a mannose receptor in the liver (10,11). As the presented method does not focus on transferrin alone, it can be applied to other N-glycosylation defects with normal transferrin profiles and is compatible with clinical laboratories due to the use of an internal standard. Since LC-QqQ mass spectrometry with multiple-reaction monitoring (MRM) is commonly used in clinical chemistry laboratories for quantitative analysis of metabolites, translation of the current ESI-QTOF method would be needed to allow a much broader implementation.

### Clinical interpretation of glycomics in the diagnostic laboratory

Even with the use of (internal) standards, a remaining issue for broad implementation is the clinical interpretation of abnormal glycomics profiles. Non-genetic factors, such as a wide variety of disease symptoms, are known to influence glycosylation profiles. Well known examples are the changes in glycosylation due to liver disease symptoms and even simple infections (12,13). These and others are frequently present in CDG patients and thus, abnormalities in glycomics profiles, maybe even the increased presence of high mannose glycans, might be caused by both genetic and non-genetic factors. For CDG diagnostics, glycomics profiling is still performed after prior detection of a CDG-I subtype by transferrin analysis. However, in the case of normal transferrin glycosylation or when using glycomics as a screening tool, this will complicate interpretation. Application of this novel methodology to much larger cohorts of CDG patients and patients with diverse disease symptoms is a prerequisite for straightforward clinical interpretation of glycomics results.

### Protein-specific glycoprofiling—future embedding in the diagnostic laboratory?

The identification of abnormal glycan structures that are not present on transferrin, is strongly suggestive for the presence of such glycan structures on other proteins in blood of CDG patients. This is in line with other studies as indicated above (6) that suggest that the glycosylation of individual proteins responds differently to disease. Therefore, it can

be assumed that the diagnostic specificity of glycan based biomarkers can be further increased by analysis of protein-specific glycosylation. This has the added advantage of obtaining information on site-occupancy otherwise lost in glycomics profiling. For MOGS-CDG, one of the known defects with normal transferrin glycosylation, it has been shown that high mannose glycans accumulate on IgG (14). Assays, in which a couple of different proteins are combined, might be of added value for diagnosis of a broader set of genetic glycosylation disorders. This could be done at the intact protein level, as is currently done for transferrin. Analysis at the glycopeptide level offers the advantage of site-specific glycosylation information. Assays have been reported using MRM (15), which would be ideal for broad integration in clinical chemistry laboratories. Holistic glycoproteomics analysis would even offer further improved specificity for diagnosis and might be able to discriminate glycan structural changes that are caused by the genetic defect and by disease symptoms. However, as this is a relatively novel discipline in glycosylation analysis, we are still a long way from implementing glycoproteomics in the clinical laboratory. Although analysis is already well possible, annotation of the glycopeptides data is highly challenging due to the lack of specialized software. But before we can even think of implementing such techniques in the diagnostic laboratory, broadly available internal glycopeptide standards are essential for quantification. The  $^{13}\text{C}$  labeled internal glycopeptide standard as introduced by Chen *et al.* is a good first stepping stone for translation of research based glycomics and glycoproteomics technologies into clinical laboratories.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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