Diagnostic and Therapeutic Approaches in Congenital Disorders of Glycosylation

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Contents

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Abstract

Congenital disorders of glycosylation (CDG) constitute an increasing group of inborn metabolic disorders, with more than 170 described diseases to date. A disturbed glycosylation process characterizes them, with molecular defects localized in distinct cell compartments. In CDG, N-glycosylation, O-glycosylation, glycosylation of lipids (including phosphatidylinositol) as well as the glycosaminoglycan synthesis can be affected. Owing to the importance of glycosylation for the function of concerned proteins and lipids, glycosylation defects have diverse clinical consequences. CDG affected individuals often present with a non-specific multivisceral syndrome including neurological involvement, intellectual disability, dysmorphia, and hepatopathy. As CDG are rare diseases frequently lacking distinctive symptoms, biochemical and genetic testing bear important and complementary diagnostic roles.

After an introduction on glycosylation and CDG, we review current biomarkers and analytical techniques in the field. Furthermore, we illustrate their interests in the follow-up of proven therapeutic approaches including D-mannose in MPI-CDG, D-galactose in PGM1-CDG, and manganese ($MnSO_4$) in TMEM165-CDG.

Keywords

Apolipoprotein C-III · Bikunin · CDG · Galactose · Glycosylation · Manganese · Mannose · MPI-CDG · PGM1-CDG · TMEM165-CDG · Transferrin

Abbreviations

2-DE	Two-dimensional electrophoresis
ALG	Asparagine-linked glycosyltransferase
apoC-III	Apolipoprotein C-III
Asn	Asparagine
Bkn	Bikunin
CDG	Congenital disorder(s) of glycosylation
CDT	Carbohydrate deficient transferrin
CE	Capillary electrophoresis
COG	Conserved oligomeric Golgi
CS	Chondroitin sulfate

DS	Dermatan sulfate
EndoH	Endo-β-*N*-acetylglucosaminidase H
ER	Endoplasmic reticulum
ESI	Electrospray ionization
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 adhesion molecule-1
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
MPI-CDG	Mannose-phosphate isomerase-CDG
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MW	Molecular weight
PαI	Pro-α-trypsin inhibitor
PGM1-CDG	Phosphoglucomutase 1-CDG
PIG-CDG	Phosphatidylinositol glycan-CDG
PMM2-CDG	Phosphomannomutase 2-CDG
PNGase F	Peptide-N-glycosidase F
Q-TOF	Quadrupole – time of flight
RFMS	Rapifluor^{R} mass spectrometry
SA	Sialic acid
Ser	Serine
Tf	Transferrin
TGN46	Trans-Golgi network protein 46
Thr	Threonine
UDP	Uridine-diphosphate
VUS	Variant of unknown significance
Xyl	Xylose

1 Introduction

Glycosylation is a co-/post-translational modification corresponding to the covalent linkage of a glycan chain to a protein or a lipid (Varki 2017). Protein-bound glycans are synthesized through sequential additions and removals of monosaccharides catalyzed by specific enzymes. In humans, glycans are mainly bound to proteins via the nitrogen of an asparagine (Asn) (N-linked glycans) or the oxygen of a serine (Ser) or threonine (Thr) (O-linked glycans). N-glycosylation begins in the endoplasmic reticulum (ER), followed by trimming and maturation in the Golgi apparatus, fueled by nucleotide-sugars synthesized in the cytoplasm (Colley et al. 2022). With regard to O-glycans, it can be distinguished as follows: mucin-type O-glycosylation, where the glycan chain is linked via an *N*-acetylgalactosamine (GalNAc); O-xylosylation, where it is linked via a xylose (Xyl) residue (leading to glycosaminoglycans chains); O-fucosylation; and O-mannosylation. Similar to the terminal steps of N-glycosylation, O-glycosylation mainly occurs in the Golgi apparatus (Brockhausen et al. 2022), and a fine regulation of the homeostasis of this organite, notably in terms of pH, trafficking, and ions transport, is mandatory (Rivinoja et al. 2009).

Over 170 inborn errors affecting glycosylation have been described and are designated as congenital disorders of glycosylation (CDG). Besides CDG affecting protein glycosylation, CDG affecting phosphatidyl inositol-linked glycans (PIG-CDG) and lipid glycosylation has also been described (Ng and Freeze 2018). The clinical phenotype of CDG greatly varies with multisystemic diseases including neurological disorders, intellectual disability, bone/cartilage defects, hepatopathy, and coagulopathy (Ondruskova et al. 2021). In view of these unspecific and broad presentations, biomarkers have been developed to study glycosylation and screen for CDG. While robust blood 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 difficult.

Inspired by a recently published (2024) synthesis work from our team (Raynor et al. 2024a), we provide here a simplified review regarding blood and cellular CDG biomarkers applicable in routine by specialized laboratories. We notably emphasized the diagnostic value of 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 defects. We also consider more recently developed glycoproteomic tools for the study of protein-specific N-glycosylation defects. In addition, we illustrate the interests of these biomarkers in the currently available proven CDG treatments including oral mannose in PMI-CDG (CDG-1b), oral galactose in PGM1-CDG, and oral manganese in TMEM165-CDG.

2 Glycosylation Types and Congenital Disorders of Glycosylation

2.1 N-Glycosylation

N-glycosylation is the most frequent glycosylation type in humans (Brockhausen et al. 2022). Here, the glycan moiety is bound to the protein via the amide function of an asparagine (Asn), classically located in a consensus sequence Asn-X-Ser/Thr (X: any amino acid except proline). N-glycosylation is a two-step process that begins in the ER and ends in the Golgi apparatus. First, the glycan moiety is synthesized and attached to the nascent proteins in the ER and, second, the N-linked glycan chain undergoes maturation in the ER and Golgi apparatus.

As shown in Fig. 1, the first step begins at the cytosolic face of the ER membrane: sugars are attached sequentially to an anchored lipid named dolichol-phosphate (dol-P) independently of the protein translation process. These monosaccharides are available as nucleotide-sugars synthesized in the cytosol and are linked, thanks to

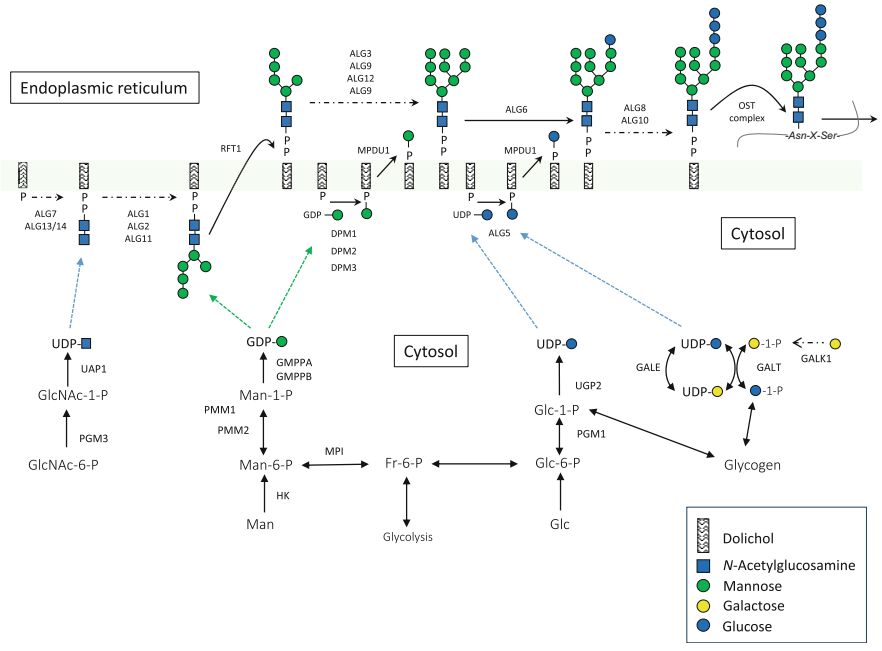

Fig. 1 First steps of N-glycosylation in the cytosol and endoplasmic reticulum. Nucleotide-sugars (UDP-GlcNAc, GDP-Man, UDP-Glc) synthesized in the cytosol through specific enzymatic machineries are the substrates for cytosolic and ER enzymes which participate in the step-by-step elongation of the precursor glycan linked to the lipidic anchor dolichol. Once synthesized, this precursor is transferred to the acceptor Asn of a nascent protein, thanks to the oligosaccharyl transferase (OST) complex. Enzymes abbreviations correspond to official gene names

the catalysis of glycosyltransferases of the ALG (Asn-linked glycosyltransferase) family. First, a *N*-acetylglucosamine-phosphate (GlcNAc-P) is attached to dol-P. Then, a GlcNAc and five mannoses (Man) are attached, yielding a Man_3-(Man)-Man-$GlcNAc_2$-PP-dolichol, which is translocated into the ER lumen. Then, four Man followed by three glucoses (Glc) are linked, yielding the biantennary 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 dol-P before translocation into the ER. Then, the biantennary polysaccharidic chain is transferred from the dol-P to the protein being translated, through the catalysis of the oligosaccharyltransferase (OST) complex (Fig. 1) (Breitling and Aebi 2013).

The second step is the maturation of the N-linked glycan (Fig. 2) that begins in the ER, where the glycoprotein undergoes folding and quality control. First, glucosidases I and II remove two terminal Glc, yielding a Glc_1-Man_3-(Man_5)-Man-$GlcNAc_2$ motif, which is recognized by calnexin and calreticulin, two lectins

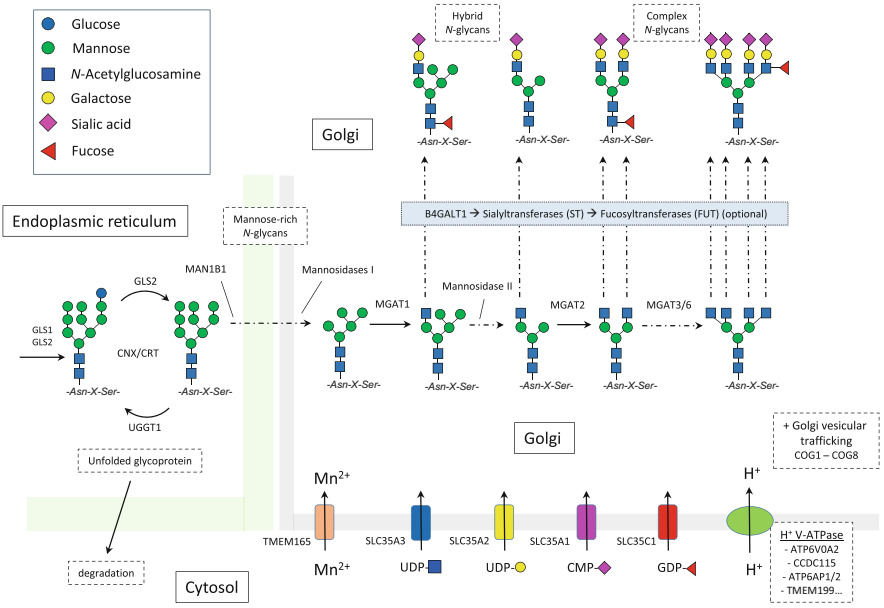

Fig. 2 Trimming and maturation of protein-linked N-glycans in the late endoplasmic reticulum and Golgi. After a quality control involving calnexin (CNX) and calreticulin (CRT), the "mannose-rich" intermediate N-glycan is trimmed by various mannosidases in the ER and the Golgi. Thereafter, GlcNAc, Gal, SA, and fucoses are successively added in this order (thanks to several glycosyltransferases), yielding a N-linked oligosaccharide of "complex-type" or "hybrid-type." Nucleotide-sugars (UDP-GlcNAc, UDP-Gal, CMP-SA, and GDP-Fuc) are synthesized in the cytosol and enter the Golgi through specific transporters of the SLC35 family. Golgi homeostasis (notably pH, Mn^{2+} concentration, and vesicular trafficking) plays a crucial role in the regulation of most molecular steps related to N-glycosylation. Enzymes abbreviations correspond to official gene names

which facilitate the glycoprotein folding (Ellgaard and Frickel 2003). At the end of the folding process, glucosidase II removes the remaining terminal Glc (Fig. 2) (Ellgaard and Helenius 2003). If the glycoprotein is incorrectly folded, the UDP-glucose glycoprotein glycosyltransferase (UGGT) adds a new Glc, and the glycoprotein can be recognized again by the lectins and undergoes a new folding process (Ellgaard and Helenius 2003). If correct folding is not achieved, three to four Man are removed and the unfolded glycoprotein is then exported to the cytosol for degradation (Hosokawa et al. 2003). If correct folding is achieved, the glycoprotein is transferred to the Golgi apparatus for further maturation (Fig. 2). Golgi mannosidases remove three Man; then, a GlcNAc transferase catalyzes the addition of a GlcNAc on one of the two antennae of the chain and mannosidase II removes 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, including (in this order) GlcNAc, galactose (Gal), terminal sialic acid (SA), and fucose (Fuc) (Stanley 2011). These sugars are available as nucleotide-sugars, which are transported from the cytosol into the Golgi apparatus, thanks to membrane transporters of the SLC35 family (Fig. 2).

In healthy individuals, N-glycans are mostly matured in "complex" N-glycans (Schachter 2014) characterized by the presence of terminal SA. Other types include "mannose-rich" and "hybrid-type" N-glycans (Fig. 2) (Huhn et al. 2009).

2.2 Mucin-Type O-Glycosylation

Mucin-type O-glycosylation takes place in the Golgi apparatus and involves the sequential building of the glycan directly on the protein. In mucin-type O-glycosylation, polypeptide-*N*-acetylgalactosamine transferases (ppGalNAcTs) first link a GalNAc on the oxygen of a Ser or a Thr (Ten Hagen et al. 2003) (no consensus sequence is yet clearly identified). Thereafter, other glycosyltransferases can attach other monosaccharides to build more complex O-glycan chains (core1 to core8 mucin-type structures) (Gill et al. 2011). In O-glycosylation, nucleotide-sugars also originate from the cytosol and are translocated to the Golgi trough SLC35 family transporters.

A mucin-type O-glycosylated protein of interest is serum apolipoprotein C-III (apoC-III) (Vaith et al. 1978). After the linkage of the GalNAc on Thr^{74}, a Gal is added by the core1 synthase (C1GalT1) (Fig. 3a). The Gal-GalNAc disaccharide constitutes the core1 motif upon which a GlcNAc can be added generating minor core2 glycoforms. For the majority core1 glycoforms of apoC-III, the addition of a first SA is catalyzed by β-galactoside α-2,3-sialyltransferase 1 (ST3GAL1), giving rise to the so-called "apoC-III_1" glycoform. Then, the optional attachment of a second SA onto the GalNAc is catalyzed by α-*N*-acetylgalactosaminide α-2,6-sialyltransferase 1 (ST6GalNAc I) leading to the "apoC-III_2" glycoform (Fig. 3a) (Wopereis et al. 2003).

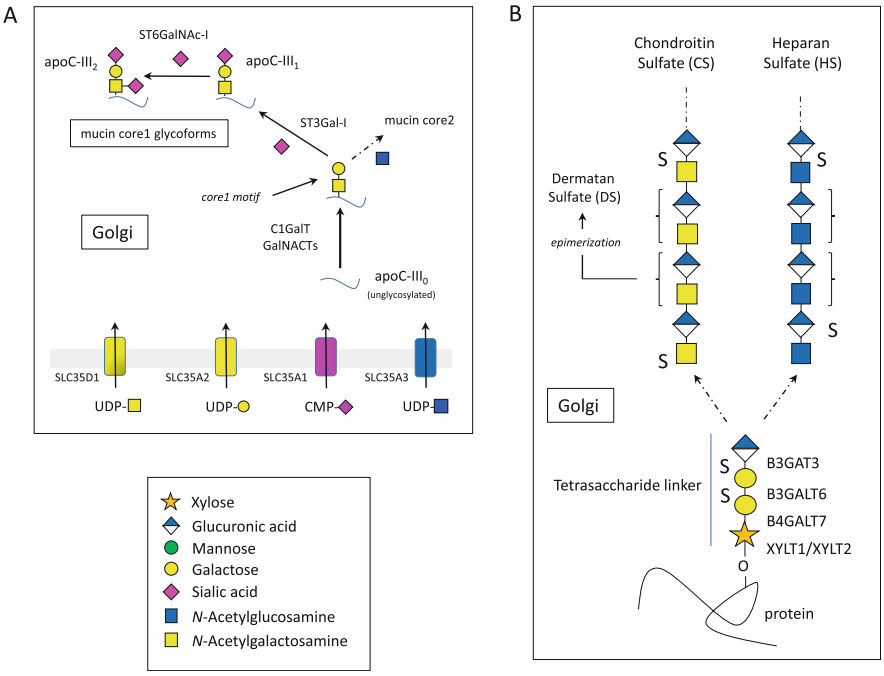

Fig. 3 Mucin core1 O-glycosylation and glycosaminoglycans biosynthesis. (**a**) For the prototype mucin core1 O-glycosylated apolipoprotein C-III (apoC-III), a GalNAc is first attached to the protein. Then, the linkage of a Gal leads to the core1 structure upon which one or two SA can be added, generating the apoC-III_1 and apoC-III_2 glycoforms. These are found in blood together with the minor unglycosylated apoC-III_0 glycoform. The core1 motif can also be substituted by a GlcNAc generating several minor mucin core2 glycoforms. Nucleotide-sugars (UDP-GalNAc, UDP-Gal, CMP-SA, and UDP-GlcNAc) are synthesized in the cytosol and enter the Golgi through specific transporters of the SLC35 family. Golgi homeostasis (notably pH, Mn^{2+} level, and membrane trafficking) plays a crucial role in the regulation of most molecular steps related to mucin core O-glycosylation. Enzymes abbreviations correspond to official gene names. (**b**) Upon the core protein-linked tetrasaccharide linkage region, GAG chain polymerization starts with the transfer of a first amino sugar (GalNAc for chondroitin sulfate [CS] and GlcNAc for heparan sulfate [HS]), followed by the addition of repeat and specific disaccharide moieties. Epimerization from CS to dermatan sulfate (DS) can occur. The GAG chains are typically heavily sulfated. Involved nucleotide-sugars are synthesized in the cytosol and enter the Golgi through specific transporters of the SLC35 family. Heparin and keratan sulfate (KS) biosynthesis pathways are not shown here. Enzymes abbreviations (here limited to the tetrasaccharide linker elongation) correspond to official gene names

2.3 Glycosaminoglycans (GAG) Synthesis

Glycosaminoglycans (GAG) chains are characterized by repetitive disaccharide motifs comprising an amino-sugar (e.g., GlcNAc or GalNAc) and a uronic acid (e.g., glucuronic acid: GlcA). In most of GAG, the chain is O-linked to the protein by a common GlcA-Gal-Gal-Xyl tetrasaccharide linker. This is the case with heparan

sulfate, chondroitin sulfate (CS), and dermatan sulfate (DS) (Fig. 3b). A notable exception is keratan sulfate (KS), which consists of a $(GlcNAc\text{-}Gal)_x$ chain linked through alternate O- or N-linkages (Casale and Crane 2024). GAG synthesis starts in the ER, at the interface with the Golgi apparatus. Once the tetrasaccharide linker synthetized, various glycosyltransferases add a variable number of repeated disaccharide motifs. Similar to other Golgi-localized glycosylation processes, sugar moieties used in GAG synthesis originate from the cytosol, where they are "activated" through their linkage to a UDP. Furthermore, GAGs are abundantly sulfated, making them very negatively charged and hydrophilic (Haouari et al. 2021) (Fig. 3b).

2.4 Congenital Disorders of Glycosylation (CDG)

The high number of enzymes involved in glycosylation, their specificity, their sensitivity to the biochemical microenvironment (pH, ions, etc.), in addition to several transporters and molecular actors, make glycosylation a complex and fragile process. CDG comprises over 170 inborn diseases linked to the inheritance of pathogenic gene variants. They are typically autosomal recessive; however, rare cases of X-linked and autosomal dominant pathogenic variants have been described (Lipiński and Tylki-Szymańska 2021). CDG are rare diseases, with one to less than a hundred described affected individuals. Causative variants can affect genes coding for enzymes (e.g., mutases, glycosyltransferases, mannosidases), Golgi trafficking proteins (e.g., COG complex subunits), nucleotide-sugar transporters (SLC35 family), or ionic transporters (e.g., V-ATPase proton pumps) (Lipiński and Tylki-Szymańska 2021). The first cases of CDG (PMM2-CDG, MGAT2-CDG, MPI-CDG, etc.) were identified on the basis of defective protein glycosylation; subsequently, cases with faulty glycosylation of lipids or glycosylphosphatidylinositol (GPI) anchors (PIG-CDG) have also been reported (Jaeken et al. 1984, 1994; Francisco et al. 2019; Pedersen and Tygstrup 1980).

Clinically, CDG are heterogeneous, typically comprising non-specific symptoms of variable severity. Most commonly, affected individuals present with neurological signs and intellectual disability, failure to thrive, hepatopathy, coagulopathy, and dysmorphia. It is believed that part of these clinical signs results from disturbed glycosylation before birth. Indeed, numerous studies have pointed to the role of glycoproteins in embryogenesis and fetal development, and more particularly in neurological and bone/cartilage development (Yanagishita 1993; Mulkey et al. 2019; Flanagan-Steet and Steet 2013; Schwartz and Domowicz 2018). Glycosylation defects could be responsible for abnormal protein folding, transport, and ligand-receptor binding, thereby altering signaling pathways essential to embryonic development (e.g., TGF-β, sonic hedgehog) and/or directly impacting protein function (e.g., proteoglycans in bone/cartilage growth).

Historically, first CDG cases were discovered based on an abnormal Tf glycosylation pattern, underlying the first classification in type I: CDG-I and type II: CDG-II (Aebi et al. 1999; Marklová and Albahri 2007). CDG-I involves the first part of the

N-glycosylation process (synthesis of the precursor N-glycan attached to dol-P, or its transfer to the nascent protein), where pathogenic variants concern 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 (CDG-Ia), belongs to this category (Jaeken et al. 1984). CDG-IIs involve the second part of the N-glycosylation process (after the linkage to the protein), where pathogenic variants are found in genes coding for ER or Golgi actors. In CDG-II, N-glycan chains are present on Tf but lack variable number of sugars, including terminal SA.

Historical CDG nomenclature was built upon this CDG-I/CDG-II classification; nowadays, each disorder is named after the mutated gene (e.g., PMM2-CDG instead of CDG-Ia) (Jaeken et al. 2009).

3 CDG Biomarkers and Related Laboratory Tools

As clinical presentations of CDG are usually unspecific, their screening/diagnosis relies on biochemical and genetic testing. Biochemical testing can highlight abnormal glycosylation, thanks to the study of specific glycoproteins, which may change in charge and/or molecular weight. Besides, characteristic patterns may orientate the diagnosis toward a specific etiology (Marklová and Albahri 2007). Genetic testing can be performed concurrently to biochemical testing, confirming the presence of causative gene variants. Both approaches are complementary, as genetic testing often results in the discovery of variants of unknown significance (VUS), which necessitate functional biochemical confirmations to assert their pathogenicity. As previously mentioned, the historical biomarker of CDG is serum transferrin (Tf).

3.1 Serum Tf Analysis by Using Isoelectric Focusing, Capillary Electrophoresis or Mass Spectrometry (N-Glycosylation Defects)

Tf is a blood-circulating N-glycoprotein synthesized by the liver and plays a key role in iron metabolism. It contains two N-glycan acceptor sites on Asn^{413} and Asn^{611} (MacGillivray et al. 1983); in healthy individuals, the two sites are occupied by complex bi-antennary N-glycans and Tf is mainly 4-sialylated (4-sialo Tf) while other glycoforms (8- to 5-, 3- and 2-sialo Tf) are present in minor proportions (Fig. 4a) (Coddeville et al. 1998).

The first technique developed to study Tf glycoforms was gel isoelectric focusing (IEF) (van Eijk et al. 1982). Tf glycoforms are gel-separated based on their charge, which depends on their terminal SA content. Elevated proportions of hyposialylated Tf glycoforms highlighted using IEF were first described in individuals with chronic alcohol consumption leading to the establishment of "carbohydrate-deficient Tf" (CDT) as a biomarker for chronic ethylism (van Eijk et al. 1983; Kapur et al. 1989). The discovery of CDG resulted from the observation of elevated % of hyposialylated Tf glycoforms in children suffering from unexplained neurological disorders (Jaeken

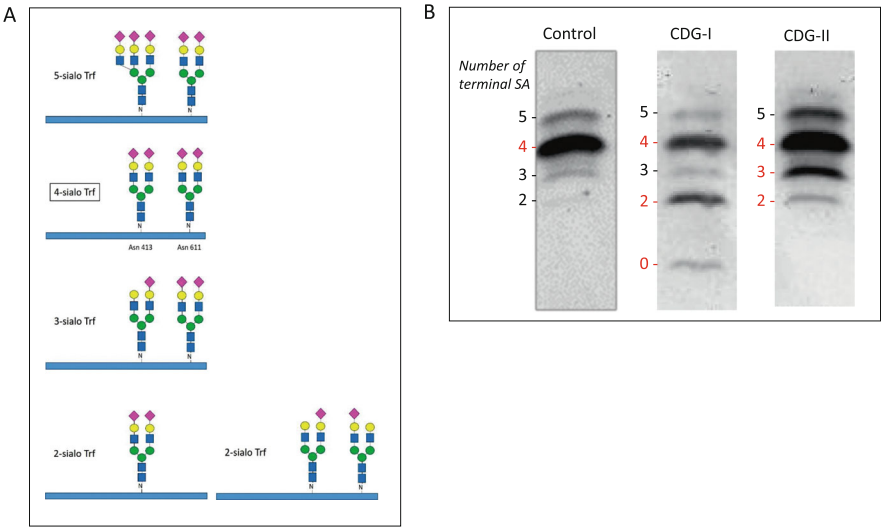

Fig. 4 Normal serum transferrin glycoforms and typical IEF profiles. (**a**) In the serum of healthy controls, the most common serum Tf glycoform is 4-sialoTf, bearing two mature N-glycan chains. 3-sialoTf and 2-sialoTf are minor glycoforms respectively missing one or two SA. The 2-sialoTf glycoform can result from the absence of a complete N-glycan chain or the loss of two terminal SA. Polyantennarity can occur, generating, e.g., the 5-sialo Tf glycoform. Although not represented, serum Tf glycoforms can also be fucosylated. (**b**) In CDG-I, the loss of one or two entire N-glycan chain(s) can be visualized after IEF with the increases of protein bands corresponding to the 2-sialo and the 0-sialo Tf glycoforms. In CDG-II, the maturation defects of the protein-linked N-glycans are associated to an increase of bands corresponding to various hyposialylated glycoforms (3-sialo and 2-sialo Tf glycoforms for the presented CDG-II profile)

et al. 1984). As previously mentioned, two types of Tf IEF patterns were described: a pattern with increased 2-sialo Tf and 0-sialo Tf (CDG-I profile), and a pattern also comprising elevated 3- and 1-sialo Tf, in variable proportions (CDG-II profile) (Fig. 4b). While most often only pointing to CDG-I or CDG-II, some peculiar Tf IEF patterns may orientate toward the causative gene, as it has been shown in MGAT2-CDG (major cathodical shift), PGM1-CDG (mixed CDG-I/CDG-II Tf pattern), or MAN1B1-CDG (major increase of the 3-sialoTf) (Jaeken et al. 1994; Rymen et al. 2013; Altassan et al. 2021).

Capillary electrophoresis (CE) is currently an interesting alternative technique for CDG screening (Parente et al. 2010; Oda et al. 1997). Here, Tf glycoforms are separated according to their electrophoretic mobility, which depends on their charge and size. The number of terminal SA affects charge, while the number and length of N-glycan chains affect size. Different commercial kits, developed for CDT measurement, were shown to provide results similar to IEF in CDG screening (Fig. 5a), with easier separation and better quantification of Tf glycoforms (Parente et al. 2010).

Both IEF and CE are sensitive to certain Tf protein polymorphisms, which more often than not modify its charge (Kamboh and Ferrell 1987) and could lead to an erroneous CDG screening. The majority of Tf polymorphisms may be detected by

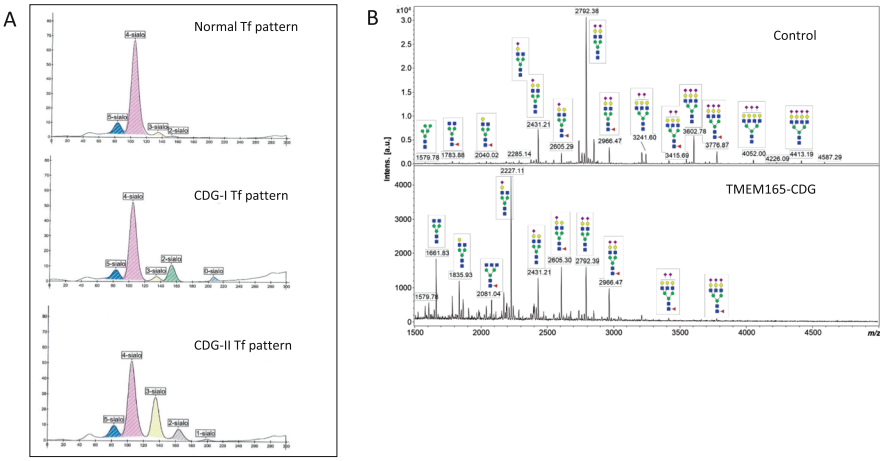

Fig. 5 Examples of serum Tf capillary electrophoresis patterns and N-glycome mass spectra. (**a**) The capillary electrophoresis (CE) pattern of a serum from a healthy individual shows a major peak corresponding to 4-sialoTf, with 5-sialo, 3-sialo, and 2-sialoTf also being present in minor proportions. The CE pattern of a CDG-I affected individual is characterized by increased proportions of 2- and 0-sialoTf at the expense of 4-sialoTf. In CDG-II, elevated proportions of various hyposialylated serum glycoforms can be visualized and accurately quantified. (**b**) MALDI-TOF mass spectra of permethylated N-glycans released through the action of PNGase F from serum samples of a healthy control and of an individual affected with TMEM165-CDG (CDG-II). Several additional peaks corresponding to major hypogalactosylation can be observed. Measurements were performed in positive-ion mode and all ions are present in sodiated form

pre-treatment of samples with sialidase, which removes all terminal SA residues from Tf N-glycan chains (Parker and Bearn 1962). In homozygous individuals, the sialidase-treated Tf pattern will display a single 0-sialo Tf band/peak, while two 0-sialo Tf moieties will be displayed in heterozygous individuals.

In some liver diseases, abnormal Tf N-glycosylation may also affect glycoforms separation. This has notably been observed (increased fucosylation and polyantennarity) in metabolic dysfunction-associated steatosis liver disease or cirrhosis. Therefore, in the context of CDG with hepatopathy, reanalysis by high pressure liquid chromatography (HPLC) could be recommended since it has been shown to permit the recovery of a significant part of uninterpretable CE profiles, thanks to better peak resolution (Stewart et al. 2010). Furthermore, a drawback in CDG screening with Tf is that neither IEF nor CE (and HPLC) is sensitive to fucosylation defects, as fucose is uncharged. Tf also lacks diagnostic value in CDG without N-glycosylation defects. Finally, we must be aware that some proven CDG linked to N-glycosylation defects could be falsely or transiently normal when analyzing Tf. Notably, this has been well described, but not clearly understood, in cases of PMM2-CDG, ATP6V0A2-CDG, COG-CDG as well as in GNE myopathy (an inherited SA biosynthesis defect) (Willems et al. 1860; Lefeber et al. 2011; Raynor et al. 2021a). Therefore, this should be kept in mind when facing unexpected

normal CDG screening tests or when considering Tf as a potential biomarker for therapeutic monitoring in CDG clinical trials (Witters et al. 2021; Taday et al. 2021).

Mass spectrometry (MS)-based methods have been developed for highlighting abnormal glycan structures attached to Tf and to more precisely orientate toward causative genes. Analytical workflows involving liquid chromatography (LC) coupled to electrospray ionization (ESI) and quadruple time-of-flight (Q-TOF) mass spectrometer have been implemented in specialized laboratories for analyzing immunopurified intact Tf (Jansen et al. 2020; Bakar et al. 2018). Of note, while the MS study of intact Tf can theoretically not distinguish between CDG sharing a partial absence of entire N-glycan chains (CDG-I), it could present a direct diagnostic value for some CDG-II with specific abnormal N-glycan structures such as MGAT2-CDG or MAN1B-CDG (Van Scherpenzeel et al. 2014; Poskanzer et al. 2021).

Nevertheless, MS analysis of intact Tf has some analytical limitations. Notably, ESI mass spectra of Tf show a distribution of multiply charged ions that needs to be deconvoluted into "zero-charge" spectra of Tf glycoforms and potential variants. This step facilitates data interpretation but should be a source of inaccuracy. Thus, high spectrum complexity due to insufficient resolution or mass measurement accuracy, low sensitivity for minor Tf glycoforms, as well as improper deconvolution parameters should be considered for good data quality and proper interpretation (Wada et al. 2022). Jansen et al. recently performed ESI-QTOF-MS analysis of intact Tf on samples from 247 patients and 40 healthy controls and obtained interpretable spectral data only for approximately half of the cases, which could be regarded as rather limiting for CDG screening (Jansen et al. 2020). Furthermore, as mentioned for other techniques, falsely normal MS Tf patterns have also been reported in some cases of MOGS-CDG (Shimada et al. 2022) as well as in CDG with fucose deficiency (e.g., SLC35C1-CDG), probably due to the low basal level of fucosylation of Tf (Hipgrave Ederveen et al. 2020).

3.2 N-Glycome Analysis

In the field of CDG screening/diagnosis, the analysis of total serum/plasma N-glycans, the so-called N-glycome, has been shown to be a useful complement to Tf glycoprofiling (Bruneel et al. 2018a; Xia et al. 2013). Serum/plasma N-glycome analysis usually starts by the release of N-glycans from circulating glycoproteins by using peptide-N-glycosidase F (PNGase F) which cleaves the bond between the first GlcNAc of N-linked oligosaccharides and the Asn residue of the carrier protein. Released N-glycans are then derivatized by using chemical (e.g., permethylation) or fluorescent tags (e.g., 2-aminobenzamide) prior to their analysis. Most of the methods used for CDG diagnosis are based on MS, matrix-assisted laser desorption/ionization (MALDI), and/or LC coupled to high-resolution ESI MS (Bruneel et al. 2018a; Beimdiek et al. 2022; Park et al. 2020). When high throughput and ease of operation and data interpretation are required, MALDI-TOF-based approaches are often preferred for N-glycan profiling (Fig. 5b). Of interest, recent approaches

involving labeling of N-glycans with a *N*-hydroxysuccinimide carbamate tag (RapiFluor-MS® or RFMS) were reported to bring high detection sensitivity and clinical specificity for CDG diagnosis when coupled to high-resolution ESI Q-TOF instruments (Messina et al. 2020; Chen et al. 2019). Lastly, N-glycomics can also be used for highlighting specific N-glycosylation defects of isolated plasma-derived glycoproteins such as Tf, IgG, or α_1-antitrypsin (Hipgrave Ederveen et al. 2020; Beimdiek et al. 2022; Sturiale et al. 2021).

Many examples thus demonstrate that total N-glycan profiling can be very informative for CDG-II diagnosis (Fig. 5b) (Hipgrave Ederveen et al. 2020; Bruneel et al. 2020). Recent reports also proved the usefulness of sensitive RFMS-based strategies for CDG-I diagnosis, for instance, through the detection of a low-intensity specific N-linked tetrasaccharide, and its fucosylated counterpart, in samples from individuals affected by different CDG-I such as ALG1-CDG (CDG-Ik), PMM2-CDG (CDG-Ia), and MPI-CDG (CDG-Ib) (Raynor et al. 2024b; Zhang et al. 2016; Abu Bakar et al. 2022).

Lastly, the use of endo-β-*N*-acetylglucosaminidase H (EndoH), which preferentially cleaves between the two GlcNAc of high-mannose and hybrid-type *N*-linked oligosaccharides, has been recently reported as facilitating the N-glycome-based diagnosis of MAN1B1-CDG or SLC37A4-CDG (Raynor et al. 2021b; Duvet et al. 2018).

3.3 Serum N-Glycoproteins Analysis by Using Western Blot or Two-Dimensional Electrophoresis

Besides Tf, the analysis of haptoglobin, alpha-1-antitrypsin, and alpha-1-acid glycoprotein by classical Western blot and/or two-dimensional electrophoresis (2-DE) can help orientate toward CDG-I or CDG-II (Bruneel et al. 2017; Seta et al. 1996). In classical Western blot, the absence of complete N-glycan chains in CDG-I will produce additional bands of lower molecular weight compared to controls (Fig. 6a), while the partial loss of monosaccharides in CDG-II will, in most cases, not significantly alter protein migration (Seta et al. 1996). Using 2-DE, additional protein spots of less acidic isoelectric point (cathodical shift) can be observed in CDG-I and CDG-II, while combined losses in molecular weight will typically be displayed in CDG-I only (Fig. 6c) (Bruneel et al. 2017). Of note, Western blot and 2-DE can be applied to very practical dried blood spot samples, by contrast with most of other described CDG screening methods.

3.4 Serum Apolipoprotein C-III Analysis (Mucin Core 1 O-Glycosylation)

Apolipoprotein C-III (apoC-III) is an O-linked glycoprotein (~10 kDa) primarily synthesized by hepatocytes and participating in the metabolism of lipoproteins (Norata et al. 2015). As mentioned above, it contains a single O-glycosylation

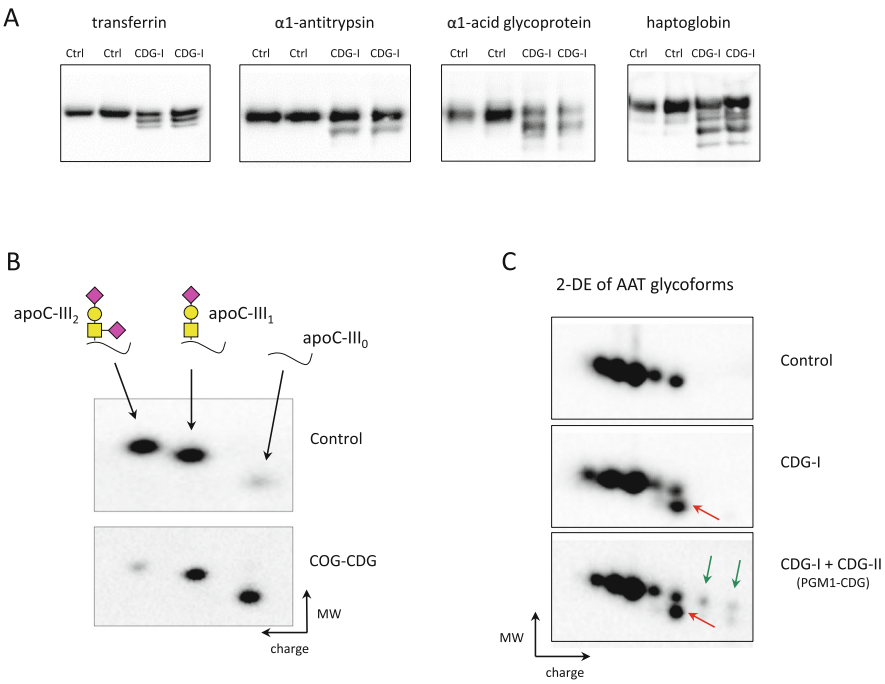

Fig. 6 Western blot and 2-DE patterns of various serum N-glycoproteins and mucin core1 O-glycosylated apolipoprotein C-III. (**a**) By comparison with controls (Ctrl), Western blot profiles of serum Tf, α1-antitrypsin, α1-acid glycoprotein, and haptoglobin in CDG-I affected individuals (CDG-I) typically display additional bands of decreased molecular weights. (**b**) In control, the 2-DE pattern of serum O-glycosylated apolipoprotein C-III (apoC-III) typically displays the major apoC-III_1 and apoC-III_2 glycoforms with trace amounts of unglycosylated apoC-III_0. In most of samples from COG-CDG-affected individuals (CDG-II with Golgi trafficking defects), a major and quasi-specific increase of the apoC-III_0 glycoform is observed. (**c**) Using 2-DE, serum α1-antitrypsin (AAT) can be separated into five major protein spots in control. In CDG-I, an additional spot (red arrow) corresponding to the loss of an entire N-glycan chain is classically observed. In PGM1-CDG (a mixed CDG-I + CDG-II), 2-DE of serum AAT displays the typical additional spot of CDG-I (red arrow) together with other additional spots typical of CDG-II (green arrows)

acceptor site on Thr^{74} (Vaith et al. 1978). In healthy individuals, this site is occupied by a mono- or bi-sialylated core1 O-glycan chain with trace amounts of asialylated apoC-III glycoforms (Wopereis et al. 2003) (Fig. 3a). The first technique developed to study the glycosylation of apoC-III for CDG screening was IEF (Wopereis et al. 2003). Three types of patterns were described: "apoC-III_0," "apoC-III_1," and "apoC-III_2," characterized respectively by increased % of 0-, 1-, and 2-sialo-apoC-III. Notably, apoC-III_0 and apoC-III_1 patterns were reported in CDG related to the vesicular Golgi trafficking (COG-CDG) or to the V-ATPase proton pump (ATP6V0A2-CDG) (Wopereis et al. 2003).

Beside IEF, 2-DE has been carried out for the analysis of apoC-III glycoforms. In 2-DE, a molecular weight-based separation is performed following IEF, which allows further characterization of core1 apoC-III glycoforms (Fig. 6b) (Bruneel et al. 2007). In addition, this technique can separate various asialylated apoC-III_0 glycoforms. These include apoC-III_{0a} (non-glycosylated apoC-III), the increase of which is strongly evocative of COG-CDG (Fig. 6b) (Yen-Nicolaÿ et al. 2015).

MALDI-TOF MS was also proposed for the study of intact core1 apoC-III glycoforms and showed good capacity to screen some CDG linked to Golgi homeostasis defects (e.g., ATP6V0A2-CDG and COG-CDG) (Yen-Nicolaÿ et al. 2015).

Lastly, in agreement with our personal experience, liver diseases, obesity and sepsis have been associated to altered apoC-III glycoforms proportions (Harvey et al. 2009). We also showed that apoC-III patterns could display typical O-glycosylation defects in some patients with CDG presenting with normal Tf patterns (Raynor et al. 2021a).

3.5 Cellular CDG Biomarkers

Although essential to characterize deficiencies and eventually test therapeutic options, robust cellular glycosylation biomarkers are relatively lacking in the routine CDG field. ICAM-1, LAMP2, and TGN46 are currently studied by Western blot to detect and characterize glycosylation deficiencies. Classically, glycosylation defects lead to abnormalities in their electrophoretic profiles and/or a decrease in their abundance. The intercellular cell adhesion molecule-1 (ICAM-1) has been identified as a useful cellular CDG-I biomarker as the lack of N-glycans leads to its degradation (He et al. 2012; Ferrer et al. 2020). Concerning N- and/or O-glycan defects, LAMP2 (heavily N-glycosylated) and TGN46 (heavily O-glycosylated) are interesting cellular biomarkers that have been widely used to identify glycosylation defects mainly in skin-derived fibroblasts of patients with Golgi homeostasis deficiencies (e.g., COG-CDG, TMEM165-CDG) (Houdou et al. 2019; Blackburn et al. 2018; Lebredonchel et al. 2019). Lastly, some of these cellular biomarkers such as LAMP2 and ICAM-1 have shown interests in the research and development of novel pharmacological agents (Ligezka et al. 2021; Durin et al. 2024).

3.6 Serum Bikunin Analysis (GAG Biosynthesis)

Bikunin is synthesized and excreted by hepatocytes under various isoforms in the blood. These include (i) the free core protein (Bkn), (ii) the proteoglycan consisting of Bkn bearing a CS chain (Bkn-CS) attached via the common tetrasaccharide linker, and (iii) Bkn-CS esterified by one or two "heavy chain" (HC) proteins leading to pro-α-trypsin inhibitor (PαI) and inter-α-trypsin inhibitor (ITI) (Fig. 7a).

We implemented serum Bkn-CS analysis as a biomarker of GAG biosynthesis defects such as linkeropathies, i.e., genetic diseases affecting the enzymes involved in the elongation of the GlcA-Gal-Gal-Xyl-O tetrasaccharide (Haouari et al. 2020;

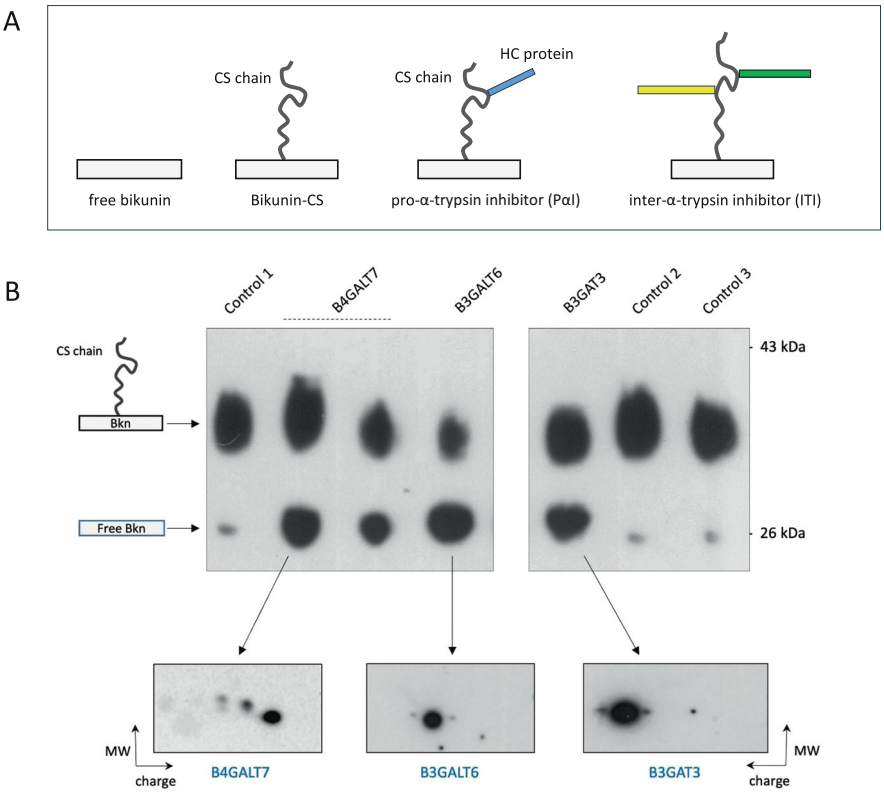

Fig. 7 Serum bikunin isoforms and Western blot patterns (1D and 2D) of linkeropathy-affected patients. (**a**) The major serum bikunin isoforms include (i) the free bikunin protein, (ii) the proteoglycan consisting of bikunin linked to a CS chain (bikunin-CS), and (iii) bikunin-CS linked to one or two heavy chain (HC) proteins leading to pro-α-trypsin inhibitor (PαI) and inter-α-trypsin inhibitor (ITI). (**b**) Compared to controls (control 1, 2, and 3), the Western blot of serum bikunin light forms (free Bkn and Bkn-CS) from linkeropathy-affected individuals (B4GALT7, B3GALT6, B3GAT3) show a large ~27 kDa band traducing a major defect in the initial biosynthesis of the CS chain. After 2-DE-based separation of the protein content of these abnormal bands, apparently gene-specific patterns could be observed

Bruneel et al. 2018b). We showed that Western blot of serum bikunin in individuals affected with linkeropathies (e.g., B4GALT7-CDG, B3GALT6-CDG) showed increased levels of abnormal light forms pointing to a defect in the tetrasaccharide linker biosynthesis (Fig. 7b). Furthermore, 2-DE of these abnormal forms displayed apparently specific patterns hinting at the causative mutated gene (Haouari et al. 2020) (Fig. 7b). While an interesting biomarker for the diagnosis of linkeropathies, Bkn-CS analysis has some limitations. Since mainly synthesized by hepatocytes, Bkn-CS analysis can only detect deficiencies in enzymes expressed in the liver. Furthermore, beside linkeropathies, the diagnostic interest of bikunin is theoretically

restricted to the diseases affecting the CS chain, excluding those related to other GAG moieties.

3.7 Glycoproteomics Approaches

As discussed above, MS-based serum N-glycoprofiling is a relevant tool for CDG diagnosis. However, one of the main limitations of this approach is associated with the difficulty (if not the impossibility) to functionally interpret the generated data because N-glycome reflects the glycosylation of all the glycoproteins present in the sample. Thus, an alteration in the glycosylation profile can originate from one or more of the 24 major glycoproteins accounting for half of the total serum/plasma protein concentration (Clerc et al. 2016). In this context, obtaining protein- and site-specific information about glycosylation site occupancy (macroheterogeneity) and glycan structure heterogeneity at a given glycosylation site (microheterogeneity) would bring functionally relevant data with potentially meaningful related biomarkers. For example, Hülsmeier et al. (2007) evaluated by LC-MS/MS the macroheterogeneity of serum Tf and α_1-antitrypsin from a dozen of CDG-I patients and found a correlation between the degree of N-glycosylation site occupancy and the severity of the disease, with a greatest dynamics in site occupancy of Asn^{611} from Tf in pathological conditions. Later, this approach has been extended to Tf, haptoglobin, IgG_2, and IgA_1 proteins, and data demonstrated reduced N-glycosylation site occupancies for liver-synthesized Tf and haptoglobin from CDG-I patients samples, whereas no change was observed with the glycopeptides from IgA_1 and IgG_2 (of non-hepatic origin) (Hülsmeier et al. 2016). Although the physiological significance of such site occupancy-related data remains to be demonstrated, these approaches hold great potentials for bringing meaningful protein- and site-specific information.

These studies also confirmed that individual glycoproteins could respond differently to CDG and that glycopeptide-based diagnostic could present greater specificity and sensitivity. This can be illustrated by a very recent work of Garapati et al. (2024) which, using a multistep MS-based N-glycoproteomics approach, highlighted that a complement-C4 derived $Man_5GlcNAc_2$ glycopeptide can be regarded as a potentially sensitive and specific biomarker of PMM2-CDG.

4 Therapeutic Approaches in CDG and Interests of Biomarkers

Most of CDG are yet incurable. However, in a few disorders, therapeutic options exist that can correct or improve glycosylation status and/or symptoms. Notably oral D-mannose (D-Man), oral D-galactose (D-Gal), and oral manganese (Mn^{2+}) therapies showed beneficial clinical and/or biological effects in MPI-CDG,

PGM1-CDG, and TMEM165-CDG, respectively (Durin et al. 2024; Verheijen et al. 2020).

4.1 Oral D-Mannose in MPI-CDG (CDG-1b)

Mannose phosphate isomerase (MPI)-congenital disorder of glycosylation or MPI-CDG (formerly named CDG-1b; OMIM #602579) is an autosomal recessive inherited defect of the N-linked glycosylation of proteins (Niehues et al. 1998; Vuillaumier-Barrot et al. 2002). The enzymatic deficiency is caused by pathogenic variants affecting the *MPI* gene on chromosome 15. Mannose phosphate isomerase is a cytosolic enzyme that catalyzes the conversion of fructose-6-phosphate into mannose-6-phosphate, one of the first steps of the GDP-Man biosynthesis (Fig. 1). The clinical phenotype significantly differs from other CDG since affected individuals mostly present hypoglycemia (of variable severity), gastrointestinal symptoms (diarrhea, vomiting, protein losing enteropathy), liver disease (elevated transaminases, progressive fibrosis), and coagulopathy (mostly thrombosis), but in the absence of neurological defects or intellectual disability (Girard et al. 2020). Furthermore, MPI-CDG is one of the few treatable CDG subtypes with a proven efficacy of oral D-mannose therapy that can bypass the enzymatic deficiency (Fig. 8a) (Niehues et al. 1998; Girard et al. 2020).

Biochemical screening of MPI-CDG can be performed by previously detailed analyses of Tf, classically showing a marked CDG-I pattern. Although simple, fast, and very sensitive, these Tf-based methods lack specificity since MPI-CDG shares the same abnormal pattern with all other CDG-I subtypes. Diagnostic suspicion can be reinforced by determining MPI enzymatic activity from skin fibroblasts or blood leukocytes (Van Schaftingen and Jaeken 1995), which typically shows a significant decrease compared to controls. The diagnosis must be imperatively confirmed by sequencing of the *MPI* gene in the affected individual and both parents.

The recommended dose of oral D-mannose varies between 150 and 170 mg/kg bodyweight, three to five times a day (0.5 to 0.75 g/kg bodyweight/day). D-mannose is well absorbed orally, and its half-life is of approximately 4 h. The normal serum concentration of mannose varies between 46 μM and 65 μM. The concentration peak is obtained 1–2 h after ingestion of 0.07–0.2 g/kg. In adults, an intake of 12–20 g/day (to be modulated according to the digestive tolerability) should be sufficient (Čechová et al. 2020).

Mannose treatment improves the general condition and the digestive symptoms of the MPI-CDG affected individual. It normalizes glycemia and coagulation factors but its efficacy on the liver fibrosis regression is not demonstrated. Diarrhea, vomiting episodes, and hypoalbuminemia have been corrected in all patients presenting a protein losing enteropathy. In most cases, mannose treatment also normalizes (at least significantly improves) the glycosylation profile of Tf. The clinical response is classically observed within the first week of treatment while biological abnormalities often persisted several weeks (at least 15 days for Tf glycosylation) or months (Girard et al. 2020). In the French experience, thrombosis

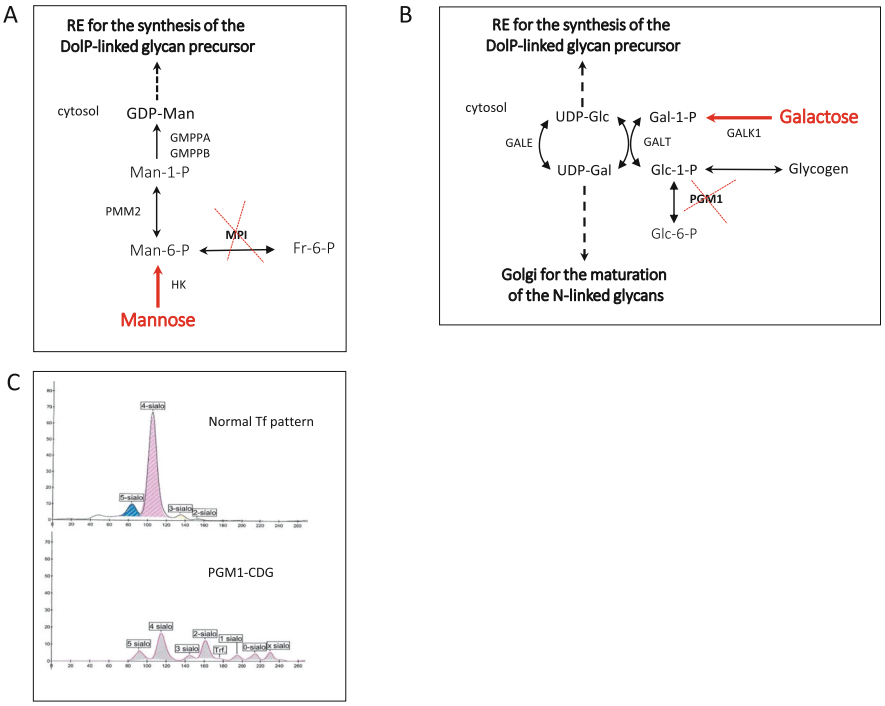

Fig. 8 Altered metabolic pathways in MPI-CDG and PGM1-CDG. Typical CE profile of untreated PGM1-CDG. (**a**) In MPI-CDG (CDG-Ib), mannose phosphate isomerase enzymatic deficiency can be bypassed by oral D-mannose supplementation that restores cytosolic biosynthesis of GDP-Man necessary to the N-glycosylation process in the endoplasmic reticulum (ER). (**b**) In PGM1-CDG (CDG-It), phosphoglucomutase 1 (PGM1) enzymatic deficiency can be bypassed by oral D-galactose supplementation, fueling in Gal-1P the biosynthesis of UDP-Gal and its conversion in UDP-Glc, both necessary to the N-glycosylation process (in the ER and Golgi). (**c**) Typical mixed CDG-I/CDG-II serum transferrin CE profile of a PGM1-CDG-affected individual compared to a control

episodes could occur at the discontinuation of the treatment (non-compliant patient), strongly suggesting that mannose must be administered for life.

The major adverse effects of oral mannose are abdominal pain and diarrhea, which could also reflect underdosage or bad compliance. Concerning this last point, we have shown a correlation between poor compliance for the oral mannose treatment and the worsening of the Tf glycosylation profile (Girard et al. 2020). The development of more convenient galenic formulations than the powder form (slow-release forms) would be a significant improvement for this very beneficial long-life treatment.

4.2 Oral D-Galactose in PGM1-CDG

PGM1-CDG (formerly CDG-It; OMIM #614921) corresponds to the inherited deficiency of the phosphoglucomutase 1 enzyme (Pérez et al. 2013) which catalyzes the cytosolic conversion of Glc-6P to Glc-1P, the latter being importantly involved in glycogen metabolism and UDP-Glc and UDP-Gal biosynthesis (Fig. 8b) (Radenkovic et al. 2019). It is an autosomal recessive CDG with biallelic mutations of the *PGM1* gene associated to defects in the N-glycosylation of proteins. Less than one hundred affected individuals have been described to date. Clinically, two distinct phenotypes can be differentiated (Altassan et al. 2021; Tegtmeyer et al. 2014) – one with a predominant muscular symptomatology, typically with an onset during adulthood, and presenting similarities to other glycogen storage diseases (acute rhabdomyolysis, muscular weakness, etc.) and the other phenotype is multivisceral with early symptoms consisting of liver disease (with elevated aminotransferases), rhabdomyolysis, hypoglycemia, congenital malformations (cleft palate, bifid uvula), coagulopathy, myopathy, and possibly severe cardiac involvement. Overall, neurological involvement and intellectual disability are less frequently observed than in other CDG. Furthermore, PGM1-CDG belongs to the rare subtypes for which an effective treatment is available. Indeed, oral galactose supplementation can bypass PGM1 deficiency by fueling in Gal-1P the biosynthesis of UDP-Gal and its conversion in UDP-Glc, both necessary to the N-glycosylation process (Fig. 8b) (Wong et al. 2017).

Besides possible specialized measurement of the PGM1 enzyme activity in skin fibroblasts (Tegtmeyer et al. 2014), biochemical screening of PGM1-CDG can be performed by previously detailed analyses of Tf, classically showing a very peculiar CDG-I/CDG-II mixed pattern (Tegtmeyer et al. 2014). More precisely, PGM1-CDG Tf patterns commonly associate elevated 2-sialo and 0-sialo Tf, reflecting the absence of entire N-glycan chains (type I; ER-localized origin), to elevated 3-sialo and 1-sialo Tf, linked to altered maturation of N-glycans in the Golgi (type II). It is tempting to hypothesize that, in this CDG, deficiencies in UDP-Glc (involved in ER steps of N-glycosylation) and UDP-Gal (involved in Golgi maturation steps) result in CDG-I and CDG-II abnormalities, respectively. Using CE, related mixed profiles appear quite puzzling with unusual abnormalities (in term of relative proportions of each Tf fractions) and frequent additional abnormal peaks (Fig. 8c). Using 2-DE of serum glycoproteins, superimposed CDG-I and CDG-II patterns can be more easily differentiated (Bruneel et al. 2017) (Fig. 6c). In our experience, such Tf patterns are quite specific since only found, in very attenuated form, in rare cases of fructosemia and chronic alcoholism. Nevertheless, isolated CDG-I or CDG-II Tf patterns have been described in a few number of PGM1-CDG cases (Altassan et al. 2021). In this context, more sensitive MS methods could perhaps bring additional clues. Furthermore, it has been suggested, using MS analysis of purified Tf, that the intensity of observed alterations (notably the aglycosylation and hypogalactosylation) could be related to the initial severity of the disease (Bakar et al. 2018). Lastly, as with other CDG, the diagnosis of PGM1-CDG must be imperatively confirmed by sequencing of the *PGM1* gene in the affected individual and both parents.

Recommended oral D-galactose doses range between 0.5 and 3 g/kg/day (to be gradually increased; single dose or divided in 4 doses daily) with a maximum of 50 g/day to avoid excessive levels of toxic Gal-1P and galactitol. Under this dosage, no significant adverse event has been noted with the exception of one patient who did not tolerate higher doses. In most of the patients, this therapy has been shown to improve several clinical and biological signs such as exercise intolerance, rhabdomyolysis, frequency of hypoglycemic episodes, as well as CK, aminotransferases, and coagulation factors levels (Altassan et al. 2021). Nevertheless, if initially present, the cardiac phenotype of the disease might be resistant to the treatment (Radenkovic et al. 2023). Concerning Tf glycosylation, the intakes of UDP-Glc and UDP-Gal related to galactose supplementation led to significant improvements (albeit without complete normalization) in the majority of cases. Lastly, as with mannose in MPI-CDG and although data about compliance to this treatment are quite scarce, galenic improvements of this oral powder D-galactose therapy would be also highly appreciated by concerned affected individuals.

4.3 Oral Manganese in TMEM165-CDG

We recently reported the ongoing manganese (Mn^{2+}) + D-galactose-based treatment in a young TMEM165-CDG-affected individual (Durin et al. 2024). In this case, TMEM165-CDG is related to a homozygous gene variant affecting the expression of the membrane transporter TMEM165 involved in the import of Mn^{2+} from the cytosol into the Golgi lumen (Fig. 2). As Mn^{2+} is crucial as a cofactor of numerous glycosylation enzymes (notably galactosyltransferases) (Durin et al. 2023; Foulquier and Legrand 2020), its TMEM165-CDG-related deficiency in the Golgi is not only accompanied by defects in N-glycosylation (maturation steps) but also in mucin-type O-glycosylation and GAG biosynthesis (Haouari et al. 2020; Foulquier et al. 2012; Khan et al. 2021).

The patient presented at 2 months old with a multivisceral syndrome combining recurrent hypovolemic shocks, diarrhea, failure to thrive, skeletal abnormalities, hepatomegaly with elevated transaminases, psychomotor delay, and abnormal coagulation. The few other described patients were diagnosed at older age and presented essentially with major skeletal abnormalities (scoliosis, osteoporosis), joint laxity, dwarfism, and psychomotor retardation (Foulquier et al. 2012; Zeevaert et al. 2013). Based essentially on the research of the team of François Foulquier on TMEM165 KO cell lines and on the skin fibroblasts of the patient, a $MnSO_4$ + D-galactose treatment was early initiated at 1 year old. Notably, it had been shown that the $MnCl_2$ treatment of TMEM165 KO cells fully rescued all abnormalities affecting N-glycosylation (LAMP2 profile), O-glycosylation (TGN46 profile), and GAG biosynthesis (Houdou et al. 2019; Durin et al. 2024; Foulquier et al. 2012; Khan et al. 2021). Furthermore, the Mn^{2+} treatment of the cultivated patient's fibroblasts showed beneficial effects on the altered migration profile of TGN46.

In first line, the biochemical screening of this TMEM165-CDG was performed by CE of serum Tf, showing a very abnormal CDG-II pattern with increased levels of all

hyposialylated Tf glycoforms, at the expense of 4-sialoTf. In second line, MS-based serum N-glycome revealed major galactosylation defects of serum N-glycoproteins (Fig. 5b), as previously described for other TMEM165-CDG cases. In combination with these N-glycosylation defects, the 2-DE pattern of apoC-III showed major defects in mucin core1 O-glycosylation, highly suggestive for a Golgi homeostasis defect affecting all types of glycosylation. Furthermore, the Western blot of serum bikunin also showed marked abnormalities suggestive for a linkeropathy-like deficiency of the GAG biosynthesis (Durin et al. 2024). Altogether, these results prompted us to urgently sequence the *TMEM165* gene, with the discovery of a new homozygous pathogenic variant.

Oral $MnSO_4$ treatment was initiated at 11 months-old in combination with D-galactose. Although previously described as only improving the N-glycosylation defects observed in TMEM165 KO cells, D-galactose was added in view of its absence of toxicity and the high degree of hypogalactosylation in patient's samples. Oral galactose therapy was started at 0.5 g/kg bodyweight/day, progressively increasing (by steps of 6 weeks) to 1.5 g/kg/day. Combined Mn^{2+} treatment (MnSO4; H_2O) was started at 2 mg/day for 4 months, then 12 mg/day for 3 months, 30 mg/day for 1.5 months, to finally reach 10 mg/kg bodyweight/day.

After 1 year of treatment, the patient's digestive symptoms completely disappeared. Skeletal abnormalities and hepatomegaly did not improve but stabilized. Discrete psychomotor delay is still present, but the patient progressed at her own pace. Body weight increased under enteral nutrition. By contrast with the mostly stable clinical phenotype, we observed major biological improvements of aminotransferases and coagulation factors (both quasi-normal after 1 year). Regarding glycosylation, results were also spectacular with a quasi-normalization of all glycosylation types as evaluated by CE of serum Tf and MS-based serum N-glycome (N-glycosylation), 2-DE of apoC-III (mucin core1 O-glycosylation), and Western blot of serum bikunin (GAG biosynthesis). Furthermore, the kinetics of improvement of these glycosylation biomarkers varied, with belated improvements of apoC-III and bikunin patterns, suggesting a time and/or dose-dependent effects (Durin et al. 2024).

Regarding the potential toxicity of manganese, overexposure to this metal element can cause "manganism," which is a Parkinson-like syndrome related to irreversible neurological damages (Zheng et al. 2011). No efficient biological biomarker of manganese toxicity has been yet identified, notably due to large discrepancies between its blood and tissular half-life (Zheng et al. 2000, 2011). In these conditions, it has been shown that magnetic resonance imaging (MRI) can reflect manganese accumulation in the brain with good sensitivity and specificity. In the case of our patient, after 1 year of treatment, brain MRI did not show any manganese accumulation. This possible adverse effect will however require cautious evaluation of the patient during follow-up consultations.

Therefore, metal (i.e., cofactor) supplementation might offer novel opportunities as pharmacological agents to improve symptomatology in other CDG yet without therapy.

5 Conclusions

The glycosylation process is a very complex modification of proteins and lipids involving a multitude of enzymes and other molecular actors, in distinct subcellular compartments. Related congenital disorders of glycosylation (CDG) constitute an ever-growing group of inborn errors of metabolism. Their biochemical screening/diagnosis still mainly relies on the analysis of serum N-glycosylated transferrin using various gel-based and/or MS techniques. Beside, additional MS-based N-glycoprofiling and other biomarkers, such as apolipoprotein C-III (O-glycosylation) and bikunin (GAG biosynthesis), are precious tools to orientate toward the causative gene defect or to assert its pathogenicity. Furthermore, new N-glycomics and glycoproteomics approaches offer exiting perspectives in term of sensitivity and specificity. Nevertheless, several important glycosylation pathways remain poorly understood and currently lack biomarkers easily usable in clinical laboratories. Established and efficient pharmacological therapies for CDG are still very rare and quite limited to oral monosaccharide supplementation, with potential for improvement in galenic formulations (e.g., late-release forms). In this context, we believe that ongoing research on new metal-based CDG therapies look promising and offer exciting prospects regarding future clinical developments.

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