# Biochemical diagnosis of congenital disorders of glycosylation

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## Abstract

Congenital disorders of glycosylation (CDG) are one of the fastest growing groups of inborn errors of metabolism, comprising over 160 described diseases to this day. CDG are characterized by a dysfunctional glycosylation process, with molecular defects localized in the cytosol, the endoplasmic reticulum, or the Golgi apparatus. Depending on the CDG, N-glycosylation, O-glycosylation and/or glycosaminoglycan synthesis can be affected. Various proteins, lipids, and glycosylphosphatidylinositol anchors bear glycan chains, with potential impacts on their folding, targeting, secretion, stability, and thus, functionality. Therefore, glycosylation defects can have diverse and serious clinical consequences. CDG patients often present with a non-specific, multisystemic syndrome including neurological involvement, growth delay, hepatopathy and coagulopathy. As CDG are rare diseases, and typically lack distinctive clinical signs, biochemical and genetic testing bear particularly important and complementary diagnostic roles. Here, after a brief introduction on glycosylation and CDG, we review historical and recent findings on CDG biomarkers and associated analytical techniques, with a particular emphasis on those with relevant use in the specialized clinical chemistry laboratory. We provide the reader with insights and methods which may help them properly assist the clinician in navigating the maze of glycosylation disorders.

## Abbreviations

| | |
|---|---|
| **2-DE** | two-dimensional electrophoresis |
| **ALG** | asparagine-linked glycosyltransferase |
| **apoC-III** | apolipoprotein C-III |
| **Bkn** | bikunin |
| **CDG** | congenital disorder(s) of glycosylation |
| **CDT** | carbohydrate deficient transferrin |
| **CE** | capillary electrophoresis |
| **CMP** | cytidine-monophosphate |
| **CS** | chondroitin sulfate |
| **DS** | dermatan sulfate |
| **EndoH** | Endo-β-*N*-acetylglucosaminidase H |
| **ER** | endoplasmic reticulum |
| **ESI** | electrospray ionization |
| **Fuc** | fucose |
| **GAG** | glycosaminoglycan(s) |
| **Gal** | galactose |
| **GalNAc** | *N*-acetylgalactosamine |
| **GDP** | guanosine-diphosphate |
| **Glc** | glucose |
| **GlcA** | glucuronic acid |
| **GlcNAc** | *N*-acetylglucosamine |
| **GPI** | glycophosphatidylinositol |
| **GSD1** | glycogen storage disease 1 |
| **HPLC** | high-pressure liquid chromatography |
| **ICAM-1** | intercellular cell adhesion molecule-1 |

| | |
|---|---|
| **ICP-MS** | inductively coupled plasma—mass spectrometry. |
| **IdoA** | iduronic acid. |
| **IEF** | isoelectric focusing. |
| **ITI** | inter-α-trypsin inhibitor. |
| **KS** | keratan sulfate. |
| **LAMP2** | lysosomal-associated membrane protein 2. |
| **MALDI-TOF** | matrix assisted laser desorption/ionization—time of flight. |
| **Man** | mannose. |
| **MS** | mass spectrometry. |
| **PαI** | pro-α-trypsin inhibitor. |
| **PGP** | protein-glycosaminoglycan-protein. |
| **PMM2** | phosphomannomutase 2. |
| **PNGase F** | peptide-N-glycosidase F. |
| **Q-TOF** | quadrupole—time of flight. |
| **RFMS** | RapifluorR mass spectrometry. |
| **SA** | sialic acid. |
| **Tf** | transferrin. |
| **TGN46** | trans-Golgi network protein 46. |
| **UDP** | uridine-diphosphate. |
| **Xyl** | xylose. |

## 1. Introduction

Protein glycosylation is a co-/post-translational modification in which a glycan chain is covalently bound to a protein, with a plurality of possible effects on its folding, targeting, secretion, interactions, and stability [1]. Protein-bound glycans are synthesized through sequential additions, and sometimes removals, of sugar residues catalyzed by highly specific enzymes called glycosyltransferases and glycosidases. In humans, glycans are mainly bound to proteins via the nitrogen atom of an asparagine (Asn) residue (N-linked glycans) or the oxygen atom of a serine (Ser) or threonine (Thr) residue (O-linked glycans). N-glycosylation is a streamlined process that occurs in the endoplasmic reticulum (ER), with the help of precursors synthesized in the cytoplasm, followed by trimming and maturation in the Golgi apparatus [2]. O-glycans present more structural diversity than N-glycans. Clinically relevant subtypes include mucin core-type O-glycosylation, where the glycan chain is linked to the protein via an *N*-acetylgalactosamine (GalNAc) residue; O-xylosylation, where it is linked via a xylose (Xyl) residue (leading to glycosaminoglycans chains); O-fucosylation, where it is linked via a fucose (Fuc) residue; and O-mannosylation, where the chain is linked via a mannose residue (Man; e.g., α-dystroglycan). Similar to the terminal trimming/maturation steps of

N-glycosylation, O-glycosylation mainly occurs in the Golgi apparatus [3]. Thus, a finely tuned regulation of the Golgi homeostasis, notably in terms of pH, vesicular trafficking, and metallic ions transport and gradient, is mandatory for correct N-/O-glycosylation [4].

Reflecting the important number of actors involved in the process, over 160 inborn errors of metabolism affecting protein glycosylation have been described. These are collectively known as congenital disorders of glycosylation (CDG). Following the description of CDG affecting protein glycosylation, CDG affecting phosphatidyl inositol-linked glycans (PIG-CDG) and lipid glycosylation have been described [5]. CDG greatly vary in clinical expression and affected individuals may present with multisystemic diseases including neurological disorders, developmental delay, coagulation defects and hepatopathy [6]. In view of these usually unspecific presentations, biomarkers have been developed to study glycosylation capacities and screen for related deficiencies. While robust serum/plasma biomarkers are available to highlight defects of N-glycosylation (transferrin, N-glycome) and mucin core1 O-glycosylation (apolipoprotein C-III), the screening/diagnosis of other types of glycosylation defects is more intricate and may require the discovery of additional biomarkers.

Here, we provide a comprehensive and critical review of our documented experience regarding CDG biomarkers, with a particular emphasis on biomarkers and techniques employed for clinical purposes. First, we provide the reader with a concise introduction regarding the fundamentals of N- and O- glycosylation. Second, we summarize knowledge on well-established biomarkers such as serum transferrin (Tf) and serum N-glycome for N-glycosylation defects, serum apolipoprotein C-III (apoC-III) for mucin core1 O-glycosylation defects and serum bikunin for glycosaminoglycans (GAG) synthesis disorders. Third, we provide an update on more recently developed biomarkers and their inputs in CDG diagnosis. We notably detail the contributions of glycopeptide analysis by mass spectrometry as emerging diagnostic tools of N-glycosylation defects. We also detail interesting cellular biomarkers such as ICAM-1, LAMP2 and TGN46. Fourth, we provide insights of ongoing and future developments in CDG biomarkers, including asialylated and fucosylated mucin core2 apoC-III glycoforms, and metabolomics. Lastly, we will provide the reader with an updated algorithm applicable to the clinical chemistry laboratory in the context of CDG diagnosis.

## 2. Fundamentals of glycosylation and related congenital disorders

### 2.1 N-glycosylation

N-glycosylation is the best-known glycosylation process, and the most frequently found in humans. It is believed that 80–90% of glycoproteins are N-glycosylated [3]. In this process, the glycan moiety is bound to the protein on the nitrogen residue of the amide function of an asparagine (Asn), most frequently located in a consensus sequence "Asn-X-Ser/Thr" (Ser: serine; Thr: threonine; X: any amino acid except proline). An alternative (albeit atypical) consensus site is "Asn-X-Cys" (Cys: cysteine; X: any amino acid except proline) [7]. N-glycosylation begins in the endoplasmic reticulum (ER) and ends in the Golgi apparatus. While historically described as a post-translational modification, the ER steps of N-glycosylation can occur concurrently to protein synthesis; therefore, it is also adequate to call N-glycosylation a co-translational modification [8]. Schematically, N-glycosylation is a two-step process: (i) in the ER, the oligosaccharide moiety is synthesized and attached to the nascent proteins; (ii) in the ER and, later, in the Golgi apparatus, it undergoes maturation consisting in trimming and elongation.

As shown in Fig. 1, the first step of the process begins on the cytosolic side of the ER membrane: sugars are attached sequentially to a specific anchored lipid named dolichol-phosphate. These monosaccharides are available in the form of nucleotide-sugars, synthesized in the cytosol by a specific enzymatic machinery. Their attachment is catalyzed by glycosyltransferases of the ALG (asparagine-linked glycosyltransferase) family. The first sugar attached to dolichol-phosphate is a *N*-acetylglucosamine-phosphate (GlcNAc-P), through a pyrophosphate (PP) bond. Then, a second GlcNAc and five Man are attached, yielding a $Man_3$-(Man)-Man-$GlcNAc_2$-PP-dolichol, which is translocated to the ER lumen through the action of the flippase RFT1 [9]. Then, four Man and three glucoses (Glc) are attached, yielding the biantennary precursor N-glycan $Glc_3$-$Man_3$-($Man_2$-($Man_2$)-Man)-Man-$GlcNAc_2$-PP-dolichol. Here, Man and Glc originate from the cytosol, where they are attached to dolichol-phosphate before translocation to the ER lumen [10]. Then, the biantennary oligosaccharide moiety is transferred from the dolichol-phosphate to the nascent protein, through the action of the oligosaccharyl-transferase (OST) complex comprising eight distinct protein subunits (Fig. 1).

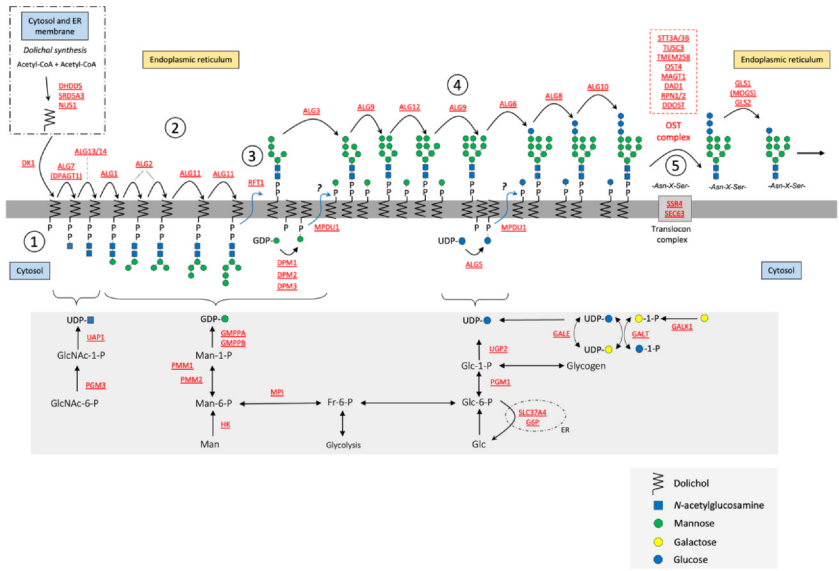


**Fig. 1 N-glycosylation: cytosol and initial endoplasmic reticulum steps.** N-glycosylation begins by (1) the reaction of a nucleotide-sugar (synthesized in the cytosol) with a dolichol-phosphate, yielding a sugar attached to a dolichol moiety via a pyrophosphate bond; (2) the addition of one *N*-acetylglucosamine (GlcNAc) and five mannoses under catalysis of specific glycosyltransferases (ALG); (3) the translocation from cytosol to the ER lumen of the dolichol-linked glycan under catalysis of the flippase RFT1; (4) the addition of four mannoses and three glucoses and (5) the transfer of the resulting oligosaccharide to the nitrogen of the lateral chain of an Asparagine (Asn) on the nascent protein, under catalysis of the oligosaccharyltransferase complex (OST), with a subsequent removal of two glucoses. Involved CDG-related molecular actors in each step are indicated in red.

The second step of the N-glycosylation process is the maturation of the glycan moiety (Fig. 2). It begins in the ER, where the glycoprotein undergoes folding and quality control. First, glucosidases I and II remove two of the three terminal Glc from the glycan chain, yielding a $Glc_1$-$Man_3$-($Man_5$)-Man-$GlcNAc_2$ motif, which is recognized by the lectins calnexin and calreticulin. These lectins, in relation with the oxidoreductase ERp57, act as chaperones which facilitate the folding of the glycoprotein [11]. At the end of the folding process, glucosidase II removes the remaining terminal Glc residue from the glycan chain (Fig. 2) [12]. If the glycoprotein is incorrectly folded, a UDP-glucose glycoprotein glycosyltransferase (UGGT) adds a new terminal Glc residue to the glycan chain and the glycoprotein can then be recognized again by the lectins and undergoes a

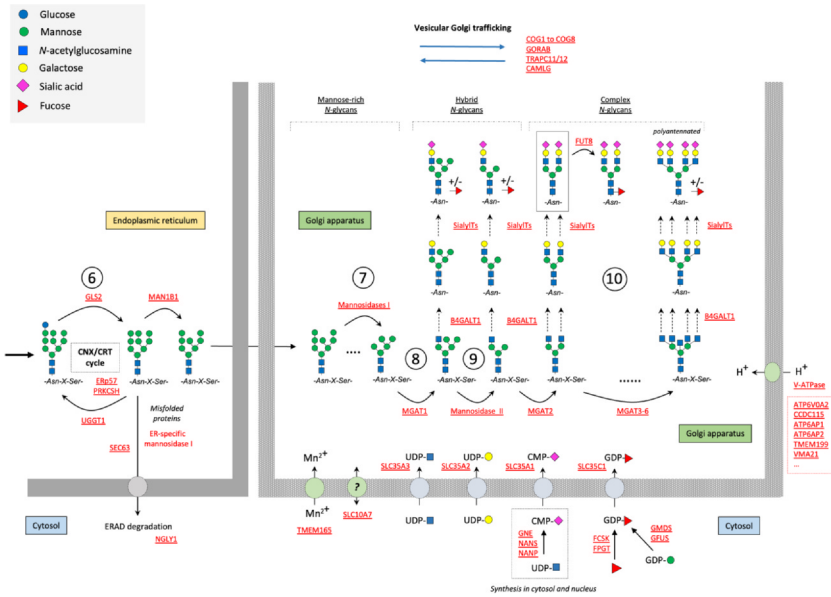


**Fig. 2 N-glycosylation: late endosplasmic reticulum and Golgi steps.** In the late endoplasmic reticulum steps, the glycoprotein undergoes a quality control with (6) the recognition by the chaperones calnexin/calreticulin (CNX/CRT), and, in case of protein misfolding, the action of the oxidoreductase ERp57. If the protein remains misfolded, it is recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which adds back a glucose leading to a new cycle of chaperone/ERp57 recognition. After a few cycles, if the protein cannot be folded properly, it is sent back to the cytosol for degradation and recycling. If properly folded, it is transported to the Golgi via vesicular trafficking and chaperones. In the Golgi apparatus, the protein-bound N-glycan (labeled "mannose-rich") can undergo maturation through sequential trimming and addition of sugars: (7) three mannoses are removed from the oligosaccharide under catalysis of Golgi mannosidases I; (8) a *N*-acetylglucosamine is added under catalysis of a GlcNAc transferase; (9) two mannoses are removed under catalysis of Golgi mannosidases II and (10) various monosaccharides are added under catalysis of multiple glycosyltransferases, yielding a mature oligosaccharide (complex-type or hybrid-type). Involved CDG-related molecular actors in each step are indicated in red.

new folding process. This cycle can be repeated several times [13]. If correct folding is not eventually achieved, three to four Man are removed from the glycan chain through the action of ER-specific mannosidase I. The glycoprotein is then transported to the cytosol for degradation and recycling [14]. If correct folding is achieved, the glycoprotein is transferred to the Golgi apparatus for additional maturation (Fig. 2). In the Golgi apparatus, Golgi mannosidases catalyze the removal of three Man from the

glycan chain. Then, a GlcNAc transferase catalyzes the addition of a GlcNAc on one of the two antennae of the chain, and Golgi mannosidase II catalyze the removal of the two terminal Man from the other antenna, resulting in a GlcNAc-$Man_2$-(Man)-$GlcNAc_2$ chain. Other sugars can then be sequentially attached to this chain, including GlcNAc, galactoses (Gal), terminal sialic acids (SA) and lateral Fuc [15]. These sugars are available in the form of nucleotide-sugars, which are transported from the cytosol into the Golgi apparatus thanks to more or less specific transporters from the SLC35 family (Fig. 2).

In healthy individuals, N-glycans are mostly matured in the form of "complex" N-glycans [16]. These are characterized by the presence of terminal SA. Other types of matured N-glycan include "mannose-rich" and "hybrid" N-glycans, which are found abundantly on immunoglobulins [17]. Mannose-rich N-glycans are of the $Man_X$-$GlcNAc_2$ form, while hybrid N-glycans include other sugars, which still bear some terminal Man (Fig. 2). In the context of CDG screening, a N-glycoprotein of major interest is serum transferrin (Tf), which bears two complex N-glycan chains.

## 2.2 Mucin type O-glycosylation

Mucin type O-glycosylation is a post-translational modification that takes place in the Golgi apparatus (Fig. 3). Unlike N-glycosylation, where the primary glycan is built on dolichol-phosphate before transfer to the protein, O-glycosylation involves the building of the glycan directly on the protein, step by step. In mucin-type O-glycosylation, polypeptide-*N*-acetylgalactosamine transferases (ppGalNAcTs) first link a GalNAc on the oxygen residue of the lateral chain of a Ser or a Thr [18]. Unlike N-glycosylation, no consensus sequence has yet been clearly established for O-glycosylation. This O-linked GalNAc constitutes the simplest form of mucin type O-glycosylation. Thereafter, other glycosyltransferases can attach other sugars to build more complex O-glycan chains, corresponding to the "core1" to "core8" mucin-type structures [19]. Sugar moieties used in this O-glycosylation originate from the cytosol, where they are linked to a nucleotide di/monophosphate (UDP-Gal, UDP-GalNAc, UDP-GlcNAc, CMP-SA), and translocated to the Golgi apparatus with the help of transporters belonging to the SLC35 family (Fig. 3).

A typical example of a mucin type O-glycosylated protein is serum apolipoprotein C-III (apoC-III) [20]. After the attachment of the first GalNAc on $Thr^{74}$, the addition of a Gal is catalyzed by the glycoprotein-*N*-acetylgalactosamine, 3-β-galactosyl transferase 1 (C1GalT1 or Core1 synthase).

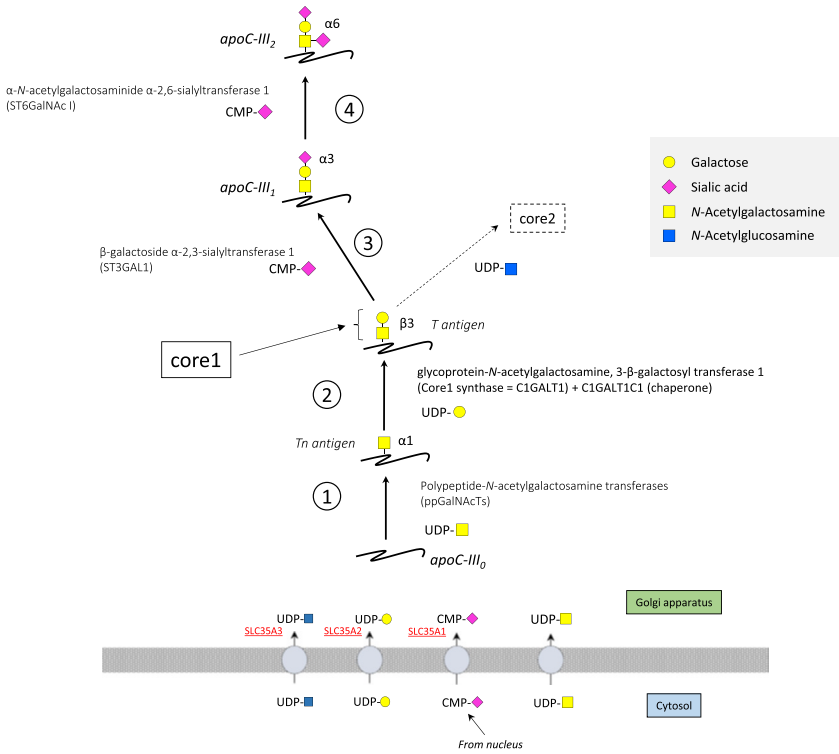


**Fig. 3 Mucin core1 O-glycosylation: the example of apolipoprotein C-III (apoC-III).** (1) First, a *N*-acetylgalactosamine (GalNAc) is added to the oxygen of the lateral chain of a threonine under catalysis of polypeptide *N*-acetylgalactosamine transferases (ppGalNAcTs), then (2) a galactose is added under catalysis of a *N*-acetylgalactosaminide β-1,3-galactosyltransferase (C1GalT1 or Core 1 synthase), forming the mucin core 1 motif. Then, (3) a sialic acid is added on the galactose under catalysis of β-galactoside α-2,3-sialyltranserase (ST3Gal1). In some cases, (4) a second sialic acid is added on the GalNAc under catalysis of an α-*N*-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc I). Asialylated, monosialylated and disialylated apoC-III proteins are labeled apoC-III$_0$, apoC-III$_1$ and apoC-III$_2$, respectively.

The Gal-GalNAc disaccharide (T-antigen) constitutes the "core1" motif, shared among many O-glycoproteins, and upon which more complex chains can then be built. In the case of apoC-III, the addition of a first SA is catalyzed by β-galactoside α-2,3-sialyltransferase 1 (ST3GAL1). ApoC-III bearing the SA-Gal-GalNAc chain is named "apoC-III$_1$". The possible attachment of a second SA onto the GalNAc is catalyzed by α-*N*-acetylgalactosaminide α-2,6-sialyltransferase 1 (ST6GalNAc I). The apoC-III bearing the SA-Gal-(SA)-GalNAc chain is named "apoC-III$_2$" [21,22] (Fig. 3).

## 2.3 Glycosaminoglycans synthesis

Glycosaminoglycans (GAG) are glycan chains defined by the presence of a repeated disaccharide motif, comprising an amino-sugar (e.g., GlcNAc) and a uronic acid (e.g., glucuronic acid: GlcA) (Fig. 4). This chain is typically attached to the bearing protein by a common (GlcA-Gal-Gal-Xyl) tetrasaccharide linker, which forms an O-xylosylation bond on an oxygen

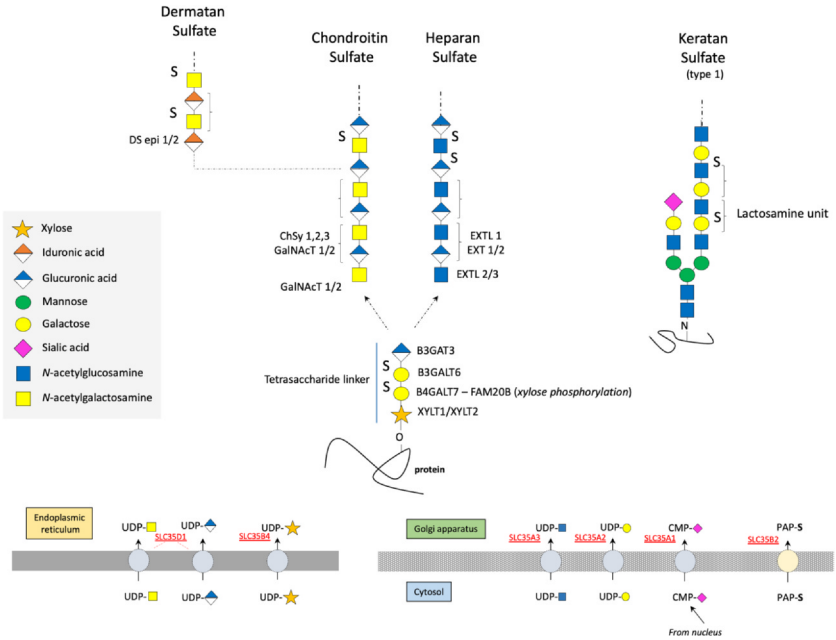


**Fig. 4 Biosynthesis and modifications of proteoglycans** (excluding heparin and keratan sulfate type 2 and type 3). Upon the core protein-linked tetrasaccharide linkage region (i.e., GlcA-Gal-Gal-Xyl), the GAG chain polymerization starts with the transfer of a first amino sugar (i.e., GalNAc for chondroitin sulfate [CS] and GlcNAc for heparan sulfate [HS]) and continues with the addition of repetitive GlcA-GalNAc for CS and GlcA-GlcNAc for HS. Epimerization from CS to dermatan sulfate (DS; IdoA-GalNAc backbone) can occur. The GAG chains are also heavily sulfated. Each pathway involves specific glycosyltransferases, sulfotransferases (not shown), and epimerases. For keratan sulfate (KS), there is three types of initiations leading to GAG chains composed of a polylactosamine (GlcNAc-Gal)x backbone. Sugar moieties used in GAG synthesis originate from the cytosol, where they are linked to a UDP, before being translocated to the ER and the Golgi with the help of transporters of the SLC35 family. Enzyme abbreviations: XylT: xylosyltransferase; β4Gal-T7: β-4-galactosyltransferase7; β3Gal-T6: β-3-galactosyltransferase6; GlcAT-I: β-3-glucuronyltransferase3; CSGalNAcT: *N*-acetylgalactosaminyltransferase; ChSy: chondroitin synthase complex; DS epi: dermatan sulfate epimerase; EXT: exostosin family protein; EXTL: exostosin-like.

residue of a Ser. This is the case with heparan sulfate, chondroitin sulfate (CS) and dermatan sulfate. Notable exceptions are keratan sulfate, which consist in a $(GlcNAc\text{-}Gal)_x$ backbone (polylactosamine) linked to the protein without the help of the common tetrasaccharide linker but via alternate O- or N-linkages [23]. GAG synthesis begins at the end of the ER, at the interface with the Golgi apparatus [24]. After the synthesis of the linker (or the first linking sugars in the case of keratan sulfate), various glycosyltransferases add a variable number of the repeated disaccharide motif. Sugar moieties used in GAG synthesis originate from the cytosol, where they are linked to a UDP (or a CMP for SA), before being translocated to the ER and the Golgi apparatus with the help of transporters belonging to the SLC35 family. Furthermore, GAG are typically abundantly sulfated, making them very negatively charged (Fig. 4).

## 2.4 Congenital disorders of glycosylation (CDG)

The high number of enzymes involved in glycosylation, their high specificities, their sensitivity to the biochemical microenvironment (pH, ionic strength, cofactors…), in addition to a plethora of transporters and molecular actors, make glycosylation a fragile process. Disorders of glycosylation can appear in the course of acquired diseases (e.g., hepatic failure, cancers), or be present from birth, linked to the inheritance of pathogenic variants. CDG comprise over 160 inborn errors of metabolism affecting glycosylation. CDG inheritance is typically autosomal and recessive, however rare cases of autosomal dominant and X-linked pathogenic variants have been described [25]. CDG are rare diseases, where most disorders have only been described in less than a hundred individuals worldwide. Pathogenic variants can be found on genes coding for enzymes (e.g. mutases, isomerases, glycosyltransferases, glycosidases), Golgi trafficking proteins, nucleotide-sugar transporters or ionic transporters (e.g. proton pumps, cation transporters) [25]. The first cases of CDG (PMM2-CDG, MPI-CDG, MGAT2-CDG…) were identified on the basis of faulty protein glycosylation; subsequently, cases with faulty glycosylation of lipids, or faulty synthesis of glycosylphosphatidylinositol (GPI) anchors have also been described [26–29]. Clinically, CDG are heterogenous diseases, typically comprising non-specific symptoms of variable severity. Most commonly, affected individuals present with neurological signs. Other common signs include growth delay, hypotonia, hepatopathy, coagulopathy and dysmorphia. CDG as a whole are yet incurable; however, in a few specific disorders (MPI-CDG, PGM1-CDG, SLC39A8-CDG) therapeutic options exist that may improve glycosylation status and/or symptoms [30].

While the pathogeny of these diseases is poorly understood at this time, it is believed that part of these clinical signs stem from faulty glycosylation before birth. Indeed, numerous studies in humans and animal models have pointed to the major role of glycoproteins in embryogenesis and fetal development, and more particularly in neurological and bone/cartilage development [31–35]. Glycosylation defects could, in this context, be responsible for abnormal protein folding, transport, and ligand-receptor binding, thereby altering key cell signaling pathways essential to *in utero* development (e.g., TGF-β, sonic hedgehog, Notch…) and/or directly impacting protein function (e.g., proteoglycans in the context of bone/cartilage growth).

As clinical presentation of CDG is typically non-specific, its screening and diagnosis relies on biochemical and genetic testing. Biochemical testing can highlight abnormal glycosylation through the study of specific glycoproteins, which may change in charge and/or weight. Besides, characteristic patterns (as described later) may orientate the diagnosis towards a specific etiology [36]. Genetic testing can be performed concurrently to biochemical testing, confirming the presence of causative variants on implied genes. Furthermore, besides helping establish diagnosis, it may serve as a base for ulterior genetic counseling. Both approaches are complementary, as genetic testing often results in the discovery of variants of unknown significance, in which case biochemical studies can hint at their pathogenicity.

Historically, biochemical CDG screening consisted in separation of serum transferrin (Tf) glycoforms by isoelectric focusing (IEF), which led to the establishment of the first CDG classification (type I: CDG-I and type II: CDG-II) [37]. CDG-I involve the first part of the N-glycosylation process (synthesis of the precursor N-glycan attached to dolichol-phosphate, and/or its transfer to the nascent protein), where pathogenic variants are found in genes coding for cytosolic or ER actors. In CDG-I, Tf loses one or two complete N-glycan chains. The most common CDG, PMM2-CDG belongs in this category [29]. CDG-II involve the second part of the N-glycosylation process (trimming/maturing of the precursor N-glycan after its linkage to the protein), where pathogenic variants are found in genes coding for ER or Golgi actors. In CDG-II, Tf N-glycan chains are present on the protein but lose a variable number of sugars, including terminal negatively charged SA.

Historical CDG nomenclature was built upon this CDG-I/CDG-II classification. Nowadays, each disorder is named after the affected gene (e.g. PMM2-CDG) [38].

## 3. Current CDG biomarkers and related laboratory techniques

### 3.1 Serum transferrin (N-glycosylation) using charge-based techniques

Tf is a 679 amino acid-long N-glycoprotein that is primarily synthesized by hepatocytes and present abundantly in serum. It is a key player in iron metabolism, carrying up to two $Fe^{3+}$ atoms through the bloodstream. Its primary structure contains two N-glycan acceptor sites on $Asn^{413}$ and $Asn^{611}$ [39]. In healthy individuals, the two sites are occupied by complex di-antennary N-glycans and Tf is mainly 4-sialylated (4-sialo Tf). Other glycoforms (notably 5-, 3- and 2-sialo Tf) are present in minor to trace proportions (Fig. 5) [40].

The first technique developed to study Tf glycoforms was gel isoelectric focusing (IEF) followed by immunofixation and staining [41]. Tf glycoforms are separated based on their isoelectric points (p*I*), which depend on their terminal sialylation status. Samples are pre-incubated with $Fe^{3+}$ to saturate of all binding sites, as differential saturation could affect migration. Elevated proportions of hyposialylated Tf glycoforms highlighted using IEF were initially described in individuals with a history of chronic alcohol consumption and the term "carbohydrate-deficient Tf" (CDT) was later established as a standard biomarker for chronic alcohol abuse screening [42,43]. The emergence of CDG resulted from the observation of elevated proportions of hyposialylated Tf glycoforms in children suffering from unexplained neurological disorders [29,44]. As mentioned above, two types of Tf IEF patterns were described: a CDG-I pattern with highly increased 2-sialo Tf and 0-sialo Tf, and a CDG-II pattern also comprising elevated 3- and 1-sialo Tf, in uneven proportions (Fig. 6). With very few exceptions, most of identified cases of CDG have been identified using this technique. While most often not specific of a peculiar subtype (only pointing to "CDG-I" or "CDG-II"), some peculiar Tf IEF patterns may however quickly orientate toward the causative gene. Typical examples are MGAT2-CDG and B4GALT1-CDG (complete cathodical shift of Tf glycoforms), PGM1-CDG (mixed CDG-I/CDG-II Tf pattern) and MAN1B1-CDG (isolated and major increase of the 3-sialo Tf fraction) [27,45–47].

While IEF remains the "gold-standard" for serum Tf glycoforms analysis in the context of CDG screening, capillary electrophoresis (CE) has been developed as an alternative technique [48,49]. In CE, Tf glycoforms

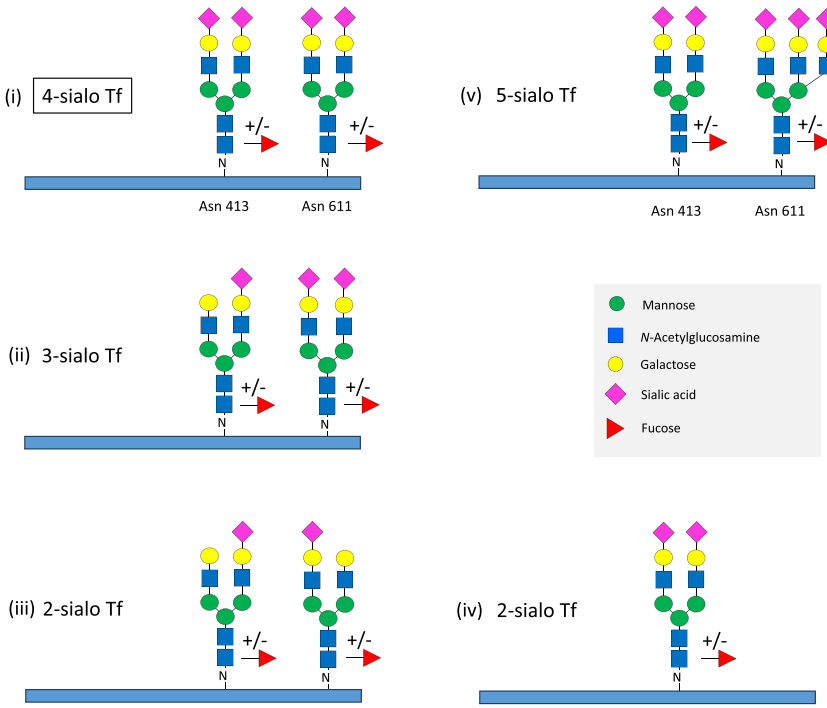


**Fig. 5 Examples of transferrin glycoforms.** (i) Tetrasialotransferrin (4-sialoTf), the most common glycoform (78–86% of total), bearing two N-glycan chains on $Asn^{413}$ and $Asn^{611}$, with a total of four terminal sialic acids; (ii) trisialotransferrin (3-sialoTf) and (iii, iv) disialotransferrin (2-sialoTf), minor glycoforms (<6% and <1.6% of total, respectively) missing one (3-sialoTf) or two (2-sialoTf) sialic acids. It must be noted that hyposialylated transferrin glycoforms can result from the presence of hyposialylated N-glycans bound to the protein (e.g., (iii)), the absence of a complete N-glycan chain (e.g., (iv)), or both. These glycoforms are typically present in higher proportions in patients with CDG. They are readily identified thanks to their lower negative charge (isoelectric focusing, capillary electrophoresis). Finally, some polyantennarity naturally occurs in healthy individuals, with for instance the presence of (v) pentasialotransferrin (5-sialoTf) (11–18% of total), bearing an extra terminal sialic acid. These Tf glycoforms can be fucosylated.

are separated based on their electrophoretic mobility, which depends on their total charge and size. The number of terminal negatively charged SA affects total charge, while the number and length of N-glycan chains affect size. Different commercial kits (originally developed for chronic alcohol abuse screening) were shown to provide results similar to IEF in CDG screening [50,51]. By contrast, CE is automated, easier to implement for routine use and allows easier separation and quantification of Tf

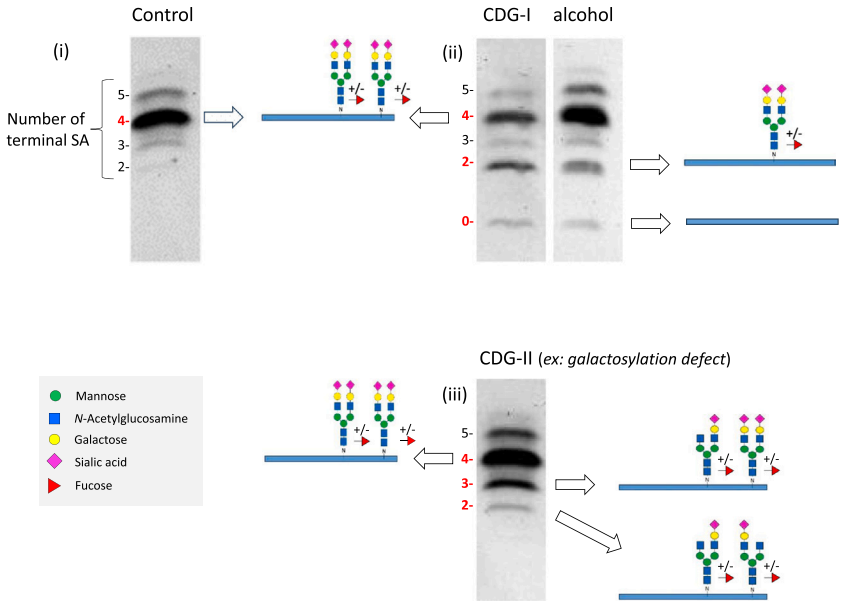


**Fig. 6 Examples of serum transferrin isoelectric focusing (IEF) patterns.** In IEF, transferrin glycoforms are separated based on their isoelectric point (p*I*), which depends on the number of terminal sialic acids. (i) Pattern of a healthy individual (control). The major band is 4-sialoTf, with additional glycoforms also present in minor proportions (5-, 3- and 2-sialoTf). (ii) Patterns of a CDG-I affected individual, and a chronic alcohol abuser. Both patterns are characterized by lowered proportion of 4-sialoTf, and increased proportions of 2- and 0-sialoTf. In both CDG-I and chronic alcohol abuse, these hyposialylated glycoforms emerge from the loss of one or two complete N-glycan chains. Thus, a differential diagnosis upon discovery of a CDG-I IEF pattern is chronic alcohol abuse. (iii) Pattern of a CDG-II affected individual (here, with a galactosylation defect). CDG-II patterns are characterized by a lowered proportion of 4-sialoTf, and variably increased proportions of 3- to 0-sialoTf. In CDG-II, these hyposialylated glycoforms emerge not from the loss of complete N-glycan chains, but from a defective trimming/maturation process, resulting in a partial loss of sugars and thus in a reduced terminal sialylation.

glycoforms. While robust, Tf CE may be affected by interferents common to protein analyses including fibrinogen, immunoglobulins, complement, hemolysis, lipemia, icterus and substances in patients with severe hepatopathy [52].

Both IEF and CE are sensitive to certain Tf protein polymorphisms, which modify its p*I*. Exceptionally, these polymorphisms may also be located on one of the N-glycan acceptor sites, resulting in the synthesis of a large proportion of 2-sialo-Tf [53–55]. The major risk in all cases is the

misattribution of the resulting pattern to CDG. Tf polymorphisms that modify its p*I* may be detected by pre-treatment of samples with neuraminidase (sialidase), which removes all terminal SA residues from Tf N-glycan chains [54,56]. In homozygous individuals, the pattern will display a single asialo Tf band/peak, while two asialo Tf bands/peaks will be displayed in heterozygous individuals (Fig. 7). Very exceptionally,

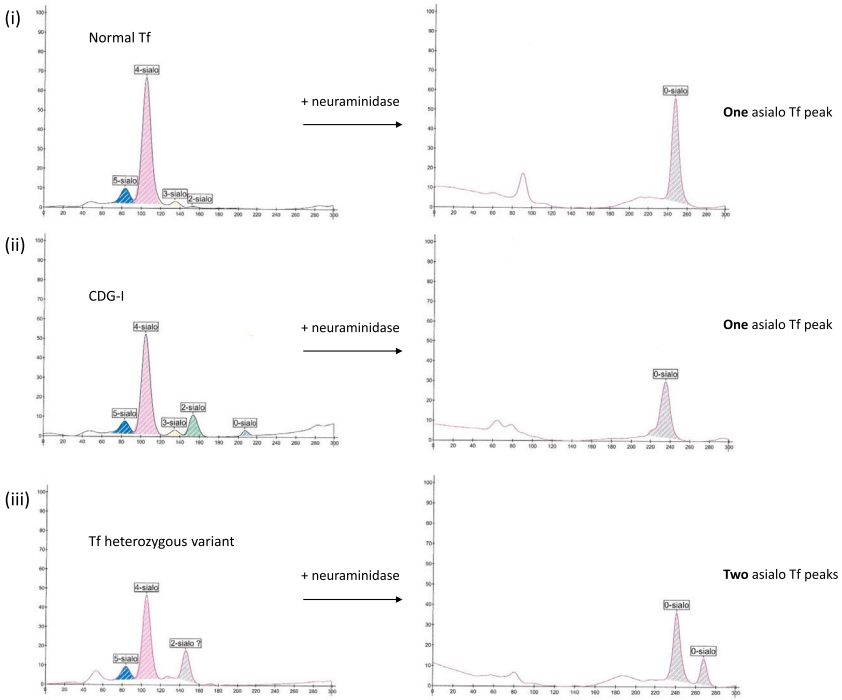


**Fig. 7 Examples of serum transferrin capillary electrophoresis (CE) patterns, and effect of neuraminidase/sialidase treatment in the detection of transferrin polymorphisms.** In CE, transferrin glycoforms are separated based on their electrophoretic mobility, which depends on the number of terminal sialic acids. (i) Pattern of a healthy individual. The major peak is 4-sialoTf, with additional glycoforms also present in minor proportions (5-, 3- and 2-sialoTf). Treatment by neuraminidase removes all terminal sialic acids on the N-glycan chains, resulting in a single 0-sialoTf peak. (ii) Pattern of a CDG-I affected individual. This pattern is characterized by lowered proportion of 4-sialoTf, and increased proportions of 2- and 0-sialoTf. Treatment by neuraminidase results in a single 0-sialoTf peak. (iii) Pattern of a healthy individual with a Tf variant at the heterozygous state. Here, a peak migrating similarly to 2-sialoTf is observed. This can sometimes lead to confusion with a CDG-I pattern. Here, however, treatment by neuraminidase will result in two 0-sialoTf peaks, each corresponding to one Tf isoform, as the amino-acid variation results in differential electrophoretic migration.

a patient may present with both CDG and Tf polymorphism, requiring cautious interpretation of their Tf CE pattern [57].

In patients with hepatopathy, aberrant protein N-glycosylation (excessive polyantennarity and fucosylation) may also affect peak/band separation (di-trisialo Tf bridging) [58]. This has notably been observed in non-CDG diseases (e.g., non-alcoholic fatty liver disease, chronic hepatitis C). Therefore, in the context of CDG with hepatopathy, or in some legal medicine cases, reanalysis by high pressure liquid chromatography (HPLC) could be of interest, as it has been shown to provide interpretable profiles in about half of cases of uninterpretable CE profiles [58,59].

Despite good sensitivity, an important limitation in CDG screening with Tf is that neither IEF nor CE and HPLC are sensitive to fucosylation defects (e.g., SLC35C1-CDG, FCSK-CDG, FUT8-CDG), as fucose bears no electrical charge [60]. Alternative biomarkers are thus required to screen for fucosylation CDG, and a promising candidate will be discussed later. Tf also lacks diagnostic value in the CDG without N-glycosylation defects. Finally, we and others reported CDG cases and subtypes where Tf patterns were unexpectedly normal (or subnormal). Among them, notable examples concern PMM2-CDG, ALG13-CDG, MOGS-CDG, SLC35A2-CDG, ATP6V0A2-CDG, COG-CDG as well as most of the CDG related to the hexosamine/sialic acid pathway (GNE myopathy, NANS-CDG…) [60–68]. Interestingly, it has also been reported that some patients may experience a spontaneous normalization in their Tf glycosylation profile over time. Improvements in coagulation parameters, transaminases and thyroid releasing hormone have also been observed [69]. However, these laboratory tests changes do not appear to be linked to positive clinical evolutions. Therefore, we think that these points must be critically considered when facing unexpected negative CDG screening tests (notably in adult individuals) and in the context of clinical trials targeting CDG patients and using Tf as a biomarker for therapeutic outcome/follow-up measurement [70,71].

## 3.2 Serum transferrin (N-glycosylation) using mass spectrometry

In that context, mass spectrometry (MS)-based methods have been adopted in some places (particularly in the US and in Japan), as approaches for precisely identify abnormal glycan structures attached to Tf and potentially more precisely orientate towards causative genes. Thus, analytical workflows involving liquid chromatography (LC) coupled to electrospray ionization (ESI) and high-resolution quadruple time-of-flight (Q-TOF) mass

spectrometer have been included in clinical routine for analyzing immunopurified intact Tf, applied to CDG screening and subtype delineation [72,73].

Of note, while the MS study of intact Tf can theoretically not distinguish between CDG-I subtypes (which all share a partial absence of N-glycan chains), it could present a direct diagnostic value for some CDG-II bearing particular abnormal structures such as MGAT2-CDG (monoantennated N-glycans), B4GALT1-CDG (complete agalactosylation) and PGM1-CDG (underoccupancy and maturation defects) [47,74]. Furthermore, although representing a particularly relevant tool for studying CDG, ESI-MS analysis of intact Tf has its own analytical drawbacks and limitations. ESI mass spectra of Tf are characterized by a distribution of multiply charged ions that needs to be deconvoluted into "zero-charge" spectra of Tf glycoforms and variants (Fig. 8A). This deconvolution step greatly facilitates data interpretation, but particular attention should be paid to avoid any inaccuracy of this processing step. High spectrum complexity with potentially overlapping peaks (e.g. presence of variants conjunctly with multiple glycoforms) due to insufficient spectral resolution or mass measurement accuracy, insufficient detection sensitivity for minor Tf glycoforms, as well as improper deconvolution parameter settings should be considered to guarantee data quality [75]. Measured masses of some intact Tf glycoforms/variants can vary by only a few Da, potentially yielding misleading interpretation for structures presenting with similar masses. For instance, van Scherpenzeel et al. reported masses of the major 4-sialo Tf glycoform ranging from 79,546 to 79,560 Da (expected theoretical mass: 79,556 Da) across a cohort of ~100 controls and patients [47]. Although such mass variability should not be problematic for Tf variant screening presenting with ~30 Da mass differences as reported by Zühlsdorf et al., it could prevent confident identification of minor glycoforms with close masses [75,76]. Jansen et al. recently performed ESI-QTOF-MS analysis of intact Tf on 247 patient samples and 40 healthy controls, and obtained spectral data of sufficient quality (for interpretation of the glycosylation profile) only for 110 and 39 of those, respectively (~52% of cases), which could be regarded as potentially problematic for routine CDG screening [73].

Besides, there are several CDG that cannot be discriminated by intact Tf glycoprofiling, this protein only displaying a limited set of N-glycans and mainly reflecting the physiological status of the liver. For instance, MOGS-CDG is a glycosylation defect frequently showing a normal Tf

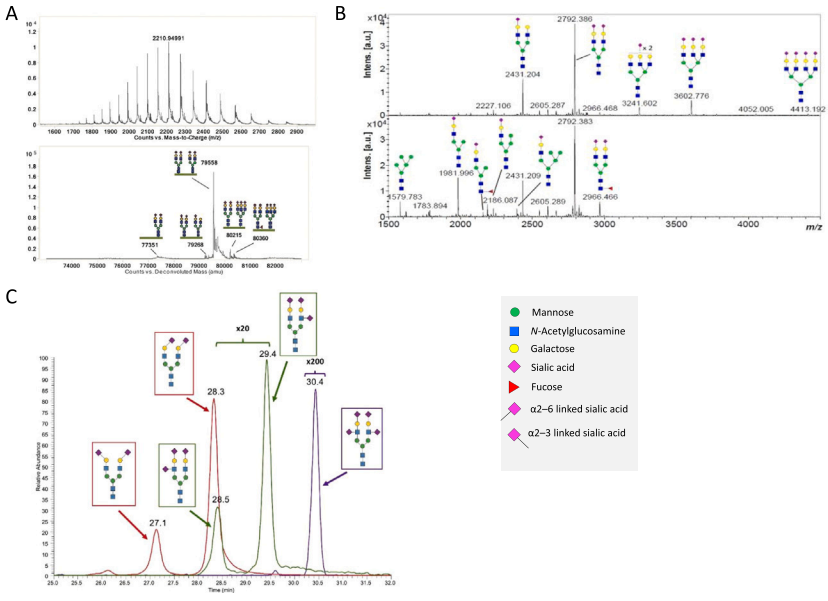


**Fig. 8 Examples of mass spectrometry techniques applied to serum transferrin and N-glycome.** (A) Multiple charged ion distribution of transferrin (top) and reconstructed mass spectrum obtained by deconvolution of the charge distribution raw data (below). (B) MALDI-TOF mass spectra of permethylated N-glycans released (PNGase) from serum sample of a healthy control (top) and a patient with a GlcNAc deficiency (below). Measurements were performed in the positive-ion mode and all ions are present in sodiated form. (C) UHPLC-ESI MS analysis of disialo-, trisialo- and tetrasialo-biantennary N-glycans from patient serum Tf pinpoints different sialo-isomers. Superimposed extracted ion chromatograms of some significant RFMS labeled Tf biantennary sialoglycans. Disialo-isomers (red chromatogram) and trisialo-isomers (green chromatogram) eluted at different retention times depending on α2–6 or α2–3 NeuAc linkage positions at the Gal residue. Ion currents associated to the tri-sialylated and tetra-sialylated species (violet chromatogram) are reported with 20-fold and 200-fold enhanced intensity, respectively. The PNGase released oligosaccharides were RFMS labeled and separated by HILIC. *(A) From M. van Scherpenzeel, G. Steenbergen, E. Morava, R.A. Wevers, D.J. Lefeber. High-resolution mass spectrometry glycoprofiling of intact transferrin for diagnosis and subtype identification in the congenital disorders of glycosylation. Transl. Res. 166 (6) (2015) 639–649.e1. doi: 10.1016/j.trsl.2015.07.005. Epub 2015 Aug 8. PMID: 26307094; (C) From L. Sturiale, M.C. Nassogne, A. Palmigiano, A. Messina, I. Speciale, R. Artuso, G. Bertino, N. Revencu, X. Stephénne, C. De Castro, G. Matthijs, R. Barone, J. Jaeken, D. Garozzo. Aberrant sialylation in a patient with a HNF1a variant and liver adenomatosis. iScience. 24 (4) (2021) 102323. https://doi.org/10.1016/j.isci.2021.102323. PMID: 33889819; PMCID: PMC8050382.*

profile while analysis of total plasma and IgG N-glycomes (see below) revealed the abnormal accumulation of oligomannose N-glycan structures [77–79]. Also, Tf carries low level of fucosylation, which makes it rather unsuitable to screen for fucosylation defects such as those potentially associated with GDP-fucose transporter deficiency (SLC35C1-CDG) with only IgG and total plasma N-glycans revealing significant lowered fucosylation [80].

To summarize, each Tf-based technique presents its pros and cons. On the one hand, charge-based techniques are rather inexpensive to operate (in both equipment and running) but provide only limited information on abnormal glycan structures. On the other hand, MS techniques offer detailed glycan structural information, but are much more expensive and difficult to operate in the context of CDG screening. Of additional note, LC-MS techniques are recommended for the follow-up of D-galactose oral treatment in PGM1-CDG, one of the few CDG with a therapeutic option to this day [46].

### 3.3 Total serum/plasma N-glycome analysis after PNGase or EndoH treatment

As mentioned above, the analysis of total serum/plasma N-glycans is a good complement to Tf glycoprofiling. Thus, multiple laboratories have reported robust and efficient methods for the clinical application of total plasma/serum N-glycome to CDG [81–85]. For instance, total plasma N-glycomics in combination with hierarchical clustering has been demonstrated to efficiently stratify patients from a cohort of 99 individuals presenting with abnormal Golgi glycosylation (47 of which being unsolved), and the data pinpointed the unexpected link between *SLC10A7* variants and particular protein glycosylation profiles from four individuals [86].

Profiling of whole plasma/serum N-glycans usually starts by the enzymatic release of all N-glycans from plasma/serum glycoproteins by using peptide-N-glycosidase F (PNGase F). PNGase F cleaves between the first GlcNAc of N-linked oligosaccharides and the Asn residue from N-glycoproteins. Traditionally, released N-glycans are then typically derivatized by using fluorescent tags (e.g. 2-aminobenzamide, procainamide, 8-aminopyrene-1,3,6-trisulfonic acid), or chemically derivatized (e.g. permethylation, sialic acid esterification) prior to their analysis. Although methods involving the coupling of LC or CE separation to fluorescence detection have been fruitfully applied, most of the methods actually deployed for clinical CDG diagnostic are based on MS, using

either or both matrix-assisted laser desorption/ionization (MALDI) and LC coupled to high-resolution ESI MS [82,85]. MALDI-TOF-based approaches are often preferred for N-glycan profiling and relative quantification for CDG diagnosis when high-throughput and relative ease of operation and data interpretation are required (Fig. 8B). One important limitation of such a workflow is that MALDI-TOF MS with any prior N-glycan separation does not distinguish isomeric species such as differently branched mannosylated structures or α2,3- and α2,6-linked SA. Linkage-specific SA derivatization can be used prior to MALDI-TOF MS to facilitate distinction between α2,3- and α2,6-linked SA [80]. Recently introduced workflows involving labeling of N-glycans with a *N*-hydroxysuccinimide carbamate tag (RapiFluor-MS® or RFMS from Waters) bring high detection sensitivity and clinical specificity for CDG diagnosis when resulting derivatized N-glycans are analyzed by high-resolution ESI Q-TOF instruments [87,88]. Direct sample analysis by flow injection (i.e. without any LC column prior to MS analysis) also enables high-throughput characteristics to the approach [87]. Separating N-glycans by hydrophilic interaction liquid chromatography (HILIC) prior to ESI-MS analysis allows to discriminate structural isomers and isomers differing in the linkage type, such as oligomannosylated N-glycans from MAN1B1 deficient patients or sialylated complex N-glycans present in the serum of TMEM199-CDG patients [78]. Lastly, similar analytical workflows are also widely used for highlighting specific N-glycosylation defects of previously isolated plasma-derived glycoproteins such as Tf, IgG or $\alpha_1$-antitrypsin (Fig. 8C) [80,85,89]. Thus, many examples demonstrate that total serum/plasma N-glycan profiling can be very informative and relevant for CDG-II diagnosis and delineation [80,90]. Recent reports also proved its usefulness for CDG-I diagnosis, for instance through the detection of a diagnostic N-tetrasaccharide (and its fucosylated counterpart) and the accumulation of mannosylated N-glycan structures in the serum of various patients presenting with CDG-I such as ALG1-CDG, PMM2-CDG, MPI-CDG, ALG3-CDG, ALG13-CDG, or DPM1-CDG [91,92].

Although efficient in diagnosing many CDG, total plasma/serum N-glycan profiling following PNGase F treatment may not be optimal for decision-making due to the low abundance of some potentially relevant N-glycan signals obtained under these conditions. Endo-β-*N*-acetylglucosaminidase H (EndoH), which cleaves between the two GlcNAc of various high-mannose and hybrid-type N-linked oligosaccharides, has been recently reported as facilitating the diagnosis

of MAN1B1-CDG or SLC37A4-CDG with the evident accumulation of hybrid-type N-glycans which yielded rather low-intensity signals after PNGase F treatment [93–95]. Thus, the development of analytical workflows involving specific enzymes other than PNGase F or chemical treatment strategies would certainly be helpful for specifically highlighting particular N-glycan structures with greater diagnostic value.

### 3.4 Other serum N-glycoproteins

Besides Tf, our team has extensively studied other serum N-glycoproteins as second-line biomarkers in the context of CDG screening. In particular, the analysis of haptoglobin, alpha-1-acid glycoprotein and alpha-1-antitrypsin by classical western blot and/or two-dimensional electrophoresis (2-DE) can complement an abnormal Tf screening to orientate towards CDG-I or CDG-II, especially in atypical cases (e.g., combined CDG and Tf variant) [57]. In western-blot, the absence of complete N-glycans in CDG-I will produce additional bands of lower molecular weight (MW) compared to a healthy control, while the partial loss of sugars in CDG-II will not significantly alter protein migration in most cases [96]. In 2-DE, additional spots of less acidic p*I* can be observed in CDG-I and CDG-II and lower MW will typically be observed in CDG-I [97]. Of note, contrary to IEF, CE and HPLC, western blot and 2-DE can be successfully applied to dried whole blood samples deposited on a Guthrie card.

Lastly, as proposed by the team of Thiel et al., IEF of alpha-1-antitrypsin may also prove useful in screening CDG in the first weeks of life, as it achieves a mature glycosylation profile earlier than Tf [98].

### 3.5 Serum apolipoprotein C-III (mucin core1 O-glycosylation)

Apolipoprotein C-III (apoC-III) is a 79 amino acids-long O-glycoprotein that is synthesized primarily by hepatocytes but also enterocytes. It participates in the metabolism of lipoproteins, mainly as an inhibitor of lipoprotein lipase [99]. As mentioned above (Section 2.2), its primary structure contains a single O-glycosylation acceptor site, on $Thr^{74}$ [20]. In healthy individuals, this site is occupied by a mucin-type core1 O-glycan chain (Gal-GalNAc-O) and apoC-III is mainly mono- and bi-sialylated [22]. Trace amounts of asialylated apoC-III glycoforms may also be detected (Fig. 3). The first technique developed to study apoC-III glycoforms for CDG screening was IEF followed by passive transfer and immunorevelation [22]. Three types of patterns were described:

"apoC-$III_2$", "apoC-$III_1$" and "apoC-$III_0$", characterized respectively by increased proportions of 2-, 1- and 0-sialo-apoC-III. Notably, apoC-$III_0$ and apoC-$III_1$ patterns were described in individuals with CDG affecting both N- and mucin-type O-glycosylation e.g., CDG related to the vesicular Golgi trafficking (COG-CDG) or to the V-ATPase proton pump (ATP6V0A2-CDG, CCDC115-CDG) [22]. In agreement with our laboratory experience, obesity, liver diseases and critical resuscitation states were notably described to significantly alter apoC-III glycoforms proportions [100]. Furthermore, apoC-III patterns could also be susceptible to protein polymorphisms, which can typically be identified after neuraminidase treatment [22]. In exceptional cases, as with Tf, patients may present with polymorphism affecting the O-glycosylation site [101].

While a performant technique, apoC-III IEF suffers from the same drawbacks as Tf IEF: it is a manual technique that requires expertize to implement in laboratories. In parallel with Tf, a CE technique was developed to separate apoC-III glycoforms from commercial solutions [102]. Although promising, its implementation on serum/plasma samples remains to be achieved before being employed for CDG screening. Additionally, MALDI-TOF MS was proposed as a standalone test for the study of intact apoC-III major glycoforms in the field of CDG (Fig. 9A). Although artefactual SA losses related to laser intensity were reported, this technique showed good capacity to screen some CDG linked to Golgi homeostasis defects (e.g., ATP6V0A2-CDG and COG-CDG) [103,104].

2-DE has been proposed as a refinement over IEF for the analysis of apoC-III glycoforms. In 2-DE, a polyacrylamide gel electrophoresis is performed following IEF, which allows further separation of apoC-III glycoforms based on their MW (Fig. 9B) [105]. Most interestingly, this technique can separate various asialylated apoC-III glycoforms. These include apoC-$III_{0a}$ (non-glycosylated apoC-III), the increase of which we showed several times to be strongly evocative of COG-CDG (Fig. 9B); apoC-$III_{0b}$ (apoC-III linked to a single GalNAc residue) and apoC-$III_{0c}$ (apoC-III linked to a Gal-GalNAc disaccharide), which in practice are detected only in a few very peculiar cases [105]. Additionally, 2-DE can reveal some additional interesting asialylated glycoforms, which will be discussed later. Lastly, we also showed that apoC-III patterns could display typical O-glycosylation defects in some patients with Golgi homeostasis CDG presenting with normal Tf patterns [61].

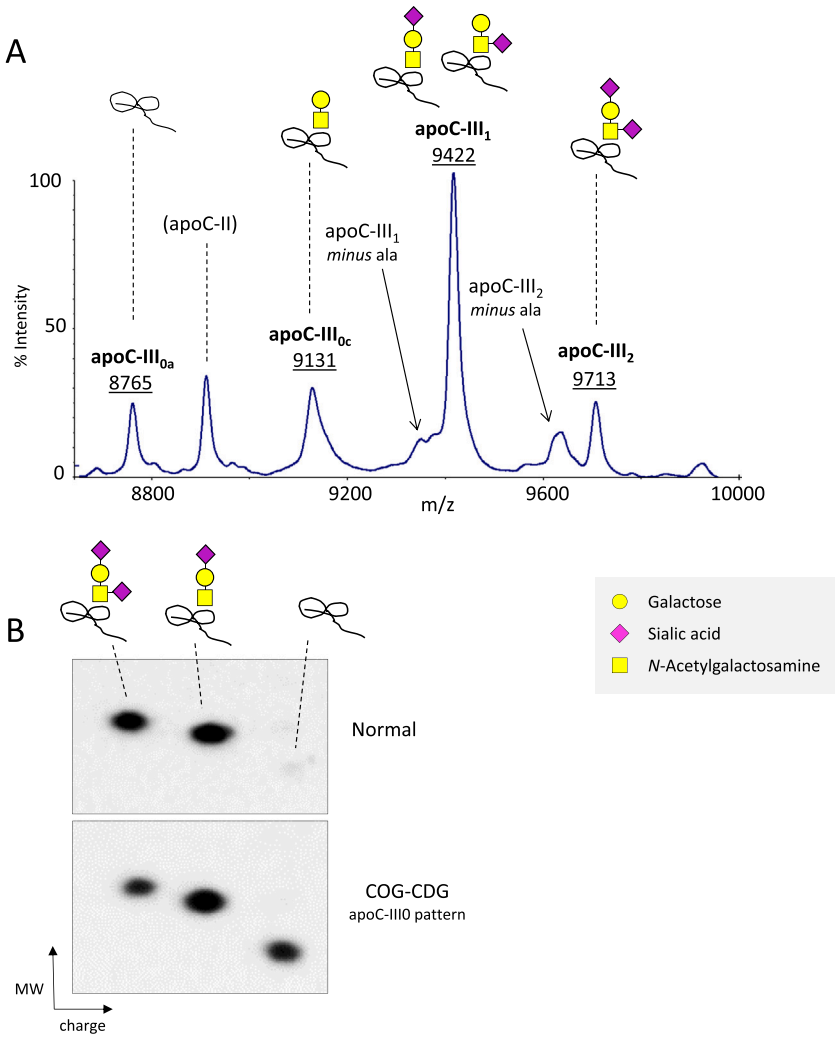


**Fig. 9 MALDI-TOF and two-dimensional electrophoresis of apolipoprotein C-III glycoforms.** (A) MALDI-TOF mass spectrum of serum/plasma O-glycosylated apolipoprotein C-III (apoC-III) glycoforms from a healthy control. Three major apoC-III glycoforms can be detected: apoC-III bearing a mucin core1 motif (Gal-GalNAc-O) substituted by one (apoC-$III_1$) or two sialic acids (apoC-$III_2$), and an aglycosylated isoform named apoC-$III_{0a}$. It has been shown that the glycoform only harboring the core1 structure (apoC-$III_{0c}$) is mostly artefactual, since its level increases in parallel to the applied laser intensity. (B) Two-dimensional electrophoresis (2-DE) pattern of serum/plasma O-glycosylated apolipoprotein C-III (apoC-III) glycoforms. After 2-DE and immunostaining of a control serum/plasma, apoC-$III_2$ and apoC-$III_1$ can be detected while apoC-$III_{0a}$ is barely detectable (top). In a sample from a COG-CDG affected individual (below), the apoC-$III_{0a}$ glycoform is typically significantly increased.

## 3.6 Cellular CDG biomarkers

Although essential to further characterize deficiencies, robust cellular glycosylation biomarkers are lacking in the CDG field. Few of them such as ICAM-1, LAMP2, TGN46 and alphadystroglycan are currently studied by Western blot to detect and better characterize glycosylation deficiencies. In general, glycosylation defects lead to abnormalities in their electrophoretic migration profiles and/ or a significant decrease in their abundance. The intercellular cell adhesion molecule-1 (ICAM-1) has been identified as an interesting cellular CDG-I biomarker as the lack of N-glycans leads to its degradation [106,107]. Regarding N- and/or O-glycan processing defects, LAMP2 and TGN46 are interesting cellular biomarkers. LAMP2 is a heavily N-glycosylated type-1 lysosomal membrane protein presenting abnormal electrophoretic migration profiles in fibroblasts of some CDG-II patients [108–110]. This marker has been widely used to identify Golgi N-glycosylation defects mainly in patients with Golgi homeostasis deficiencies (e.g., COG-CDG, TMEM165-CDG). Similarly, TGN46 is a Golgi glycoprotein which is mainly O-glycosylated for which abnormal electrophoretic migration profiles have been observed in specific CDG subtypes (e.g., TMEM165-CDG) [111]. This marker allowed to specifically identify Golgi O-glycosylation defects in fibroblasts of CDG patients. Regarding alpha-dystroglycan, this unique O-mannosylated muscular glycoprotein is used as a cellular biomarker of rare alpha-dystroglycanopathies (e.g., POMT1/2-CDG, B3GALNT2-CDG) [112,113]. Of note, the robustness of these biomarkers highly depends on the deficiency. In our experience, some deficiencies clearly impact the glycosylation of these markers and some not, certainly due to different compensatory mechanisms according to cell types.

Besides diagnostic applications, glycosylation cellular biomarkers could be of interest in the research and development of novel pharmacological agents (e.g., epalrestat in PMM2-CDG) [114].

## 3.7 Serum bikunin (O-xylosylation of glycosaminoglycans)

Bikunin is a "protein-glycosaminoglycan-protein" (PGP) that is synthesized by hepatocytes as various isoforms found at high concentrations in serum. These are involved in many physiological processes, including extracellular matrix stabilization, inhibition of inflammatory proteases and fetal development [24]. The major serum bikunin isoforms include (i), the free core protein (Bkn), (ii) Bkn bearing a chondroitin sulfate chain (Bkn-

CS) attached via the O-linked GlcA-Gal-Gal-Xyl-O linker tetrasaccharide and (iii), Bkn-CS esterified by one or two "heavy chain" (HC) proteins leading respectively to the so-called "pro-α-trypsin inhibitor" (PαI) and "inter-α-trypsin inhibitor" (ITI) (Fig. 10A).

Our laboratory developed serum Bkn-CS as a biomarker of GAG defects such as linkeropathies i.e., inherited diseases affecting the enzymes involved in the synthesis of the GlcA-Gal-Gal-Xyl-O tetrasaccharide [115,116]. More precisely, we showed that bikunin western-blot in patients with linkeropathies (e.g., B4GALT7-CDG, B3GAT3-CDG) systematically displayed highly increased levels of abnormal light forms pointing to a defect in the protein-linked tetrasaccharide (Fig. 10B) [115,116]. Furthermore, 2-DE of these abnormal forms displayed apparently signature patterns hinting at the causative mutated gene [115]. Lastly, we recently showed that 2-DE of serum bikunin could present, in peculiar experimental conditions, great interests in the screening of inherited diseases affecting the important sulfation of GAG [117].

While a powerful tool for the diagnosis of linkeropathies, Bkn-CS analysis has some limitations. Indeed, since Bkn-CS is mainly synthesized by hepatocytes, we can only detect deficiencies in enzymes expressed in the liver. For instance, XYLT1-CDG affected individuals displayed normal Bkn-CS profiles as xylosyltransferase 1 (XYLT1) is absent in hepatocytes and replaced by xylosyltransferase 2 (XYLT2) for the first step of the tetrasaccharide linker synthesis [116]. In addition, the elongation of the CS chain linked to bikunin is theoretically not impacted in diseases specifically affecting dermatan sulfate, heparan sulfate and keratan sulfate GAG synthesis.

## 4. Emerging approaches as sources of new or more informative CDG biomarkers

### 4.1 Implementation of glycoproteomics for protein- and site-specific information

As discussed above and exemplified, MS-based total plasma/serum N-glycan profiling represents a particularly relevant qualitative or (semi-) quantitative tool for CDG diagnosis and delineation. One of the main drawback or limitation of this approach is associated with the difficulty to functionally interpret the generated data and is linked to the fact that total plasma/serum N-glycan profile reflects the glycosylation of all the glycoproteins present in the sample. Thus, an alteration in the overall

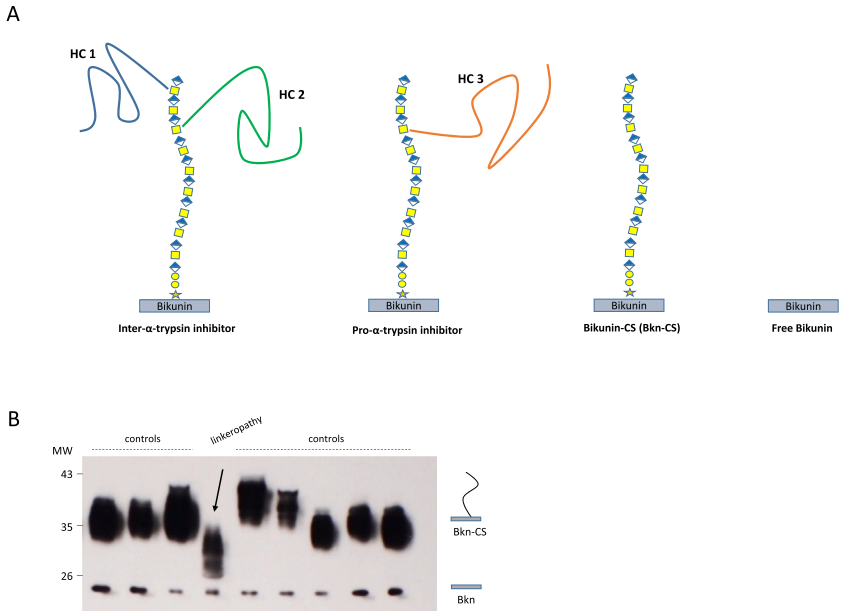


**Fig. 10 Serum/plasma bikunin for the screening of some inherited GAG biosynthesis disorders.** (A) Simplified structure of bikunin isoforms. The heavy forms: inter-α-trypsin inhibitor (ITI) and pro-α-trypsin inhibitor (PαI) result from the esterification of the glycoproteins HC1, HC2 and HC3 with the chondroitin sulfate (CS) chain of the bikunin (Bkn) core protein. The light forms correspond to Bkn-CS and the core protein Bkn. The CS chain consists of 15+/−3 [GlcA-GalNAc] and is sulfated at several GalNAc residues and at the second Gal residue of the linker region (not shown). The HCs are linked to the Bkn-CS chain via ester bonds. (B) Western-blot of serum/plama bikunin light forms. Compared to controls, the western-blot of bikunin light forms from serum/plasma of a linkeropathy-affected individual shows a large band of decreased molecular weight (MW), traducing a major defect in the initial biosynthesis of the CS chain.

glycosylation profile can originate from one or more of the 24 highly abundant glycoproteins accounting for half of the total serum/plasma protein concentration [118]. Obtaining protein- and site-specific information about glycosylation site occupancy (macroheterogeneity) and glycan structure heterogeneity at a given glycosylation site (microheterogeneity), would bring meaningful functionally relevant data. Hulsmeier et al. quantitatively evaluated the macroheterogeneity of Tf and $\alpha_1$-antitrypsin by LC-MS/MS in the serum from a dozen of CDG-I patients [119]. Interestingly, they found that (i) a clear correlation could be

observed between the degree of N-glycosylation site occupancy and the severity of the disease, (ii) Tf was more prone to underglycosylation than $\alpha_1$-antitrypsin and (iii) that the $Asn^{611}$ site showed the greatest dynamics in site occupancy in pathological conditions (~40 to 90% site occupancy). Later, this approach has been extended to Tf, haptoglobin, $IgG_2$ and $IgA_1$ proteins and the data demonstrated reduced N-glycosylation site occupancies for the ten studied CDG-I patient samples with liver synthesized Tf and haptoglobin, whereas no change was observed with the glycopeptides from the immunoglobulins $IgA_1$ and $IgG_2$ [120]. In addition, hypoglycosylation of haptoglobin has been shown to reach to as low as 3% site occupancy in the most extreme situation. Although the physiological significance of such glycosylation site occupancies remains to be demonstrated, these MS-based approaches hold great potential and promise for bringing meaningful protein- and site-specific information. These studies also confirmed that individual glycoproteins respond differently to CDG, glycopeptide-centric diagnostic can therefore hold great(er) specificity and sensitivity. One could imagine extending further such experimental workflow to many other glycoproteins, for example through the quantitative monitoring of 100 glycosylation sites from 50 serum/plasma glycoproteins as recently proposed by Li et al. [121]. Recently, Wessels et al. performed a LC-HRMS profiling of 191 glycoforms from 58 glycopeptides originating from 34 circulating plasma proteins, in 40 controls and 74 CDG affected individuals [122]. In these conditions, the authors were able to observe site-specific N-glycosylation variations. Interestingly, in MAN1B1-CDG individuals, they noted a specific change in Tf glycosylation at $Asn^{432}$, while $Asn^{630}$ was unaffected (*note: these positions include the 19 amino acid-long signal peptide*).

The development of absolute quantification approaches of glycans and glycoproteins/glycopeptides in human fluids using MS and stable isotope labeling thus appears highly desirable for a more sensitive and specific CDG diagnostic. Besides, previous studies have shown that although human plasma/serum N-glycome is rather stable within an individual, it can be significantly influenced by genetic and environmental factors [123–125]. For instance, concentrations of acute phase glycoproteins (e.g. haptoglobin, $\alpha_1$-acid glycoprotein) can be significantly impacted by inflammation occurring in many diseases, this would therefore consequently modify the N-glycan patterns of related samples [126]. Here also, absolute quantification would allow defining reference values for glycans/glycoproteins, which in turn may help revealing pathophysiological

changes at an early stage. Such an objective is clearly not out of reach as could be judged from recent literature (see for instance the recent review of Patabandige et al. [127]).

## 4.2 Fucosylated and asialylated mucin core2 O-glycoforms of apoC-III

Beside mucin core1 glycoforms, minor asialylated and fucosylated mucin core2 apoC-III glycoforms have been detected in serum. As schematized Fig. 11A, the mucin core2 motif is a trisaccharide moiety corresponding to the core1 (Gal-GalNAc-O) laterally substituted by a GlcNAc residue as catalyzed by various core2 GlcNAc transferases (C2GnTI, II, III). Following this GlcNAc linkage, the nascent polysaccharidic chain is further elongated thanks to the sequential actions of various galactosyltransferases (β1–4GalT5, β1–4GalT4) and of GlcNAc transferase 7 (β1–3GlcNAcT7), giving rise to a poly-*N*-acetyl-lactosamine (-Gal-GlcNAc-)$_x$ chain of variable length. Furthermore, this one can be fucosylated on GlcNAc and/or terminal galactose residues by numerous fucosyltransferases (FUT3 to FUT7; FUT9). Using ultra-sensitive MS methods, it has been reported up to six apoC-III-linked mucin core2 structures harboring two to four fucose residues [128]. Using 2-DE, we were able to detect, beside non-glycosylated apoC-III$_0$, up to three additional spots probably corresponding to the major circulating asialylated and fucosylated mucin core2 glycoforms (Fig. 11B). Thus, we think that ultrasensitive MS or perhaps more convenient 2-DE based analysis of apoC-III could provide interesting serum biomarkers for the screening of still largely unexplored fucosylation and/or mucin core2 O-glycosylation defects. Indeed, despite the important pathophysiological roles played by these two frequent and very diverse types of glycosylation, very few related CDG have been described so far (FCSK-CDG, SLC35C1-CDG, FUT8-CDG for fucosylation defects), notably due to the current lack of convenient biomarkers.

## 4.3 Metabolomics

Metabolomics studies the whole set of small molecule metabolites (the metabolome) found in a biological system. It is recognized as one of the omics discipline that is closest to the phenotype [129]. Metabolomics can provide a comprehensive view of the metabolites present in complex matrices such as human biofluids and can be used to identify a set of metabolites that are associated with physiological or pathological

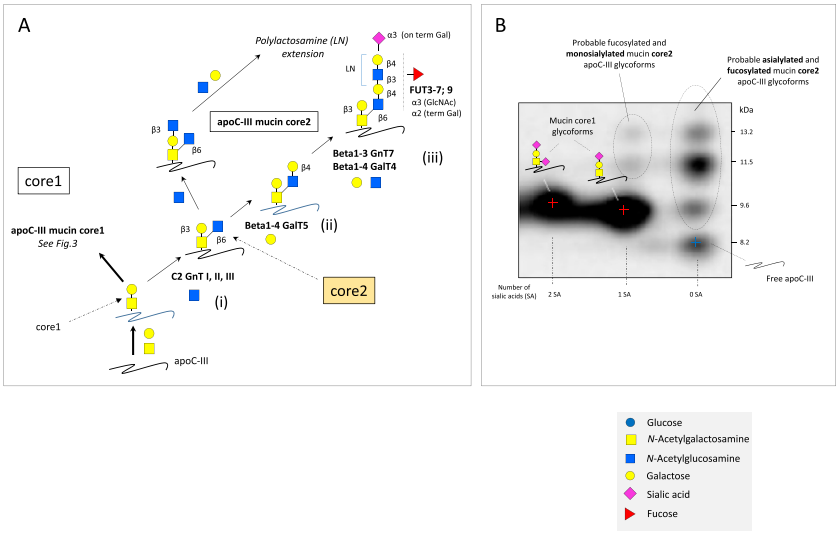


**Fig. 11 Mucin core2 O-glycosylation of apolipoprotein C-III and probable 2-DE-separated asialylated and fucosylated related glycoforms.** (A) Beside the major mucin core1 O-glycosylation (left), minor mucin core2 apoC-III glycoforms could emerge (right) from the action of (i), core2 GlucNAc transferases (C2GnTI, II, III; on the core1 motif), (ii) β1–4 galactosyltransferase 5 (β1–4 GalT5) and (iii), β1–3 GlucNAcT7 and β1–4 GalT4 both sequentially elongating a polylactosamine $(Gal\text{-}GlcNAc)_x$ chain. Furthermore, this later chain can be fucosylated (by fucosyltransferases FUT3–7;9) on internal GlcNAc residues and on terminal Gal. Lastly, the terminal Gal can be sialylated. (B) Two-dimensional electrophoresis (2-DE) followed by western-blotting allowed us to detect up to three asialylated (and possibly fucosylated) serum apoC-III glycoforms, probably corresponding (according to their measured MW) to mucin core2 O-glycosylation. Furthermore, two additional faintly stained monosialylated glycoforms can be noticed.

conditions. The focus of the field has often been to identify biomarkers or dysregulated pathways. Interestingly, metabolites not only reflect the metabolic activity of tissues but, because many metabolites have biological activity on critical pathophysiological processes, they can also influence the clinical phenotype [130].

Therefore, large-scale (untargeted) metabolomics profiling can be helpful to get deeper insight into the pathophysiological biochemical mechanisms underlying CDG as well as potentially pointing to innovative targets for further biomarker discovery or drug development. At the time of our precedent literature review on that topic in 2020 [90], only sporadic applications of metabolomics targeting a limited set of metabolites were published but already demonstrated the potential of the approach to

highlight relevant metabolic dysregulations. For instance, Dimitrov et al. used targeted metabolomics to observe that the metabolism of amino acids as well as lipid homeostasis were significantly dysregulated in ATP6AP1-CDG [131].

To the best of our knowledge, only a few studies involving MS-based metabolomics have been published since then. For instance, by performing tracer-based metabolomics and targeting 45 metabolites in PMM2-CDG patient-derived fibroblasts, Radenkovic et al. suggested that PMM2 deficiency affects intracellular glucose flux, resulting in polyols (sorbitol, mannitol) increase. The selective inhibition of aldose reductase by a pharmacological agent (i.e., epalrestat) was suggested to divert glucose flux from polyol production toward the synthesis of GDP-mannose to ultimately improve glycosylation, which could be beneficial to PMM2-CDG affected individuals [132]. This study offers an exciting display of how metabolomics and flux analysis experiments can provide a better understanding of underlying biochemical mechanisms and thus propose a relevant therapeutic option. Zdrazilova et al. also studied PMM2-CDG derived fibroblasts using metabolomics [133]. The data presented suggested bioenergetics redirection together with suppressed glycolysis in PMM2-CDG patient cells, thereby causing alterations of the tricarboxylic acid cycle in connection with the mitochondrial electron transport chain [133].

These two relevant papers are representative of published studies dealing with metabolomics application to CDG. Although highly relevant and providing meaningful biological information, these articles both targeted a limited number of metabolites and involved human fibroblasts. There is no doubt that they pave the way to further studies with broader metabolome coverage performed on more clinically relevant material (e.g. animal models) and later, on human samples. This could be exemplified by a recent paper which showed that classical galactosemia significantly impacts several metabolic pathways such as glycolysis, the pentose phosphate pathway, and nucleotide metabolism in patients' erythrocytes [134]. As an example of what could be done, untargeted plasma metabolomics was used to study the metabolic defects associated to glycogen storage disease type I (GSD1) and highlighted significant alterations of many metabolic pathways including pathways of fuel metabolism and energy generation, lipids and fatty acids, amino acid and methyl-group metabolism, the urea cycle, and purine/pyrimidine metabolism (Fig. 12) [135].

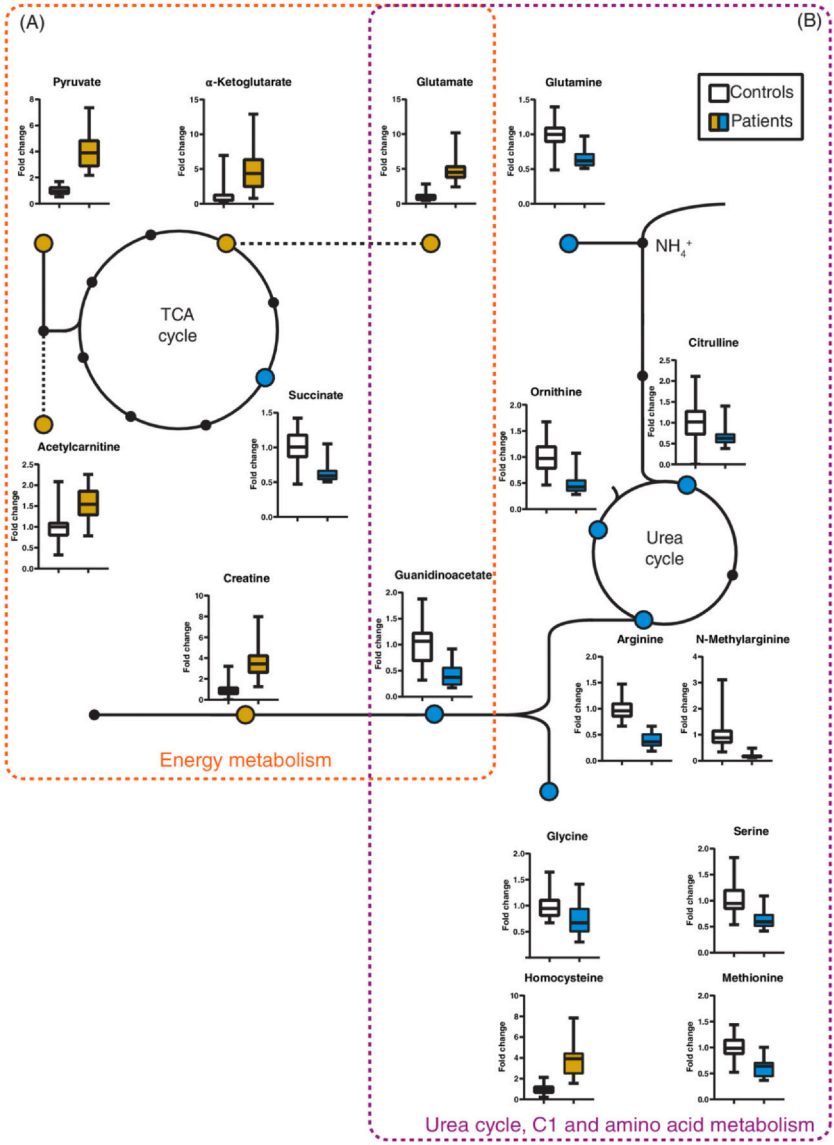


**Fig. 12 An example of untargeted plasma metabolomic approach (in glycogen storage disease I) potentially transposable to CDG.** Significantly altered metabolites associated with energy metabolism (A) as well as amino acid, C1 and urea cycle metabolism (B). Metabolites that are increased in GSD1 patients compared to controls are shown in yellow, decreases in blue. *From T. Mathis, M. Poms, H. Köfeler, M. Gautschi, B. Plecko, M.R. Baumgartner, M. Hochuli. Untargeted plasma metabolomics identifies broad metabolic perturbations in glycogen storage disease type I. J. Inherit. Metab. Dis. 45 (2) (2022) 235–247. https://doi.org/10.1002/jimd.12451. Epub 2021 Nov 10. PMID: 34671989; PMCID: PMC9299190.*

### 4.4 Metallomics

Particular attention has been paid on the quantification of metals since their recent involvement in glycosylation regulation [136]. Hyphenated techniques combining separation techniques with elemental detection such as inductively coupled plasma (ICP) MS or, more rarely, molecular detection (ESI or MALDI) can be used. This can be done on cells, body fluids and tissues and allows the quantification of various metals. These techniques are highly sensitive and can be of interest to characterize the nature of the deficiency and potential dysregulations of metal homeostasis. At the cellular level, this methodology can be particularly powerful to follow differential metal homeostasis in different compartments. We recently used a digitonin cell permeabilization based approach, coupled to ICP-MS analysis, to investigate and quantify the differential amounts of metals in the cytosol and membrane-bound organelles of control and isogenic KO cells for the manganese transporter TMEM165 [137]. With the recent identification of new CDG characterized by impaired cellular metals homeostasis, these innovative analytical techniques could undoubtedly find their place in the clinical chemistry laboratory in the future.

## Summary and perspectives

Congenital disorders of glycosylation (CDG) are one of the fastest growing groups of inborn errors of metabolism, involving a plethora of molecular actors. Parallel to the continuous identification of new CDG, several novel biomarkers and analytical techniques applicable to the diagnostics of these complex diseases were developed. Currently, the study of Tf glycoforms remains the foremost analysis in the context of CDG screening. Classical analytical techniques (IEF, CE, HPLC), which are robust and affordable, are most popular in Europe, while novel techniques based on MS (e.g., ESI-MS of immunopurified Tf) have become commonly used in the United States and in Japan. These offer extensive details on structures of Tf-bound N-glycans, with the benefit of pointing to a precise genetic defect in certain cases. Other biomarkers, such as apolipoprotein C-III and bikunin, can help evidence defects in other glycosylation pathways (O-glycosylation and GAG synthesis), which is informative in the context of diagnosis of certain entities (e.g., CDG-II with Golgi homeostasis defects, or linkeropathies).

In parallel, novel approaches based on the study of whole plasma/serum N-glycome by various MS techniques (MALDI-TOF, ESI-QTOF…) have been developed, offering a global picture of the N-glycosylation status. These can highlight defects on glycoproteins other than Tf, which is especially useful in some CDG characterized by normal Tf profiles. Finally, new "omics" approaches (metabolomics and metallomics) offer exciting prospects in both clinical practice (biomarkers for precision diagnostics, disease follow-up, therapy management) and in basic and translational research (better understanding of the role of actors in glycosylation, application to the development of novel pharmacological agents). Therefore, the clinical chemist must be aware of the strengths and limitations of each biomarker and technique, so to optimally assist the clinician in navigating the maze of glycosylation disorders, and to participate in the furthering of knowledge in this group of yet poorly understood diseases. A proposed updated flowchart for laboratory CDG diagnosis is presented as a Supplementary file.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/bs.acc.2024.03.001.

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