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## **CDG biochemical screening: where do we stand?**

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## **Abstract**

**Background:** Glycosylation is one of the most complex post-translational modifications of proteins and lipids, notably requiring many glycosyltransferases, glycosidases and sugar transporters encoded by about 1-2 % of all human genes. Deleterious variants in any of them may result in improper protein or lipid glycosylation, thus yielding the so-called ‘congenital disorders of glycosylation’ or CDG.

**Scope of review:** We first review the current state of knowledge on the common blood and cellular glycoproteins used in the biochemical screening of CDG, as well as the emerging ones for an improved diagnosis. We then provide an overview of the current state-of-the-art methodologies ranging from gel electrophoresis to mass spectrometry to measure improper glycosylation. Finally, we discuss how additional tools such as metabolomics and microfluidics can be added to the current toolbox to better diagnose and delineate CDG.

**Major Conclusions:** Combining several biochemical indicators and related methods is often required to cope with the large clinical heterogeneity of CDG and establish a definitive diagnosis.

**General significance:** This review aims to critically present current available CDG biochemical biomarkers and dedicated methods in the context of highly diverse glycosylation pathways and related inherited diseases.

**Abbreviations:**

A1AT:	$\alpha$ 1-antitrypsin
AGP:	$\alpha$ 1-acid glycoprotein
ALP:	alkaline phosphatase
ApoC-III:	apolipoprotein C-III
B3GALT6:	beta-1,3-galactosyltransferase 6
B3GAT3:	beta-1,3-glucuronyltransferase 3
B4GALT7:	beta-1,4-galactosyltransferase 7
Bkn:	bikunin
CDG:	congenital disorder(s) of glycosylation
CHSY1:	chondroitin sulfate synthase 1
COG:	conserved oligomeric Golgi complex
Cp:	ceruloplasmin
CS:	chondroitin sulfate
CZE:	capillary zone electrophoresis
EndoH:	endo- $\beta$ -N-acetylglucosaminidase H
ER:	endoplasmic reticulum
ESI:	electrospray ionization
FACS:	flow cytometry
FLAER:	fluorescent aerolysin
Fuc:	fucose
GA:	Golgi apparatus
GAG:	glycosaminoglycan
Gal:	galactose
GALNT2:	N-acetyl-galactosamine-transferase 2
Glc:	glucose
GlcA:	glucuronic acid
GlcN:	glucosamine
GalNAc:	N-acetylgalactosamine
GlcNAc:	N-acetylglucosamine
GPI:	glycosylphosphatidyl inositol
HC:	heavy chains
Hex:	hexose
HPLC:	high-pressure liquid chromatography
Hpt:	haptoglobin
ICAM-1:	intercellular cell adhesion molecule-1
IF:	immunofluorescence
Ig:	immunoglobulin
IGDs	inherited GPI biosynthesis deficiencies
IMERs:	immobilized enzyme reactors
LAMP2	lysosomal associated membrane protein 2
LOQ:	limit of quantification
MALDI:	matrix assisted laser desorption/ionization
Man:	mannose
MOGS:	mannosyl-oligosaccharide glucosidase
MPI:	mannose phosphate isomerase
MS:	mass spectrometry
NGS:	next generation sequencing
PIG:	phosphatidylinositol-linked glycan
PG:	proteoglycan
PMM2:	phosphomannomutase 2
PNGase F:	peptide-N-glycosidase F
TBG:	thyroxin-binding globulin
TIEF:	transferrin isoelectric focusing
Trf:	transferrin

WES: whole exome sequencing  
Xyl: xylose

## **Introduction**

Approximately half of the human proteins are glycosylated (1). Protein glycosylation influences protein structure, stability and function and notably plays major roles in cellular interactions, immune response and cancer. This abundant post-translational modification mainly includes N-glycosylation where an oligosaccharide chain is linked to an asparagine (Asn) residue within an N-X-S/T motif (X being any amino acid except proline), and the O-glycosylation involving a glycosidic linkage to a serine (Ser) or a threonine (Thr) residue. N-glycosylation concerns most of the circulating and membrane glycoproteins (2). N-glycans share a common chitobiose core ( $\text{Man}_3\text{GlcNAc}_2$ ) to which a variety of monosaccharides and other glycan modifications can be added to the non-reducing termini with particular linkages. Concerning the highly heterogeneous O-glycosylation, three main types of O-glycoproteins have been particularly studied, namely the mucin family (O-GalNAc linkage), the proteoglycans (PGs) carrying distinct sulfated glycosaminoglycan (GAG) chains (O-Xyl linkage) and the alpha-dystroglycan ( $\alpha$ DG) protein bearing specific O-mannosylated structures (3). Moreover, other types of glycosylation occurring in GPI-anchored protein synthesis, O-galactosylation (collagens) and O-GlcNAcylation are also relevant in human pathophysiology.

Congenital disorders of glycosylation (CDG) constitute an ever-growing group of genetic diseases affecting the glycosylation of proteins and lipids. With few exceptions, these are multisystem disorders. Since the first report by Jaeken and col. in 1984 (4), more than 150 distinct CDG have been described, in agreement with the diversity and complexity of glycosylation (5). It is important to note that in a quite restricted way, the first denomination of the so-called 'CDG' (for 'carbohydrate deficient glycoprotein') only referred to N-glycosylation defects. On this basis, type I CDG (CDG-I) and type II CDG (CDG-II) were

defined according to the impact of the underlying variants based on the N-glycosylation pattern of serum transferrin (Trf).

In CDG-I, gene variants (~ 40 identified to date) partially affect the N-glycan biosynthesis before or during the 'en-bloc' transfer of the  $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$  structure from the dolichol phosphate lipid anchor to an Asn residue of a nascent protein. Such defects lead to partial occupancy of Trf N-glycosylation sites characterized by typical Trf isoelectric focusing (TIEF) type 1 pattern with the normal fully glycosylated glycoform co-existing with underglycosylated ones missing entire N-glycan chains. Noteworthy, similar Trf N-glycosylation defects are present in untreated galactosemia and hereditary fructose intolerance (secondary CDG). In CDG-II, the defects concern the N-glycans biosynthesis steps following the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  transfer. Thus, CDG-II-causing variants (several tens identified to date) directly or indirectly affect the trimming and/or processing of protein-linked oligosaccharides leading to a type 2 TIEF pattern. Besides these N-glycosylation defects are also O-glycosylation defects (in mucin-type O-glycosylation, GAG biosynthesis and O-mannosylation), GPI anchor synthesis defects and lipid glycosylation defects.

As rare but potentially highly severe inherited diseases, CDG need to be rapidly detected in order to limit diagnostic wavering and to introduce as fast as possible curative treatment (when available) and/or symptomatic support therapies (6)(7)(8). Furthermore, a rapid identification of an index CDG patient should enable a prenatal diagnosis for future at-risk pregnancies in the related family.

The biochemical biomarkers are at the crossroad in the CDG diagnosis. Indeed, in the 'classical pathway' i.e. 'from the clinics to the genetic diagnosis', the first-line CDG screening is usually performed by interrogating available blood and/or cellular CDG biomarkers in suggestive patients. Moreover, with the advent of powerful next generation sequencing (NGS) genetic tools such as whole exome sequencing (WES), the always-



increasing ‘inverse pathway’, i.e. ‘from the genetic screening to the firm definitive diagnosis validation’, the biochemical CDG biomarkers are frequently necessary to assert the causality of suspected variants.

An arsenal of techniques is often necessary to cope with the huge clinical heterogeneity of CDG and end with a definitive diagnosis. Based on our CDG screening field experience, this review summarizes the current state-of-the-art about the available CDG biochemical biomarkers and the commonly and emerging methods used for CDG diagnosis. First, the relevant glycan structures of most of them are presented and envisaged in the CDG context. Then, the current screening methods are critically described. Lastly, outlooks for new CDG biomarkers and for promising dedicated microfluidics devices are discussed.

## **1. Biomarkers of N-glycosylation defects**

### **1.1. Transferrin**

Serum transferrin (Trf) has been, is, and will probably remain for a long time, the first-line biomarker for the routine screening of CDG with an N-glycosylation defect (4)(9). Trf is an abundant 79 kDa serum/plasma glycoprotein of liver origin extensively studied as the main iron transporter, a negative inflammation protein (whose blood level decreases under inflammation) and a biomarker for chronic alcoholism (10)(11). In terms of glycosylation, Trf carries two Asn-linked bi-antennary N-glycan chains. In physiological conditions, Trf N-glycosylation macroheterogeneity is limited, with full occupancy of both N-glycosylation sites at Asn<sup>413</sup> and Asn<sup>611</sup> (2). Concerning N-glycosylation microheterogeneity, ~80 % of the two Trf-linked complex-type oligosaccharides are fully mature and terminated by two negatively charged sialic acids (SA), thus leading to the major tetra-sialo Trf (4-sialo Trf) glycoform. While the global poly-antennary glycoforms (5-sialo Trf and 6-sialo Trf) relative amounts could vary between 10 % to 20 % of total Trf, the hyposialylated forms, namely 3-

sialo and 2-sialo Trf, represent minor entities with normal values at ~4 % and ~1 %, respectively (12) (**Fig.1**). For the assessment of chronic alcohol abuse, the primary target analyte for the calculation of the so-called ‘Carbohydrate Deficient Trf’ percentage value (CDT %) has been internationally defined as the 2-sialo Trf (13). In CDG-I, the partial lack of entire N-glycan normal chains generates a relative decrease of the 4-sialo Trf and the marked relative increases of the 2-sialo and asialo Trf glycoforms (**Fig.1**). Furthermore, these CDG-I related variations are accompanied by molecular weight (MW) differences of ~2.2 kDa (one missing N-glycan chain) and ~4.4 kDa (2 missing N-glycan chains) compared with fully glycosylated Trf glycoform. In CDG-II, deficient N-glycans trimming/processing often leads to more or less combined increases of the 3-sialo to asialo Trf glycoforms with generally discrete MW differences of a few hundred Daltons (9)(14). Besides terminal SA-related heterogeneity, Trf-linked oligosaccharides can be physiologically more or less fucosylated (15). Lastly, Trf protein genetic polymorphisms are not uncommon (~2-3 %) and can generate confusing charge variations. Classically, the occurrence of Trf polymorphisms can be corroborated after the removal of negatively charged terminal SA using neuraminidase treatment (16).

## **1.2. $\alpha$ 1-antitrypsin**

Alpha-1-antitrypsin (A1AT) is an acute-phase glycoprotein, essentially of liver origin, and normally N-glycosylated at both Asn<sup>46</sup>, Asn<sup>83</sup> and Asn<sup>247</sup> (17). Although individual N-glycan macroheterogeneity (glycosylation site occupancy) and microheterogeneity (changes in the N-glycan structural repertoire) levels are similar to those of Trf, the presence of 3 glycosylation sites can generate more (up to 8) differentially charged normal A1AT glycoforms (18). Glycosylation pattern of A1AT can currently be proposed as an alternative CDG biomarker, notably during the first weeks of life (up to the third month) where Trf-linked N-glycans chains are sometimes incomplete (19) (20). In addition, in CDG-I patients, it has been shown

that glycosylation sites at Asn<sup>83</sup> and Asn<sup>247</sup> were preferentially unoccupied while Asn<sup>46</sup> was never deglycosylated (**Fig.S1**) (18). Thus, as for Trf, detected MW differences in CDG-I account for ~2.2 kDa (one missing N-glycan chain) and ~4.4 kDa (2 missing N-glycan chains), compared to fully glycosylated A1AT.

### **1.3. Haptoglobin and $\alpha$ 1-acid glycoprotein**

The  $\beta$ -chain of haptoglobin (Hpt) and  $\alpha$ 1-acid glycoprotein (AGP) can be used as routine CDG protein biomarkers (21)(22). However, since Hpt and AGP have 4 and 5 N-glycosylation sites, respectively; their glycosylation/sialylation states are complex to decipher and cannot be easily examined using charge-based separation techniques. Furthermore, the existence of additional unglycosylated  $\alpha$ -chains for Hpt and highly acidic isoelectric points (pI) for AGP glycoforms, significantly complicate the implementation of such techniques. Thus, Hpt and AGP can be mainly used for the screening of CDG-I, where the losses of entire N-glycan chains can be efficiently detected using MW-based protein separation methods.

### **1.4. Other N-glycoproteins including the coagulation factors**

Other serum N-glycoproteins have been used or considered for CDG screening, including thyroxin-binding globulin (TBG), anti-thrombin (AT) and ceruloplasmin (Cp), with no evident added value as first-line biomarkers when compared to Trf and A1AT (23)(24)(25). Nevertheless, in case of uninterpretable (low Trf level) or ambiguous Trf pattern (e.g. protein polymorphism), or during the first weeks of life, they may have some interests similarly to A1AT, Hpt and AGP. The targeted study of serum IgG glycosylation has also attracted interest for CDG detection (26). Indeed, being of non-hepatic origin (contrary to the glycoproteins mentioned above), IgG concentration level and glycosylation are less affected by liver and/or inflammatory diseases frequently encountered in CDG. For example, IgG glycosylation is altered in MOGS-CDG (mannosyl-oligosaccharide glucosidase deficiency)

despite normal Trf glycosylation pattern (27). However, the very high complexity and variability of IgG glycosylation hitherto hampered the development of robust and reliable routine screening tests. Lastly, coagulation factors activities, notably those of AT and factor XI, are frequently markedly decreased in CDG (28). Moreover, such deficiencies can sometimes be the only biochemical sign of the disease (29)(30). Hence, we strongly think that more or less isolated and unexplained decreased procoagulant or anticoagulant factors should suggest a CDG.

### **1.5. Total serum N-glycans**

The mass spectrometry (MS) analysis of whole serum/plasma N-glycans enzymatically released from N-glycoproteins (**Fig.S2**) is currently mainly used for second-line characterization of N-glycan defects associated to a CDG-II Trf profile (31). However, protein N-glycan profiling has been recently proposed as a sensitive, robust and semi-quantitative method for the screening of CDG characterized by unusual N-glycan signatures (32). The removal of protein-linked N-glycans is classically achieved by peptide-N-glycosidase F (PNGase F), which cleaves between the first GlcNAc of complex-type oligosaccharides and the Asn residue from N-glycoproteins. Furthermore, Endo- $\beta$ -N-acetylglucosaminidase H (EndoH), which cleaves between the two GlcNAc of various high-mannose and hybrid-type N-linked oligosaccharides, has been successfully used for the detection/diagnosis of CDG affecting the early steps of N-glycan processing in the Golgi apparatus (GA). For example, in MAN1B1-CDG, serum N-glycomics (following EndoH cleavage) revealed the accumulation of hybrid-type N-glycans that are not usually present in control subjects (33). Interestingly, a recent study by Ashikov *et al.* (34) demonstrated that plasma N-glycomics in combination with hierarchical clustering could efficiently stratify patients when applied to a cohort of 99 individuals with abnormal Golgi glycosylation (47 of which being unsolved). This approach proved relevant by segregating a subgroup of four

patients characterized by a shared specific glycomic profile, characterized by decreased sialylation, an increase of high-mannose glycans and in glycans lacking GlcNAc, reflecting unexpected variants in the *SLC10A7* gene as further confirmed by gene sequencing.

## **1.6. Other cellular CDG biomarkers (N-glycosylation)**

Besides circulating CDG biomarkers, there is a need for corresponding molecules at the cellular level. In peculiar cases (post-mortem diagnosis...), such cellular biomarkers may be useful for initial CDG screening. More importantly, they can be used to test candidate defective gene complementation and/or potential treatments in N-glycosylation deficient cells. Intercellular cell adhesion molecule-1 (ICAM-1) has been identified as an interesting cellular CDG-I biomarker based on (i), its significant decrease observed in 31 CDG-I fibroblasts and (ii), on its clear recovery observed after PMM2-CDG cells gene complementation and mannose treatment of MPI-CDG cells (35). In the same way for CDG-II, the lysosomal associated membrane protein 2 (LAMP2) glycosylation pattern was shown to be dramatically altered in TMEM165-CDG cells, with major improvement under galactose treatment (36).

## **2. Biomarkers of O-glycosylation defects**

### **2.1. Apolipoprotein C-III: a blood biomarker of mucin core1 O-glycosylation defects**

Apolipoprotein C-III (apoC-III) is a relatively abundant mucin core1 O-glycosylated serum protein (~0.1-0.5 mg/mL), synthesized in the liver and intestine, and importantly implied in lipid metabolism (37). The major normal apoC-III glycoforms carry at Thr<sup>74</sup> one (Gal-GalNAc) disaccharide (i.e. core1) substituted by one SA (apoC-III<sub>1</sub>; ~60 % of total apoC-III) or two SA (apoC-III<sub>2</sub>; ~40 %). Besides these two sialylated glycoforms, the minor asialylated one (apoC-III<sub>0</sub>) circulates at percentage below ~10 % of the total glycoform pool (**Fig.2A**). In addition, very small amounts of fucosylated and asialylated mucin core2 apoC-III glycoforms have been described (38)(39)(40). For CDG screening, the major interest of apoC-III is that it

is O-glycosylated in the GA. Thus, marked alterations in the apoC-III<sub>0/1/2</sub> relative abundances can reflect a GA overall defect. When investigated alone, the specificity of apoC-III towards CDG screening is poor since acquired conditions such as obesity and liver diseases are frequently accompanied by altered patterns (41). In contrast, if considered in the second-line after a CDG-II Trf pattern, an associated abnormal apoC-III glycoform profile can orientate towards gene variants affecting the GA homeostasis. Illustrative examples are relative increases of apoC-III<sub>0</sub> ('apoC-III<sub>0</sub>' patterns) found in COG-CDG (GA trafficking defects) or the 'apoC-III<sub>1</sub>' patterns found in ATP6VOA2-CDG or CCDC115-CDG (GA acidification defects) (42)(43). Noticeably, GALNT2-CDG (N-acetyl-galactosamine-transferase 2 deficiency) is to date the only CDG specifically affecting mucin-type O-glycosylation with markedly abnormal apoC-III<sub>0</sub> profiles. (44).

## **2.2. Bikunin: a promising blood biomarker of defects in O-xylose GAG**

Bikunin (Bkn) is a serum proteoglycan (PG) synthesized in the liver. The protein core bears at Ser<sup>10</sup> a chondroitin sulfate (CS) chain consisting in the GAG common tetrasaccharide linker, i.e. (GlcA-Gal-Gal-Xyl-O) further elongated by 15+/-3 (GlcA-GalNAc) sulfated disaccharide motifs (**Fig.2B**). Additionally, one or two glycoprotein(s) named 'heavy chain(s)' can bind the CS chain, thus generating serum inter- $\alpha$ -trypsin inhibitor (ITI) and pro- $\alpha$ -trypsin inhibitor (PaI), respectively (45). It has been shown that abnormal serum Bkn light forms can be detected in patients with linkeropathies, i.e. rare osteoarticular genetic diseases due to biosynthesis defects in the initial tetrasaccharide linker (46). More precisely, samples from patients with mutated *B4GALT7*, *B3GALT6*, *B3GAT3* and *CHSY1*, showed the presence of abnormal Bkn isoforms (**Fig.2B**). Noteworthy, one *XYLT1*-mutated patient's sample did not show abnormality since the corresponding enzyme, i.e. xylosyltransferase-1, is not expressed in the liver and replaced by xylosyltransferase-2 (47).

### **2.3. Alpha-dystroglycan: a cellular biomarker of O-mannosylation defects**

Alpha-dystroglycan ( $\alpha$ DG) is a cell surface glycoprotein belonging to the dystroglycan complex. In muscles, the dystroglycan complex interacts with the membranous sarcoglycan complex and bridges extracellular laminin to the actin-associated subsarcolemmal dystrophin (48). A part of  $\alpha$ DG carries a unique recently elucidated O-glycosylated structure consisting in [(Ribitol-phosphate)<sub>2</sub>-GalNAc(phosphate)-GlcNAc-Man-O-] followed by repeated (GlcA-Xyl) disaccharide motifs (**Fig.2C**) (49). Variants in genes encoding for enzymes directly or indirectly (GDP-mannose biosynthesis) implied in the building of this O-mannosylated structure cause abnormal  $\alpha$ DG glycosylation leading to congenital muscular dystrophies termed alpha-dystroglycanopathies (alpha-DGpathies) (50). Alpha-dystroglycan, the sole identified glycoprotein bearing O-linked mannosylation, is a cellular biomarker of alpha-DGpathies when investigated in muscle biopsies using appropriate antibodies (51). Moreover, markedly decreased enzymatic measurements in lymphoblasts from patients with alpha-DGpathies have been reported (52). Lastly, since some alpha-DGpathies-related genes code for enzymes involved in the GDP-mannose metabolism (e.g., DPM1, DPM2, DPM3, DOLK...), corresponding variants can be associated to CDG-I serum Trf profiles (53)(54).

### **3. Biomarkers of GPI anchor defects**

Glycophosphatidyl inositol (GPI) anchors are glycolipids consisting of phosphatidyl-inositol (PI) linked to (Man-Man-Man-GlcN-) (where GlcN is glucosamine) elongated by ethanolamine phosphate (**Fig.2D**). Once synthesized at the ER membrane, this structure is linked to the C-termini of newly synthesized proteins, further remodeled in ER and GA, and finally attached to the plasma membrane, forming GPI-anchored proteins (GPI-APs) (55). In humans, are concerned more than 150 GPI-APs, which play important roles in embryogenesis, development and cellular interactions. With the rise of WES, numerous

deleterious variants in genes involved in the biosynthesis, protein attachment and remodeling of the GPI anchor are increasingly identified and can be grouped in a new CDG subclass with a large variety of symptoms (56). As an example among 22 identified inherited GPI biosynthesis deficiencies (IGDs), hypomorphic variants in *PIGM*, coding for one component of the enzyme complex responsible for the first Man linkage to the GlcN, were associated to severe vein thrombosis and epilepsy in 3 patients from two unrelated families (57).

Current and relatively accessible biomarkers for IGDs include GPI itself and distinct GPI-APs variably expressed at the surface of patient's blood cells/fibroblasts and of genetically modified cell lines. Indeed, protein-linked GPI can be stained by the fluorescent inactivated toxin aerolysin (FLAER) whereas the classically explored GPI-APs mainly comprise CD16, CD24, CD55 (or DAF for decay accelerating factor) and CD59 differentially expressed and inconstantly modulated on granulocytes, erythrocytes, fibroblasts or lymphoblastoid cell lines. In addition, a recently described (58) monoclonal antibody (mAb), generated from *Toxoplasma gondii* and termed 'T5-4E10 mAb', has been shown to specifically recognize free GPIs at the cell surface with promising potentialities for the detection of IGDs affecting the protein linkage to the GPI anchor (59). In view of the great diversity of explored GPI-APs and related modulations, we propose in the **Supplementary Table 1** an overview of the majority of reported variations in the 22 IGDs described to date.

It must be also underlined that alkaline phosphatase (ALP), whose biosynthesis involves a GPI anchoring step, is persistently increased in the serum (hyperphosphatasia) of a number of IGDs. More precisely (**Supp. Table 1**), described deficiencies in *PIGB*, *PIGO*, *PIGV*, *PGAP2* and *PGAP3* genes are clearly accompanied by hyperphosphatasia whereas *PIGC*, *PIGG*, *PIGH*, *PIGK*, *PIGM*, *PIGS*, *PIGU*, *PGAP1* and *GPAA1* deficiencies share normal serum ALP. For *PIGA*, *PIGL*, *PIGN*, *PIGP*, *PIGQ*, *PIGW*, *PIGY* and *PGAP1* defects, data about



serum ALP are less clear needing additional patients' recruitment . Lastly, all the described PIGT-CDG individuals strikingly had decreased or normal serum ALP levels (56)(60).

#### **4. Orphan CDG lacking reliable biomarkers and false-negative CDG**

In agreement with the complexity of glycosylation and notably its regulation paths, an important number of CDG currently lack an appropriate blood biomarker. In particular (see ref (14) for a more complete list), the described deficiencies in the GlcNAc/SA pathway (e.g. PGM3-CDG, NANS-CDG or GNE-myopathy) were unexpectedly not associated with evident hyposialylation of serum/plasma Trf and/or apoC-III (61). The absence of blood/muscular biomarkers for GNE myopathy was considered to have contributed, among other causes, to the recent failure of a phase 3 clinical trial with SA (62). In addition, false negative screening tests have been reported in various CDG including PMM2-CDG (notably in adult patients), ATP6V0A2-CDG and SLC35A2-CDG (63)(64). The more commonly evoked explanation for these discrepancies points to the liver-restricted origin of the common CDG biomarkers. Hence, the existence (in case of non-null variants) of a sufficient hepatic residual protein activity could have permitted the normal sialylation of Trf/apoC-III, at least under basal synthetic requirements. Lastly, these cases must encourage (i) repeating the CDG screening tests when confronted to unexpected negative results and (ii), continuing research efforts dedicated to new CDG biomarker discovery.

#### **5. Current CDG screening methods and glycosylation profiles**

##### **5.1. Isoelectric focusing**

First introduced in 1984 (4), Trf isoelectric focusing (TIEF) is considered as the gold-standard method for CDG screening. IEF allows the SA related charge-based separation of various protein glycoforms in a pH gradient generated through either immobilines or prefocused

ampholytes in an agarose or polyacrylamide gel. For Trf, iron-saturated glycoforms are then classically detected after in-gel immunofixation and Coomassie-blue staining (65). Although time-consuming (~48h), labor-intensive, poorly reproducible and quite poorly quantitative, TIEF is a very resolutive technique for the accurate separation of Trf glycoforms. TIEF requires low sample volume (~10  $\mu$ L) but is not compatible with EDTA as anticoagulant because it chelates iron. It can also be implemented, depending on storage conditions, to total blood on Guthrie card (66). As illustrated in **Fig.3**, TIEF allows separating 6-sialo to 2-sialo Trf in controls with a major band for 4-sialo Trf. In CDG, it shows typical patterns with increased 2-sialo and 0-sialo Trf in most CDG-I samples and of 3-sialo to 0-sialo Trf in most CDG-II samples. In case of ambiguous TIEF profiles suggesting a CDG, the existence of a potential protein variant needs to be ruled out after neuraminidase treatment (67). Indeed, once the terminal SA enzymatically removed, the presence of only one 0-sialo Trf band demonstrates that Trf isoform charge heterogeneity is only related to glycosylation, thus excluding a protein variant. By contrast, the presence of two 0-sialo Trf bands is strongly suggestive of a Trf genetic polymorphism (**Fig.3**). TIEF of the samples of patient's parents (if available) can also permit to exclude a Trf variant. Sometimes, a CDG coincides with a Trf protein variant.

Regarding apoC-III, IEF-separated glycoforms are transferred by diffusion onto a nitrocellulose sheet followed by a classical antibody-based detection (38). Besides being poorly quantitative, this technique does not permit to distinguish the unglycosylated apoC-III form, elevated in COG-CDG, between the various asialylated glycoforms. Lastly, IEF can also notably be applied to AAT (68) and other CDG serum biomarkers such as TBG and AT (24).

## **5.2. HPLC**

High-performance liquid chromatography (HPLC) with anion-exchange column and detection at 470 nm allows the efficient separation of 6-sialo to 0-sialo iron-saturated Trf glycoforms according to differences in SA-related charge and glycan structure. A typical HPLC analysis is usually performed in around 20 minutes and semi-automation allows the analysis of long series. Required sample volumes are quite low (~100 µL) and most of the anticoagulants, including EDTA, are compatible. The limit of quantification (LOQ) is ~0.1 % allowing, in contrast to TIEF, a quantitative and objective interpretation of Trf patterns. Furthermore, measured within-day and between-day % CVs are satisfactory (69). In view of its analytical performances, HPLC is now positioned as the reference procedure for the alcohol-related CDT % measurement (13) and has been extensively used for CDG screening.

Helander et al (70) first reported in 2008 the HPLC Trf analysis of 9 CDG-I and 4 CDG-II cases. In all tested CDG-I patients, typical increased relative amounts of 2-sialo and 0-sialo Trf were observed compared to controls (n=174). Furthermore, mean % values of 2-sialo and 0-sialo Trf in CDG-I patients were clearly higher than those of heavy alcohol drinkers (n=74). In CDG-II, relative increases of 3-sialo to 0-sialo Trf were observed compared to controls whereas mean % values of 3-sialo, 1-sialo and 0-sialo Trf (but not 2-sialo) were higher than those of alcohol drinkers. In addition, HPLC and TIEF showed a good correlation for CDG screening although HPLC was suggested to be less resolutive notably for the detection of some Trf protein variants (71)(72). Concerning this latter point, it should be mentioned that the HPLC method can be carried out after neuraminidase treatment to check for a Trf genetic polymorphism.

### **5.3. Capillary zone electrophoresis**

Capillary zone electrophoresis (CZE) coupled to UV detection at 200 nm is an automated, fast and accurate separation/quantification technique for the iron-saturated Trf glycoforms. As for HPLC, Trf glycoform separation is based on the glycan sialylation level and structure

variability. CZE has short analysis times (up to 10 min. per sample), very satisfactory analytical performances and good robustness. Thus, for CDT % measurement in chronic alcohol abuse, CZE is strongly correlated to the reference HPLC procedure (73), although higher 2-sialo Trf % values were often reported for the latter one (72). In the field of CDG, the main available CZE methods (Capillarys<sup>®</sup> CDT kit, Sebia and CDT reagent kit CEofix<sup>®</sup>, Analis) allow the efficient separation of the 5-sialo to 0-sialo Trf glycoforms, with a LOQ ~0.5 % and an excellent correlation with TIEF and HPLC for the screening of CDG-I, CDG-II and mixed CDG-I+II (74)(75)(76) (**Fig.4**). Furthermore, CZE is compatible with neuraminidase treatment in case of Trf polymorphism suspicion and can be implemented for the analysis of small sample volumes (5 µL in nanoVials; kit CEofix) and to serum extracted from Guthrie cards. To our knowledge, EDTA anticoagulant is not CZE-compatible.

For the separation of apoC-III glycoforms and detection of mucin core1 O-glycosylation defects, a CZE method has been recently developed showing promising results on distinct batches of purified human plasma-derived apoC-III (77). However, apoC-III glycoform CZE analysis from crude samples still needs optimization.

#### **5.4. One-dimensional western blot analysis**

Classical western blotting after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) allows MW-based separation and sensitive detection of the most usual blood CDG biomarkers. Western blot can be relatively standardized using ready-to-use precast gels, well-defined transfer and antibody-based detection procedures. It allows the analysis of up to 30 samples in one run and is fully compatible with all anticoagulants as well as with whole blood recovered from Guthrie cards (with less stringent storage conditions than TIEF). In healthy patients, Trf and A1AT are separated in the form of one protein band while Hpt β-chain shows two bands. For heavily N-glycosylated AGP, a large and diffuse band is observed (**Fig.5A**). In CDG-I, the partial lack of entire ~2.2 kDa N-glycans chains typically

leads to the appearance of lower additional bands together with persisting normal one(s) (21). Thus, for Trf, two additional bands are usually detected in CDG-I, corresponding to the loss of one or two entire N-glycan chains in significant amounts when compared to the native protein (**Fig.5A**). In the majority of CDG-II, western blot patterns are usually normal since the associated mono-/poly-saccharide(s) losses do not generate clearly detectable MW differences compared to controls. However, important exceptions notably concern MGAT2-CDG (formerly CDG-IIa) and B4GALT1-CDG (CDG-IIId) where the enzyme deficiencies are quasi-complete, generating at least one lower band and the disappearance of the normal one(s) (**Fig.S3**) (31). Lastly, one-dimensional western blot analysis of Trf is interestingly not subjected to genetic polymorphism since related amino acids changes generally do not significantly affect the Trf MW.

Although already applied to apoC-III glycoforms (78), western blot does not permit their efficient separation for CDG-II characterization. For the bikunin PG, western blot applied to control serum/plasma allows to distinguish the core protein at ~25 kDa and the isoform carrying the CS chain between 30 kDa and 37 kDa. In samples from patients with linkeropathies, abnormal forms (~27-29 kDa) are clearly detectable reflecting an enzymatic blockade in the earlier steps of the GAG chain biosynthesis (**Fig.5B**) (46).

Cellular biomarkers, ICAM-1, LAMP2 and alpha-DG can be investigated using western blot procedures. In fibroblasts of CDG-I patients, ICAM-1 is absent or considerably reduced; when hypoglycosylated, it fails to localize at the plasma membrane (35). In TMEM165 mutated cells, western blot of the heavily glycosylated protein LAMP2 shows a major MW decrease secondary to hypoglycosylation (79). Concerning  $\alpha$ DG, western blot is usually applied to protein extracts from muscles biopsies and detection is achieved by using glycosylation-specific VIA4\_1 and/or I1H6C antibodies (80). In  $\alpha$ DGpathies, since O-

mannosylation is affected,  $\alpha$ DG western blot staining is classically absent or severely reduced compared to control (81).

### **5.5. Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis (2-DE) is a gel-based protein separation method combining IEF and SDS-PAGE, in this order. It has been extensively applied to the CDG study (82) and, although frequently termed as ‘old fashioned’ (83), it keeps interests for CDG screening. When coupled to western blotting, it can be used for the simultaneous detection of various blood CDG biomarkers including Trf, A1AT, Hpt (**Fig.6A**) and apoC-III glycoforms (**Fig.6B**). Noteworthy, 2-DE enables the unambiguous separation of the Hpt  $\alpha$ -chains and N-glycosylated  $\beta$ -chains, with potentially improved CDG screening performances for the latter ones (22). Regarding the O-glycosylated apoC-III, it notably separates distinct asialylated glycoforms, currently not accessible using the charge-based IEF techniques (39) (**Fig.6B**). As HPLC and classical western blot, 2-DE is compatible with whole blood on Guthrie cards.

### **5.6. Mass spectrometry (MS) techniques**

MS techniques are among the most powerful methods for the sensitive and comprehensive analysis of glycoproteins as well as for the evaluation of glycosylation sites and determination of oligosaccharide structures (84)(85). The contribution of MS (especially high-resolution techniques) to the characterization and diagnosis of CDG has long been recognized for the analysis of both N- and O-glycosylation defects. MS-based analysis of protein glycosylation for CDG screening can be accomplished at different levels (listed in a descending order of use): i) on glycans previously released from proteins (glycomics), ii) at the intact protein level or iii) on glycopeptides following glycoprotein proteolysis (glycoproteomics, **Fig.7**). MS-based serum/plasma N-glycomics and Trf/apoC-III glycosylation assessed at the intact protein level represent the most common approaches in the clinic. For some CDG-specific examples,

the reader is referred to the recent review of Abu Bakar et al (86). MS analysis of released glycans yields direct compositional information, i.e. the number of constituting monosaccharides. Glycan stereochemistry and branching can be deeply investigated using efficient chromatographic separation, tandem MS experiments, or ion mobility spectrometry structures (84)(85). Protein and site-specific glycans can also be simultaneously characterized from glycoproteomics experiments. Matrix-assisted laser desorption/ionization (MALDI) and liquid chromatography coupled to electrospray ionization (ESI) provide versatile, complementary ionization methods that are used for glycomics and glycoproteomics approaches.

#### **5.6.1. N-glycosylation MS analysis**

Since CDG have been initially typed according to observed N-glycosylation defects (87), MS-based methods have been first used in CDG research to analyze released N-glycans from specific isolated serum/plasma proteins such as Trf (88) or A1AT (18), or directly from total serum/plasma proteins (89). Nowadays, the analysis of N-glycans from whole serum/plasma glycoproteins, i.e. N-glycomics, is becoming widespread for CDG diagnosis since it represents a good complement to the more traditional methods (see above). This approach relies essentially on the analysis of N-glycans enzymatically released by the enzyme PNGase F from serum/plasma glycoproteins (see paragraph 1.5). Chemical derivatization of the released N-glycans (e.g. permethylation) is often required for improved detection sensitivity by MS. Although both MALDI-MS and ESI-MS can be used in that context (90), MALDI-MS seems to be the preferred approach for N-glycan profiling and relative quantification for CDG diagnosis (86), essentially due to its speed and ease of operation. Although more suitable for CDG-II diagnosis (91), serum/plasma N-glycomics also proved efficient for some CDG-I such as ALG1-CDG (92). One limitation of total serum/plasma N-glycan profiling

strategies is that they do not provide information about the protein to which the N-glycans were attached.

Many examples demonstrate that total serum/plasma N-glycan profiling thus proves very informative and relevant for CDG diagnosis (31)(32)(86)(93). However, reliable data interpretation requires a good understanding of the various factors that might affect N-glycan profiles. Modifications of the total serum/plasma N-glycan profile reflect alterations in the glycosylation present in one or more of the 24 highly abundant glycoproteins accounting for about half of the total serum/plasma protein concentration. A schematic representation of the relative protein contribution to each specific glycan composition is reported in **Fig.8** (2). Such theoretical reconstructed profile compares well with the experimentally observed MALDI-MS and UHPLC-fluorescence ones. These proteins are essentially acute-phase liver-produced glycoproteins and Igs (94). Therefore, total serum/plasma N-glycan profiling can also be affected by fluctuations in the concentration of some of these particular proteins. Inflammatory conditions that occur in a large panel of pathologies often yield an acute-phase response and thus can lead to significant changes in the concentration of acute-phase proteins. Besides potentially disturbing the production of acute-phase proteins by the liver, chronic inflammation can also alter the N-glycan patterns of these glycoproteins (95). Concentration of Igs can also be modified in case of infections (96). Although the serum/plasma N-glycan profile is rather stable within an individual, population-wide studies show some interindividual variability, for instance with genetic factors affecting the N-glycome, especially regarding glycans linked to Igs (97).

To encompass some of these limitations, ESI-MS-based analysis of protein-specific glycoprofiling of intact immunopurified Trf has been proposed for CDG diagnosis and demonstrated excellent diagnostic capabilities and easy-to-interpret data for both CDG-I and CDG-II screening (15)(98). Due to the low fucosylation level of the Trf N-glycans, this



approach appears not well suited to monitor the fucosylation degree. In this case, serum/plasma N-glycome would be more suitable (87), thus demonstrating the complementarity of both approaches.

Contrary to system-wide studies (99), glycopeptide MS analysis after proteolytic digestion is not widespread in CDG research and diagnosis. Hulsmeier et al first described an LC-ESI-MS/MS method to quantify the N-glycosylation site occupancy of the glycoproteins Trf and A1AT, and found a clear correlation in CDG-I between the degree of N-glycosylation site occupancy and the severity of the disease (100). Later on, the same team devised a simpler and faster approach (in terms of sample preparation by omitting the immunopurification step) for quantifying glycosylation site occupancy of the serum/plasma glycoproteins Trf, Hpt, IgG<sub>2</sub> and IgA<sub>1</sub> (101). Such LC-ESI-MS/MS could be extended to many other glycoproteins as recently proposed by Li et al for the quantitative monitoring of 100 glycosylation sites from 50 serum/plasma glycoproteins (102). Although highly relevant for simultaneously monitoring the glycosylation status of many serum/plasma-derived glycoproteins, the main drawback of such an approach would be the time-consuming and complex data treatment that is for the moment not compatible with clinical applications. Also, of note, the glycosylation status often drastically diminishes the glycopeptide MS signal compared to the unglycosylated peptide. To the best of our knowledge, no application to CDG-II diagnosis has been published so far.

None of these biochemical CDG tests can screen for all CDG, and should not be used independently from each other but rather conjunctly (which is rarely performed) for getting deeper and more precise insight into biochemical mechanisms leading to abnormal protein glycosylation, as exemplified in **Fig.9** (103).

### **5.6.2. O-glycosylation MS analysis**

Contrarily to N-glycans, O-glycans cannot be enzymatically released due to the lack of a broad spectrum O-glycosidase. For profiling O-glycans, a chemical treatment step such as *beta*-elimination is needed. MALDI-TOF-MS analysis of both permethylated O-glycans released by NaOH treatment was successfully applied (in conjunction to N-glycans) to serum samples from CDG-II patients (104). The study of Xia et al also demonstrated that structural analysis and quantitation of combined N- and O-glycan profiles (as assessed by MALDI-TOF-MS and LC-ESI-MS/MS) proved reliable diagnostic tools for CDG (105). Although relevant, those approaches find relatively poor applications in a clinical context, probably due to the complexity of sample preparation. MS-based screening of O-glycosylation disorders is more often performed by analyzing serum/plasma apoC-III glycosylation using MALDI-MS (31)(42)(106) or ESI-MS approaches (107)(108). Krishnan et al reported an LC-ESI-MS/MS to measure apoC-III glycosylation in a site-specific and quantitative manner through the monitoring of tryptic glycopeptides (109). Overall, MALDI-MS appears nevertheless as the method of choice to analyze intact serum/plasma-derived apoC-III glycosylation.

### **5.7. Other CDG screening/confirmation methods**

Flow cytometry (FACS) analysis of FLAER and of many GPI-anchored proteins, including CD16, CD24, CD55 (DAF), and CD59, was applied to various cell types for the functional validation of IGDS (**Supp.Table 1**). With regard to the preanalytic, blood cells should be analyzed within two days. Furthermore, great variability in normal fluorescence intensities in healthy controls has been frequently highlighted (110).

Lastly, immunofluorescence (IF) staining is frequently the preferred method notably for the assessment of muscle  $\alpha$ DG (using VIA4\_1 / I1H6C antibodies) in  $\alpha$ DGpathies (111) as well as ICAM-1 in CDG-I (35).

## **6. CDG screening: where are we going?**

Thus, the clinical heterogeneity and number of CDG pose a serious diagnostic challenge. Therefore, several complementary methodological approaches might be required to better diagnose and delineate CDG. In the next following paragraphs, we discuss the emerging strategies and technical developments that could prove highly relevant in the context of CDG screening.

### **6.1. Metabolomics**

Although poorly described so far, metabolomics approaches can be highly relevant for phenotyping CDG patients, but not for first-line CDG screening using standard methodologies. Metabolomics can highlight specific metabolic changes and biochemical pathways dysregulations in CDG. This might be particularly helpful to get deeper insight into the pathophysiological and biochemical mechanisms underlying CDG as well as potentially pointing innovative targets for further biomarker discovery or drug development. For example, Dimitrov et al showed on patient fibroblasts and using targeted metabolomics that deficiency of the accessory subunit ATP6AP1 of the V-ATPase (ATP6AP1-CDG) specifically leads to dysregulation of amino acid as well as lipid homeostasis (112), which can represent a new source of biomarkers for stratifying or diagnosing patients. Radenkovic et al recently showed by using tracer-based metabolomics, that galactose treatment of PGM1-CDG fibroblasts metabolically modified their sugar metabolism, and the level of galactose-1-phosphate as well as UDP-glucose and UDP-galactose nucleotide sugars. The galactose in UDP-galactose was also shown to be into newly synthesized glycans (113). This study underlines the potential of monosaccharide therapy for CDG.

### **6.2. Microfluidic devices for CDG screening**

While analytical (electrokinetic, chromatographic, immunoassays and MS) methodologies for glycoform and glycan profiling for CDG screening purpose are close to a mature state,

continued efforts have been spent for performance improvement of forefront sample pretreatment techniques. Indeed, target glycoproteins should be well isolated and pre-concentrated from bio-fluids first prior to downstreamed separation and detection with the aforementioned approaches (see section 5). In the case of glycan analysis, their enzymatic release from glycoproteins is a key step but their enrichment is also necessary to increase detection sensitivity. Among all practical sample handling techniques (114)(115), particular attention is paid to the following directions, from the authors' point of view. First, monoliths, beads and gold nanoparticles of different chemistry have been used as supports for glycosidase immobilization for the preparation of micro-reactors to replace the widely-used in-solution deglycosylation (116)(117). These low-volume immobilized enzyme reactors (IMERs) have gained increasing popularity to bring not only reusability but also the possibility to be integrated in a micro-scale system for coupling of in-line sample treatment and glycoprotein/glycan separation. This in turn offers faster digestion with high efficiencies. Second, manual batchwise protocol using magnetic microbeads for glycan release and fluorescent labeling prior to their capillary electrophoresis separations (118) could be translated into a microfluidic droplet format (119). These microfluidic configurations are expected to offer higher throughput (via automation), lower sample consumption and less risk of cross-contamination encountered in batchwise protocols. Last, in-line preconcentration can be integrated in between the aforementioned sample treatment approaches and the separation module for further improvement of detection sensitivity, which is prerequisite for screening of glycoprotein-/glycan- biomarkers at trace amounts in biological matrices (120). Overall, a compact and unified platform integrating all modules (i.e. sample treatment, analyte preconcentration, separation and detection) for facile utilization (for instance operation in hospitals by non-expert users) would be desired and anticipated for CDG screening purpose, especially in a clinical context.

## **7. Conclusions**

Although initially restricted to N-glycosylation defects, the ‘CDG’ denomination now encompasses also O-glycosylation defects, GPI anchor synthesis defects and lipid glycosylation defects. Despite the description of several biomarkers and analytical tools for their measurement, reliable CDG diagnosis is still not an easy task. This is essentially due to the fact that there are many CDG and many CDG phenotypes. In this review essentially based on our personal experience, we summarized the current knowledge on CDG biomarkers, related glycosylation pathways and corresponding analytical tools, used independently or in combination. We also underline some new analytical avenues that can improve CDG delineation and diagnosis. For instance, we strongly believe that metabolomics should be evaluated further as a particularly relevant tool for phenotyping CDG patients.

## Figure Legends

### Figure 1. Glycosylation status of serum transferrin (Trf) in controls and CDG-I

The two Trf-linked poly-antennated complex-type N-glycans account for 6-sialo and 5-sialo Trf (~15-20 % of total Trf in controls). The major Trf glycoform (~80 % in controls), i.e. 4-sialo Trf, bears two bi-antennated and bi-sialylated complex-type N-glycans and is markedly decreased in CDG-I. The 3-sialo Trf (~4 % in controls) classically results from the loss of one terminal SA. The 2-sialo Trf, classically lacking one complete N-glycan, is found at low level (~1 %) in controls and is markedly increased in CDG-I. The 0-sialo Trf, classically lacking two entire N-glycans is undetectable in controls and typically elevated in CDG-I. The 1-sialo Trf is not shown since poorly relevant in controls and CDG-I.

### Figure 2. Biomarkers of various O-glycosylation defects and structure of the GPI anchor

**A)** Apolipoprotein C-III (apoC-III) is a serum core1 (i.e. Gal-GalNAc) O-glycosylated protein bearing either two terminal SA (apoC-III<sub>2</sub>), one SA (apoC-III<sub>1</sub>) or no glycan chain (apoC-III<sub>0</sub>).

**B)** Bikunin (Bkn) is a serum proteoglycan bearing a chondroitin sulfate (CS) chain consisting in the common GAG tetrasaccharide linker elongated by CS-specific repeated disaccharide motifs. The CS chain can be esterified by 'heavy chain' (HC) proteins.

**C)** Alpha-dystroglycan ( $\alpha$ DG) is the unique cellular protein carrying (in muscles) the presented original O-mannosylated glycan structure.

**D)** Details of the glycan structure of the glycosylphosphatidylinositol (GPI) anchor; GPIAP: GPI-anchored protein.

### Figure 3. Transferrin isoelectric focusing (TIEF) patterns in controls and CDG

**A)** In controls, TIEF separates 6-sialo to 2-sialo Trf with 4-sialo Trf being the dominant glycoform. In CDG-I (e.g. PMM2-CDG and MPI-CDG), the 2-sialo and 0-sialoTrf glycoforms are classically markedly increased at the expense of 4-sialo Trf. In CDG-II (e.g. PGM1-CDG, a mixed CDG-I+II), increases of 3-sialo to 0-sialo Trf are typically observed.

**B)** In case of a Trf variant, two dominant glycoforms are present and can suggest a CDG. Neuraminidase treatment allows to alleviate the doubt by revealing either two marked 0-sialo Trf bands (Trf variant) or only one 0-sialo Trf band (absence of Trf variant).

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#### **Figure 4. Capillary zone electrophoresis (CZE) Trf profiles in control and CDG**

CZE separates Trf glycoforms according to charge and structure variability. In control, the 4-sialo Trf glycoform bearing two complex-type bi-antennated N-glycans is the dominant form. In CDG-I, increases of 2-sialo Trf (one missing N-glycan chain) and 0-sialo Trf (two missing N-glycan chains) are observed. In CDG-II, mixed increases of 3-sialo Trf to 0-sialo Trf are classically observed traducing the absence of terminal SA.

#### **Figure 5. Western blot patterns of various serum CDG biomarkers**

**A)** Western blot of Trf, A1AT, AGP and Hpt in control (left) and CDG-I (right). In CDG-I, additional lower bands are observed, traducing the increased levels of glycoforms missing complete N-glycan chains.

**B)** Western blot of serum/plasma bikunin showing, when compared to controls (C1 to C4), the marked increase of an abnormal ~27 kDa form (arrow) in a linkeropathy case (LK). Bkn: bikunin core protein; CS: chondroitin sulfate.

#### **Figure 6. Two-dimensional electrophoresis (2-DE) of various serum CDG biomarkers and apolipoprotein C-III (apoC-III) in controls and CDG**

2-DE separates serum glycoproteins according to charge and to molecular weight (MW).

**A)** In control, Trf, AAT and Hpt glycoforms appear as distinct spots with variable charge and MW. Very acidic AGP is not visible. In the presented CDG-II<sub>d</sub> case (B4GALT1-CDG), a marked cathodical shift is observed for all glycoproteins (including now visible AGP) traducing major terminal SA losses.

**B)** In control, apoC-III can be separated into bi-sialylated (apoC-III<sub>2</sub>), mono-sialylated (apoC-III<sub>1</sub>) and not glycosylated (apoC-III<sub>0</sub>) glycoforms with apoC-III<sub>1</sub> being majority. In COG-CDG, the apoC-III<sub>0</sub> glycoform level is classically elevated.

#### **Figure 7. Schematic overview of the various MS-based approaches for the analysis of protein glycosylation in CDG screening.**

Site-specific data for Trf and apoC-III were from Clerc et al (2) and Wopereis et al (38), respectively.

### **Figure 8. Serum/plasma N-glycomics**

**A)** Schematic representation of the relative protein contribution to each specific glycan composition. *Figure reproduced from Clerc et al (2) under Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).*

**B)** MALDI-TOF mass spectrum of permethylated N-glycans released from a plasma sample of a healthy subject. Measurements were performed in the positive-ion mode and all ions are present in sodiated form  $[M + Na]^+$ . Green circles, Man; yellow circles, Gal; blue squares, GlcNAc; red triangles, Fuc; purple diamonds, Neu5Ac. Sample preparation, permethylation and analysis were performed as described before (31)(121).

**C)** Representative chromatogram of total human plasma N-glycans as separated by HILIC UPLC-Fluorescence (Excitation and emission wavelengths were set at 250 and 428nm, respectively). Plasma N-glycans were enzymatically released from proteins by PNGase F, and fluorescently labeled with 2-aminobenzamide (2-AB). Color of the boxes under chromatographic peaks account for the expected main glycoprotein sources of the corresponding N-glycans. *Reprinted from Zaytseva et al (97) with permission.*

### **Figure 9. N-Glycosylation analysis of samples from patients with hemizygous missense variants in ATP6AP1.**

**A)** MALDI-TOF analysis of permethylated N-glycans obtained from total serum proteins.

**B)** Analysis of intact serum transferrin by nanoLC-chip-QTOF mass spectrometry. See legend of Figure 8 for N-glycan color-coding. *Figure reproduced from Jansen et al (103) under Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).*

**Supplementary Table 1. Overview of the reported variations of GPI-APs and sALP in known IGDs.**

**Supplementary Figures.**



**Figure S1. N-glycosylation of A1AT in CDG-I.** In CDG-I, the fully glycosylated normal A1AT glycoform (3 bi-antennated N-glycan chains) co-exists with abnormally high levels of glycoforms missing one (2 possibilities) or two entire N-glycan chains.

**Figure S2. Enzymatic activity of PNGase F and EndoH.** PNGase F cleaves between the first GlcNAc of complex-type oligosaccharides and the occupied Asn residue from N-glycoproteins. EndoH cleaves between the two GlcNAc of various high-mannose and hybrid-type N-linked oligosaccharides. The generated glycan structures are then analyzed using MS for the characterization of CDG-II-associated glycosylation defects.

**Figure S3. Western blot profile of serum Trf in B4GALT1-CDG (formerly CDG-IId).** The B4GALT1-CDG associated enzymatic deficiency is quasi-complete generating a unique Trf band with clearly lower MW compared to controls (Ctrl).

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Figure 1

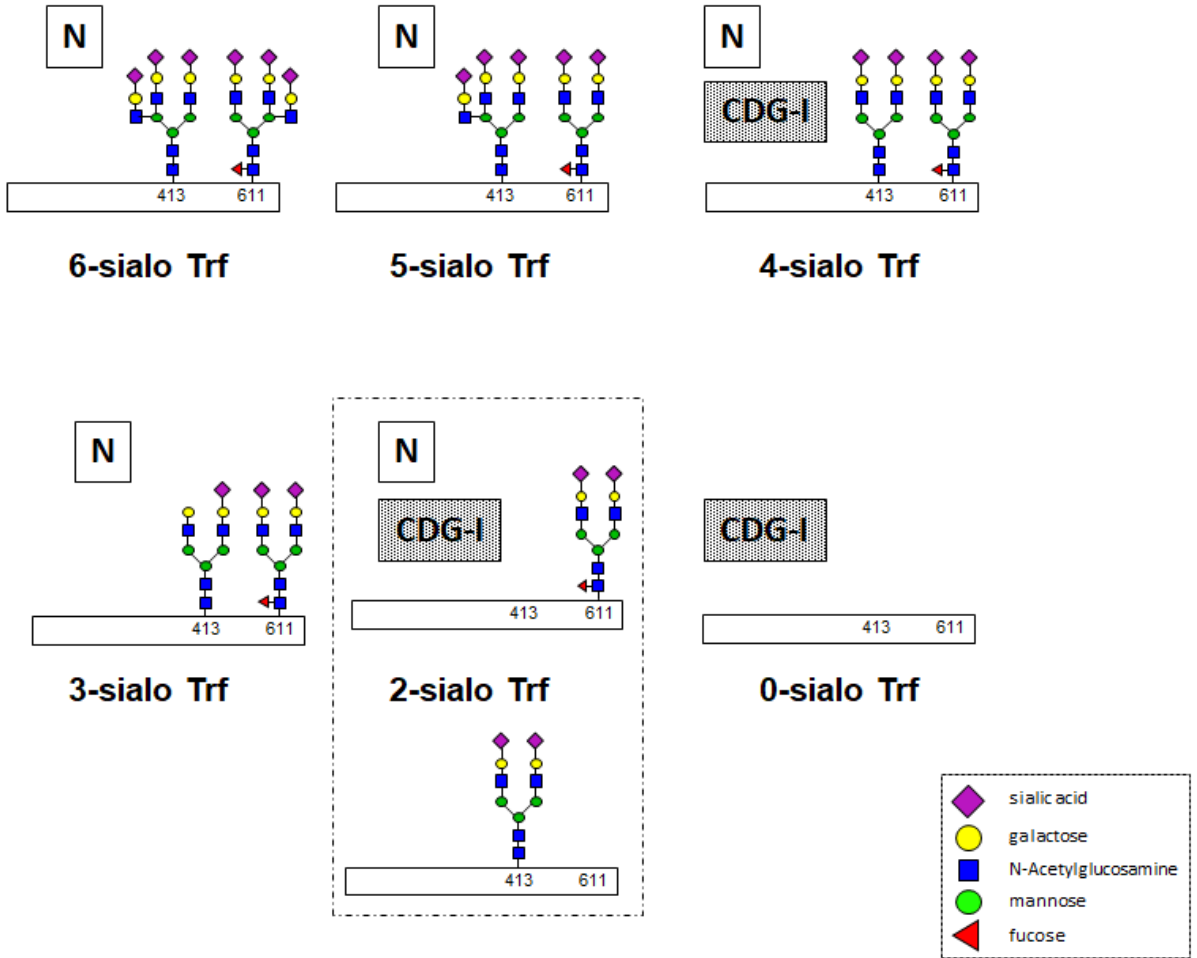


Figure 2

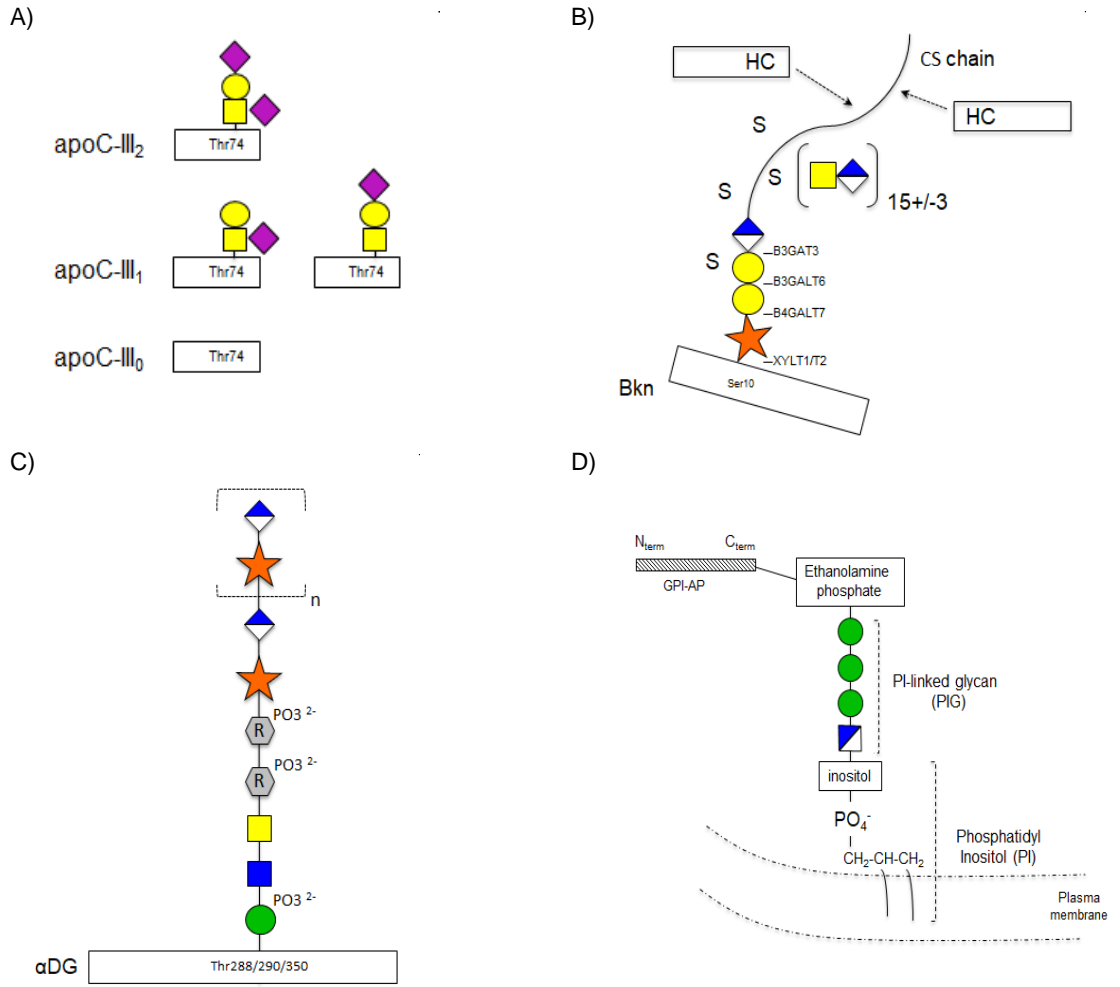


Figure 3

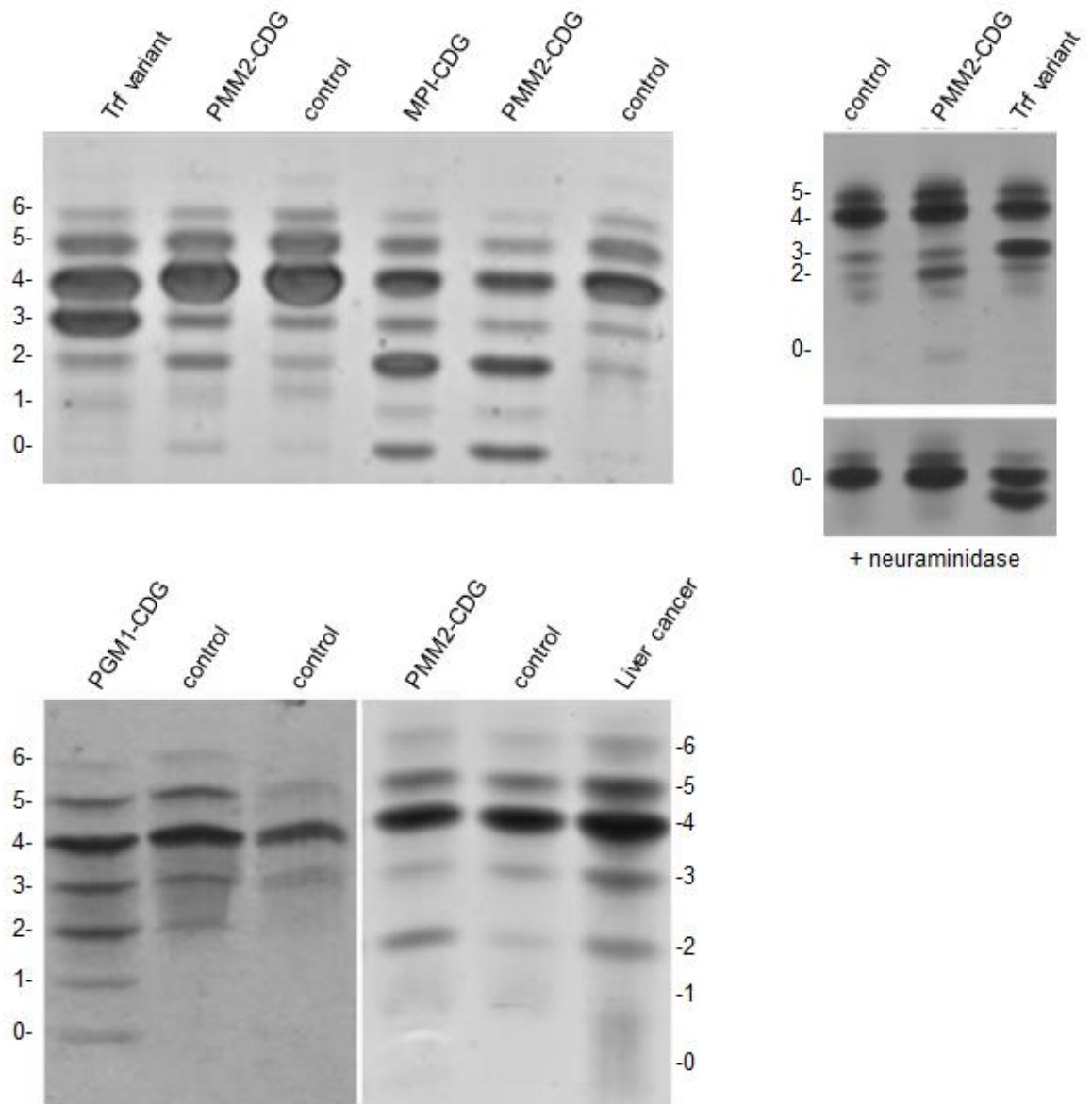


Figure 4

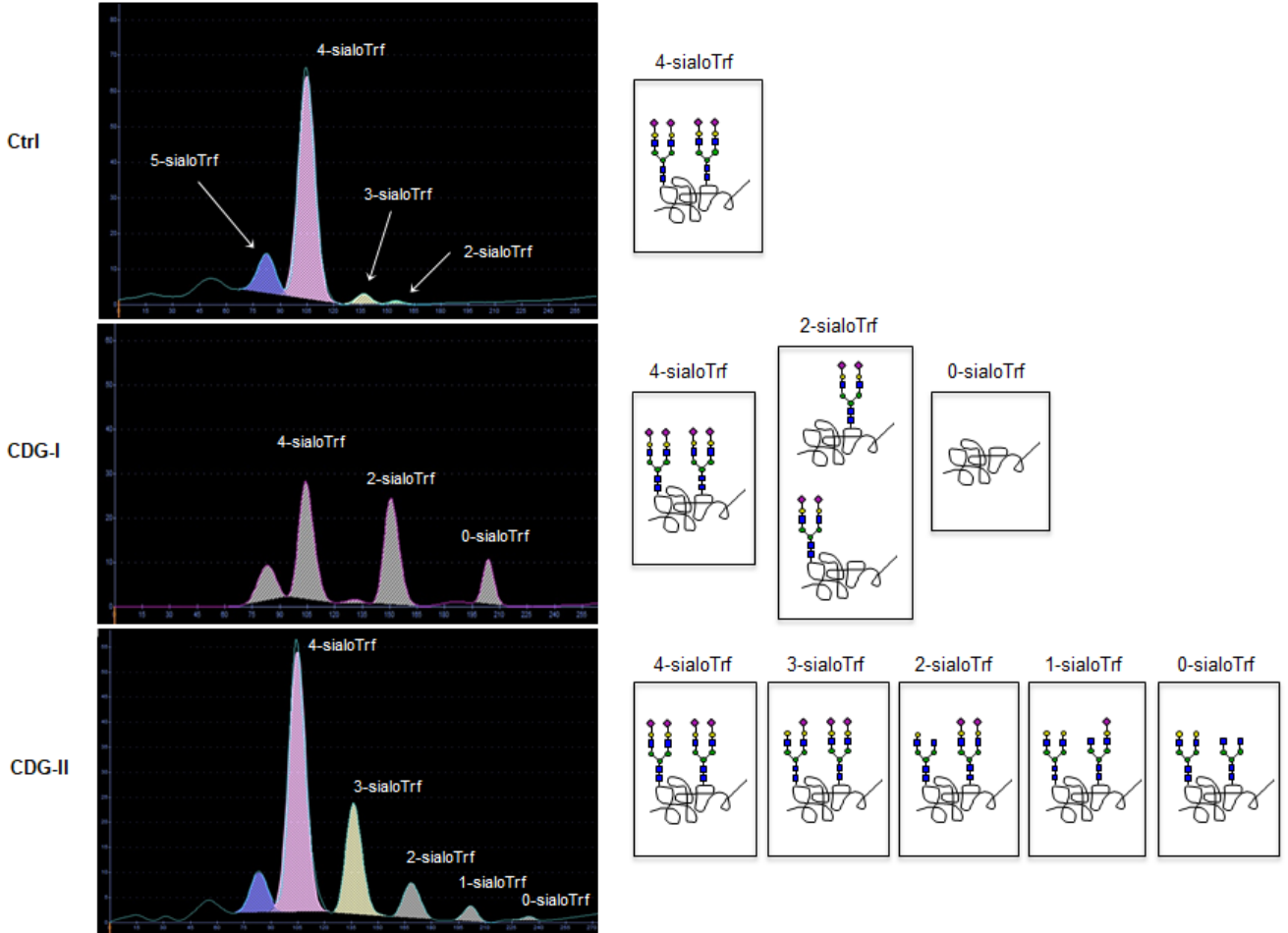
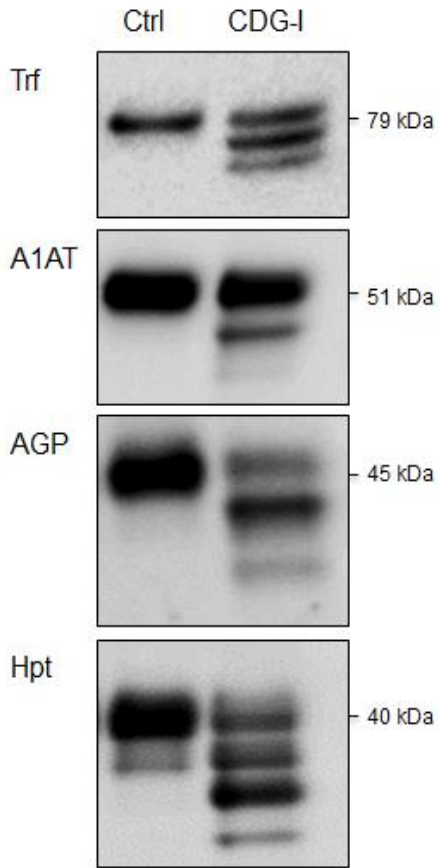


Figure 5

A)



B)

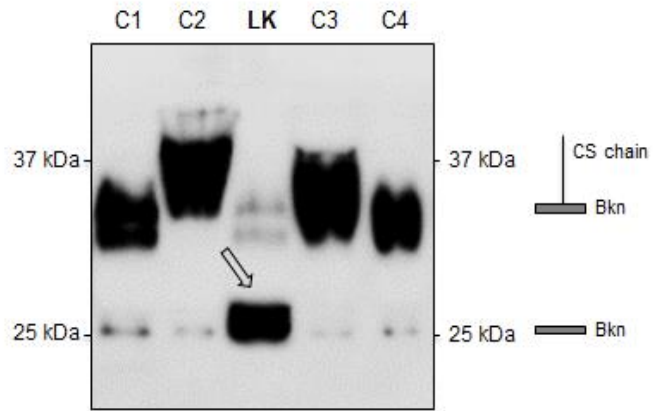




Figure 6

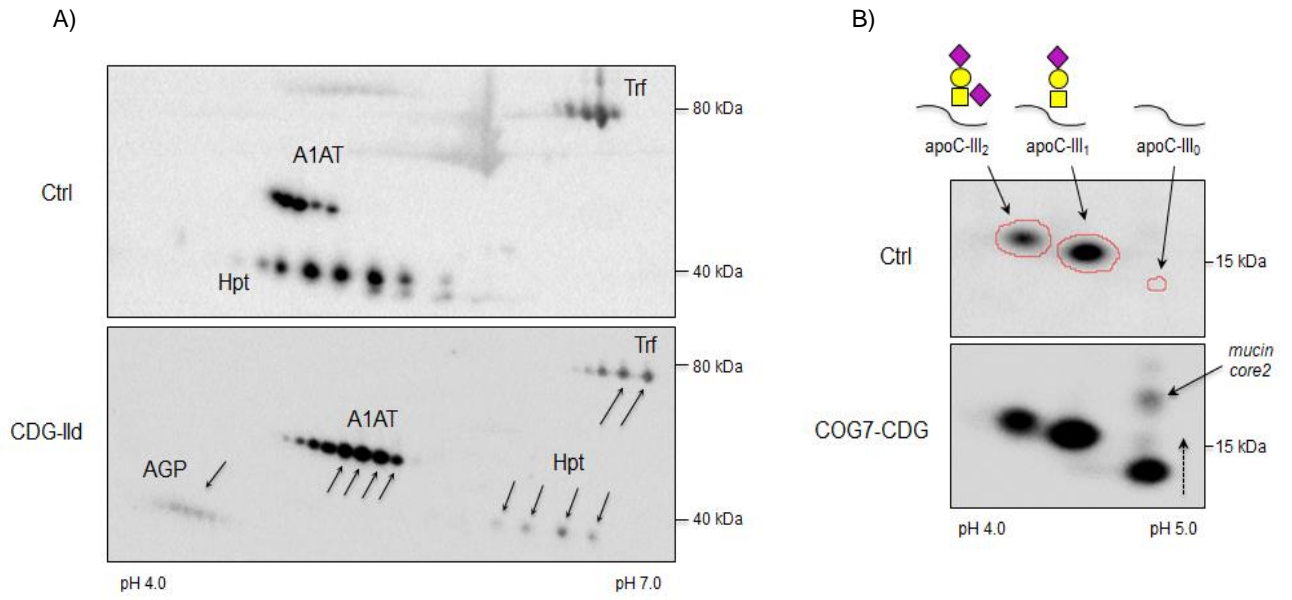


Figure 7

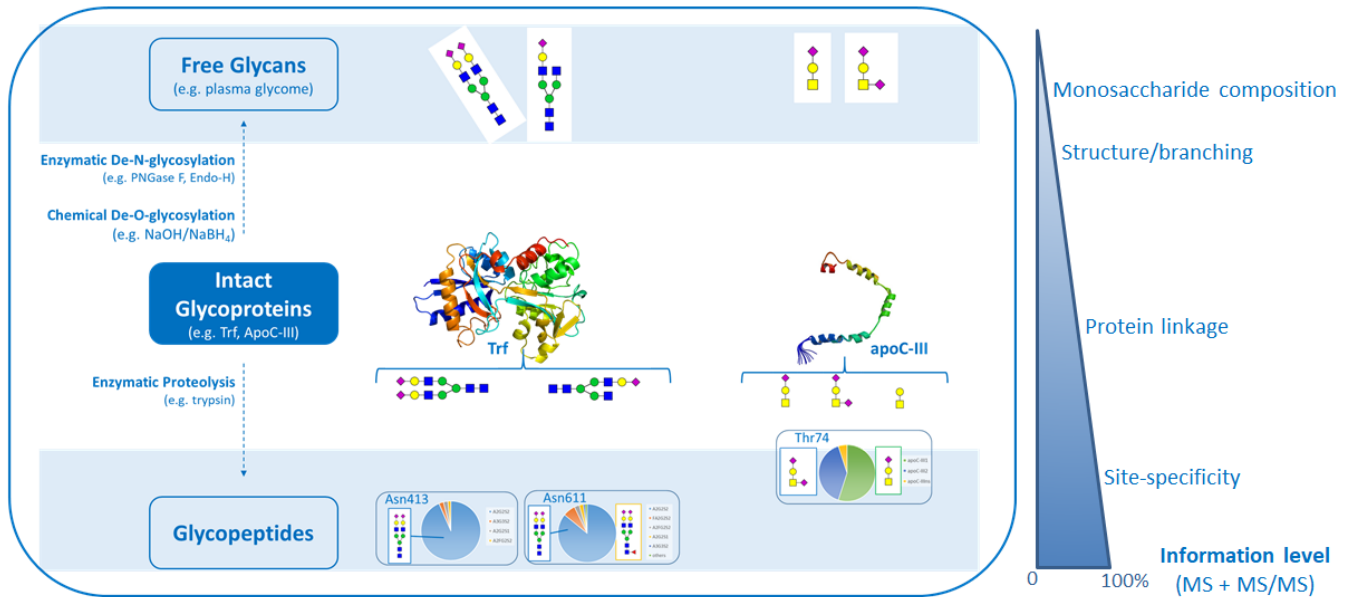


Figure 8

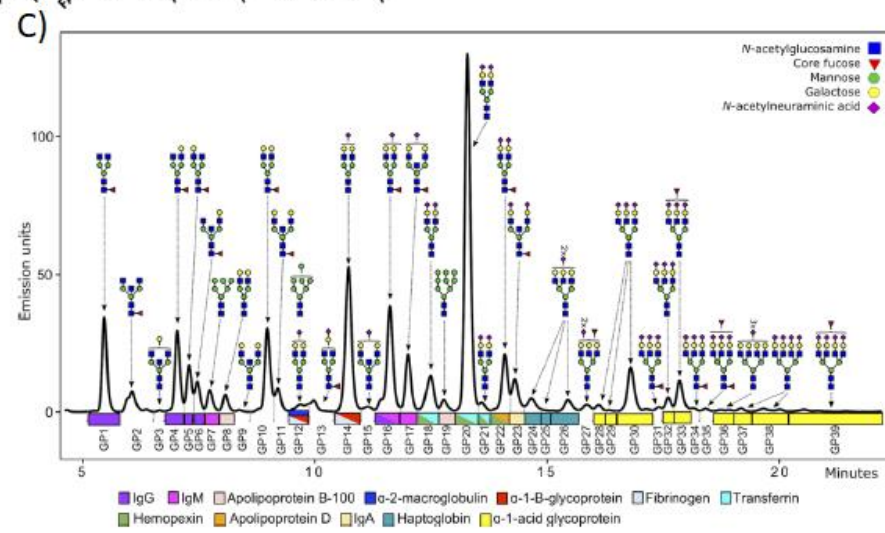
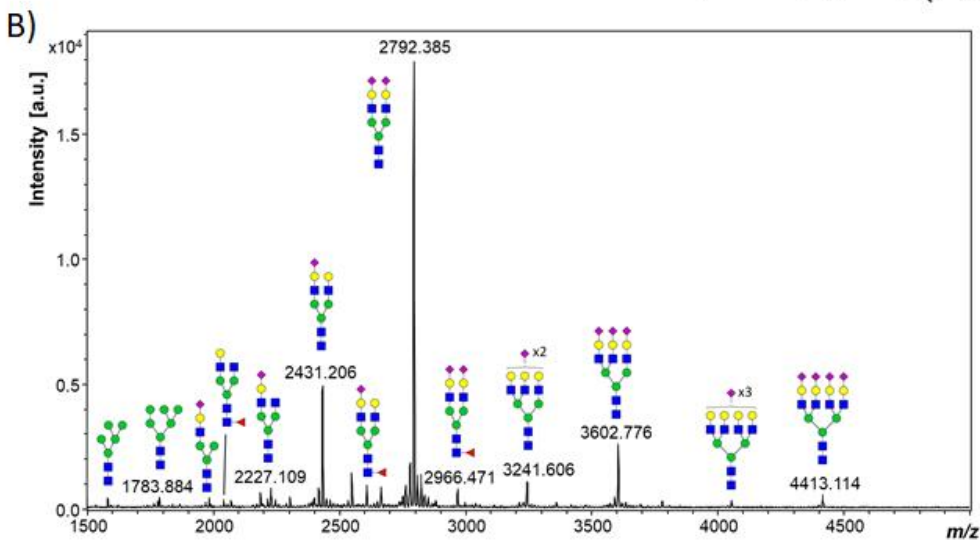
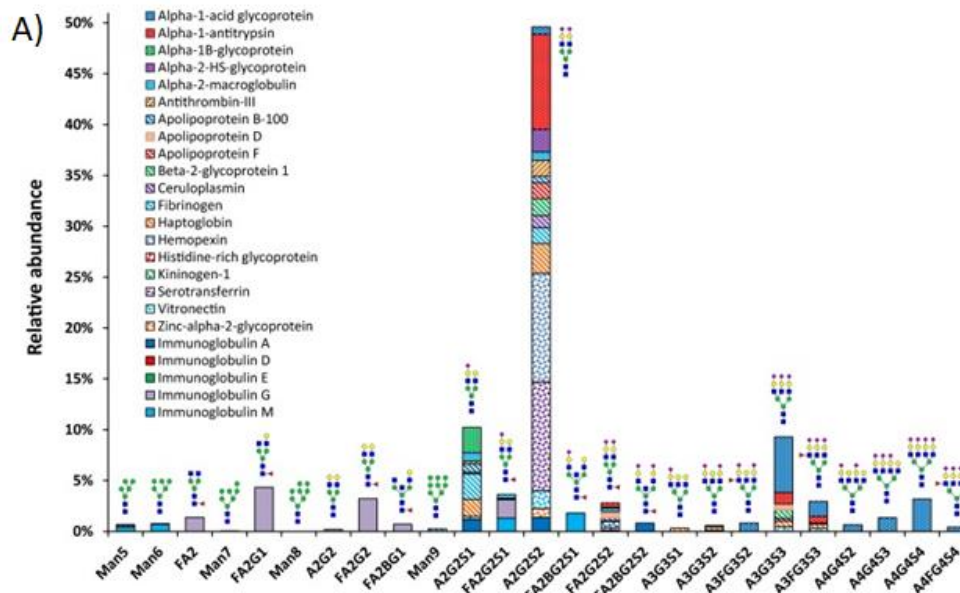


Figure 9

