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Department of Clinical Genetics, Institute of Clinical Medicine, University of Tartu, Estonia

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I **Vals MA**., Morava E., Teeäär K., Zordania R., Pajusalu S., Lefeber DJ., Õunap K. Three families with mild PMM2-CDG and normal cognitive development. Am J Med Genet A. 2017 Jun;173(6):1620–1624.

II **Vals MA**., Pajusalu S., Kals M., Mägi R., Õunap K. The Prevalence of PMM2-CDG in Estonia Based on Population Carrier Frequencies and Diagnosed Patients. JIMD Rep. 2018; 39: 13–17.

III **Vals MA**., Ashikov A., Ilves P., Loorits D., Zeng Q., Barone R., Huijben K., Sykut-Cegielska J., Diogo L., Elias AF., Greenwood RS., Grunewald S., van Hasselt PM., van de Kamp JM., Mancini G., Okninska A., Pajusalu S., Rudd PM., Rustad CF., Salvarinova R., de Vries BBA., Wolf NI., EPGEN Study, Ng BG., Freeze HH., Lefeber DJ., Õunap K. Clinical, neuroradiological and biochemical features of SLC35A2-CDG patients. J Inher Metab Dis (accepted 2018).

IV **Vals MA**., Joost K., Maipuu L., Õunap K. Congenital disorders of glycosylation: an overview of the literature and a case report. [In Estonian] Eesti Arst (Estonian Medical Journal) 2014; 93(1): 41–46.

My contributions to the original articles:

Paper I: participation in study design, performing serum transferrin isoelectric focusing, collecting and analyzing the data, writing the manuscript.

Paper II: participation in study design, performing serum transferrin isoelectric focusing, data analysis, performing statistical analysis, writing the manuscript.

Paper III: participation in study design, performing serum transferrin isoelectric focusing in Estonian patient, collecting and analyzing the data, writing/co-writing the manuscript.

Paper IV: analyzing the case history, writing the manuscript.

ABBREVIATIONS

ACMG	American College of Medical Genetics and Genomics
ApoC-III	apolipoprotein C-III
AST	aspartate aminotransferase
ATIII	antithrombin III
CDG	congenital disorders of glycosylation
CMP	cytidine monophosphate
COG	conserved oligomeric Golgi
DD	developmental delay
Dol	dolichol
Dol-P	phosphorylated dolichol
EGCUT	Estonian Genome Center at the University of Tartu
ER	endoplasmic reticulum
ERGIC	Endoplasmic reticulum-Golgi Intermediate Compartment
FSH	follicle-stimulating hormone
Fuc	fucose
Gal	galactose
GDP	guanosine diphosphate
GH	growth hormone
GlcNAc	N-acetylglucoseamine
Glu	glucose
gnomAD	Genome Aggregation Database
GPI	glycosylphosphatidylinositol
ID	intellectual disability
ICAM-1	intercellular cell adhesion molecule 1
IGF-1	insulin-like growth factor 1
IEF	isoelectric focusing
LCSH	long contiguous stretches of homozygosity
LH	luteinizing hormone
LLO	lipid-linked oligosaccharide
MALDI-TOF	matrix-assisted laser detection desorption ionization–time-of-flight
Man	mannose
MRI	magnetic resonance imaging
MS	mass spectrometry
NGS	next-generation sequencing
Sia	sialic acid
SNAREs	Soluble *N*-ethylmaleimide-sensitive factor Attachment protein Receptors
TBG	thyroxine-binding globulin
Tf	transferrin
TSH	thyroid-stimulating hormone
UDP	uridine diphosphate

UGT	UDP-Gal transporter
WES	whole exome sequencing
WISC	Wechsler Intelligence Scale for Children
WGS	whole genome sequencing

Abbreviations for N-glycosylation related enzymes and transporters

ALG1	beta-1,4-mannosyltransferase
ALG2	alpha-1,3/1,6-mannosyltransferase
ALG3	alpha-1,3-mannosyltransferase
ALG6	dolichyl pyrophosphate $Man_9GlcNAc_2$ alpha-1,3-glucosyltransferase
ALG8	probable dolichyl pyrophosphate $Glu_1Man_9GlcNAc_2$ alpha-1,3-glucosyltransferase
ALG9	alpha-1,2-mannosyltransferase
ALG11	glycolipid 2-alpha- mannosyltransferase
ALG12	alpha-1,6-mannosyltransferase
ALG13/ALG14	UDP-GlcNAc transferase
ATP6AP1	accessory protein AC45 of the V-ATPase
ATP6V0A2	V0 subunit A2 of V-ATPase
B4GALT1	beta-1,4-galactosyltransferase 1
CCDC115	coiled-coil domain containing protein 115
COG1…8	conserved oligomeric Golgi components 1…8
DHDDS	dehydrodolichyl diphosphate synthase
DDOST	oligosaccharyltransferase subunit (dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit)
DPAGT1	dolichyl-phosphate N-acetylglucoseamine phosphotransferase
DPM1	dolichol-phosphate mannosyltransferase subunit 1
DPM2	dolichol-phosphate mannosyltransferase subunit 2
DPM3	dolichol-phosphate mannosyltransferase subunit 3
DOLK	dolichol kinase
GANAB	glycosidase II subunit alpha
GMPPA	guanosine diphosphate mannose pyrophosphorylase A
GMPPB	guanosine diphosphate mannose pyrophosphorylase B
MAN1B1	mannosyl-oligosaccharide alpha-1,2-mannosidase
MGAT2	beta-1,2-N-acetylglucosaminyltransferase II
MOGS	mannosyl-oligosaccharide glucosidase
MPDU1	mannose-P-dolichol utilization defect 1 protein
MPI	mannose-6-phosphate isomerase
NUS1	Nogo-B receptor (subunit of cis-prenyltransferase)
OST	oligosaccharyl transferase
PGM1	phosphoglucomutase
PMM2	phosphomannomutase 2
PRKCSH	glycosidase II subunit beta

RFT1	RNA polymerase-associated protein, flippase
SLC35A1	CMP-sialic acid transporter
SLC35A2	UDP-galactose transporter
SLC35A3	UDP-GlcNAc transporter
SLC35C1	GDP-fucose transporter
SLC39A8	manganese and zinc transporter
SRD5A3	steroid 5-α-reductase 3
STT3A	oligosaccharyltransferase subunit
STT3B	oligosaccharyltransferase subunit
TMEM199	transmembrane protein 199
TUSC3	oligosaccharyltransferase subunit (tumor suppressor canditate 3)

1. INTRODUCTION

Congenital disorders of glycosylation (CDG) are an expanding group of inherited metabolic diseases caused by primary hypoglycosylation of proteins and lipids. Glycosylation is a process in which monosaccharides are attached to an acceptor molecule. It occurs in every cell, and it is very important for normal functioning of many circulating, membranous and intracellular proteins. CDG was first presented as a new syndrome in identical twin sisters by Jaeken and colleagues in 1980 (Jaeken et al 1980). Fifteen years later, decreased activity of phosphomannomutase 2 (PMM2) was described as a cause of CDG type I (Van Schaftingen and Jaeken 1995), which is now named as PMM2-CDG, and known as the most frequently diagnosed CDG worldwide.

Since 1980, over 125 types of CDG have been identified. Overall, about ~2% of all genes in the human genome are considered to be involved in glycosylation (Ng and Freeze 2018). Most of CDG are inherited in autosomal recessive pattern, fewer are autosomal dominant or X-linked disorders (Jaeken and Peanne 2017; Ng and Freeze 2018). Earlier, the patients were classified either as having CDG-I or CDG-II depending on the pattern found with the serum transferrin (Tf) isoelectric focusing (IEF), a method used frequently for CDG screening. Small Latin letters indicated the order in which disorders were discovered (e.g. CDG-Ia, CDG-Ib). Since 2009, new nomenclature is in use where official gene symbol is followed by '-CDG', as this disease group does not only include disorders of protein N- and O-glycosylation, but also glycosylation defects in glycosphingolipid and glycosylphosphatidylinositol (GPI) anchor pathways as well as in multiple glycosylation and other pathways (Jaeken et al 2008; Jaeken et al 2009). Nevertheless, disorders in the N-glycosylation pathway are most common, and according to the data from twelve laboratories in Europe, 94% of molecularly confirmed patients had CDG-I and 6% had CDG-II (Peanne et al 2017).

The synthesis of N-glycans is complex and involves many enzymes. Variants in genes can severely reduce the activity of different enzymes (e.g. glycosyltransferases, glycosidases) or impair the function of other proteins important in the glycosylation pathway (e.g. transporters), which ultimately lead to defective synthesis of glycoproteins. Also, the cellular environment and homeostasis are important for the correct glycosylation and any deviation can influence this process.

The clinical presentation and severity of different CDG is wide and often unspecific. It can be multisystem disease and/or involve only the nervous system. It is emphasized that CDG should be considered and screened in every patient with unexplained neurological symptoms, especially if accompanied by multiorgan abnormalities. Tf IEF still remains the main screening method for CDG. As screening with Tf IEF can only be positive with disorders that affect N-glycosylation, negative results are present in more than half of different CDG (Jaeken and Peanne 2017). In addition, several types of CDG with usual

positive screening can show age-dependent false-negative results with Tf IEF. Therefore, the diagnostics for CDG can be challenging. In earlier days, different CDG were confirmed with biochemical studies when possible, and/or single gene sequencing. Nowadays it is not unusual that more and more patients are diagnosed with CDG with different next-generation sequencing (NGS) techniques that have helped to solve suspected, or discover novel CDG. This has in turn raised the question whether all suspected variants are pathogenic and influence glycosylation, and it stresses the importance of functional assays (Ng and Freeze 2018).

In 2007/2008, the first patient with PMM2-CDG was diagnosed in Estonia (Vals et al 2014). The male newborn presented with multisystem disease and peculiar phenotype, and died on the 6th day of life. His serum and skin fibroblasts were collected and sent to metabolic laboratory of Amsterdam Free University Medical Center in the Netherlands. His serum Tf IEF and enzymatic analyses referred PMM2-CDG, which was later molecularly confirmed. It was naive to believe that this newborn was the only CDG patient in Estonia. Unfortunately, before 2012 the screening of CDG was limited to a very small group of patients as the serum Tf IEF was done in foreign laboratories. This made the diagnostic methods for CDG less available and may have been one reason why this disorder was under-diagnosed.

The aim of this study was to implement Tf IEF in clinical practice and to study the occurrence of N-glycosylation defects among Estonian patients in a three-year screening period. Positive findings were followed by a study about the prevalence of the most common CDG type, PMM2-CDG, in Estonian population, and the characterization of Estonian patients with PMM2-CDG. In addition, two other types of CDG detected with Tf IEF during the study period will be described. First, a phenotype of SLC35A2-CDG is characterized based on the international cohort of patients (including Estonian patient), and secondly, an overview of a new, previously undescribed CDG possibly caused by homozygous variant in *STX5* gene is given.

2. LITERATURE REVIEW

2.1. N-glycosylation

2.1.1. Synthesis of N-glycans

N-glycosylation involves three main stages: formation of nucleotide-linked sugars, assembly, and processing of oligosaccharide. The synthesis takes place in cytosol, endoplasmic reticulum (ER) and in the Golgi, and requires many enzymes. In addition to N-glycosylation specific enzymes, many other proteins and complexes are needed for N-glycan synthesis. The latter influence the normal course of N-glycosylation but also other pathways (e.g. protein O-glycosylation). Therefore, it is important to include these proteins in this overview as well.

In Figures 1 and 2, the illustration of N-glycosylation pathway is given to follow the metabolic steps described in this paragraph.

2.1.1.1. Dolichol-phosphate synthesis

Defects in dolichol (Dol) synthesis and metabolism are classified under the defects in multiple glycosylation and other pathways (Jaeken et al 2009; Morava and Lefeber 2011) but they carry also an important role in N-glycosylation.

Dol is an alpha-saturated polyisoprenoid alcohol and a membrane lipid like component. *De novo* biosynthesis of Dol occurs in cytoplasmic side of ER. The first steps of Dol synthesis are common with cholesterol synthesis. Its intermediate product farnesyl pyrophosphate is elongated with molecules of isopentenyl pyrophosphate by dehydrodolichyl diphosphate synthase (DHDDS) and Nogo-B receptor (NUS1) complex and subsequently, polyprenol pyrophosphate is synthesized. Polyprenol pyrophosphate is dephosphorylated into polyprenol and subsequently reduced to Dol by steroid 5-α-reductase 3 (SRD5A3). Finally, Dol is phosphorylated (Dol-P) by dolichol kinase (DOLK) (Figure 3).

In N-glycosylation process, Dol-P acts as a lipid carrier for oligosaccharide. In addition to *de novo* biosynthesis, Dol can be re-used after oligosaccharide transfer from Dol onto protein (Chapter 2.1.1.4.). Dol-P-P is dephosphorylated into Dol-P, which returns to the cytoplasmic side of ER.

Dol-P also serves as a glucose (Glu) and mannose (Man) donor (Dol-P-Glu and Dol-P-Man respectively) in ER, after lipid-linked oligosaccharide (LLO) is flipped in the lumen of ER. Four Dol-P linked to a Man (enzymes dolichol-phosphate mannosyltransferase subunits 1, 2 and 3; DPM1, DPM2, DPM3 respectively) and three Dol-P linked to a Glu are also flipped in the lumen of ER, and serve as sugar carriers for further LLO elongation. The mannose-P-dolichol utilization defect 1 protein (MPDU1) locates in ER and is needed for bioavailability and utilization of Dol-P-Man and Dol-P-Glu.

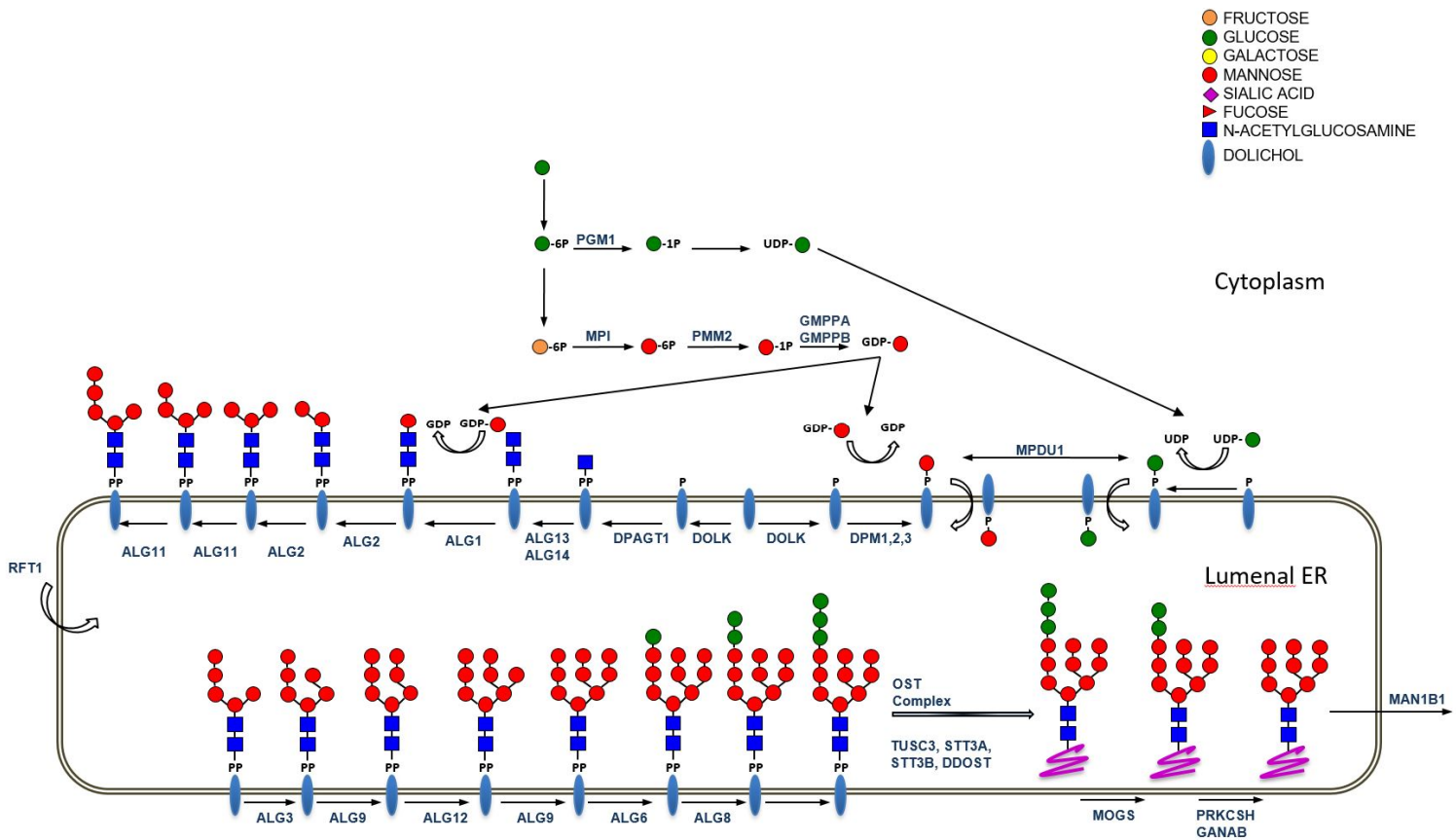

Figure 1. N-glycosylation in cytoplasm and ER.

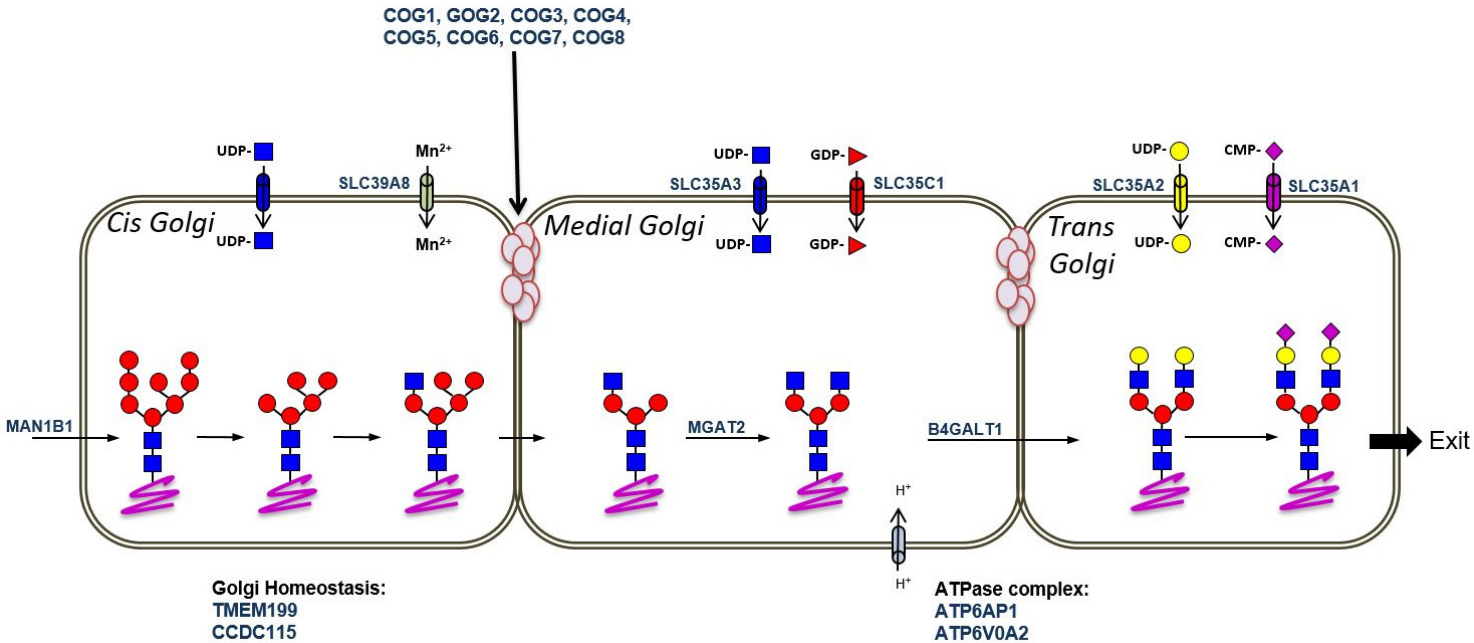

Figure 2. N-glycosylation in the Golgi.

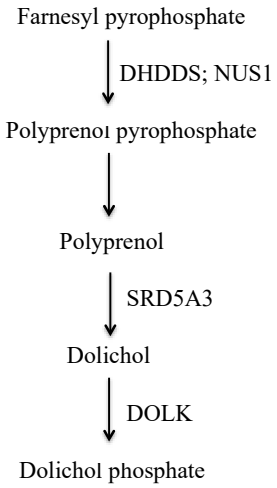

Figure 3. Schematic overview of *de novo* biosynthesis of dolichol phosphate from the intermediate farnesyl pyrophosphate.

The lack of end product Dol-P leaves glycoproteins with unoccupied N-glycosylation sites and causes reduction of glycosylation in case of SRD5A3 and DOLK deficiency. With the deficiency of DHDDS and MPDU1, truncated LLOs are characteristic.

As Dol-P is needed as a Man donor also in O-glycosylation and GPI-anchor synthesis, these processes are affected, when defects are present (Marquardt and Denecke 2003; Cantagrel and Lefeber 2011).

2.1.1.2. Synthesis of nucleotide-linked sugars

Monosaccharides act as substrates in the process of N-glycosylation and are synthesized and activated in the cytoplasmic side of ER by guanosine diphosphate (GDP) or uridine diphosphate (UDP), or in nucleus by cytidine monophosphate (CMP). Following sugars are needed in the N-glyosylation process: Man, Glu, N-acetylglucoseamine (GlcNAc), fucose (Fuc), galactose (Gal), and sialic acid (Sia). They are transferred and attached to oligosaccharide in the form of activated nucleotide-linked sugars (GDP-Man, UDP-Glu, UDP-GlcNAc, GDP-Fuc, UDP-Gal and CMP-Sia). Activated nucleotide-linked sugars can only be used in the cytosolic side of ER and ER membrane is impermeable for them. In the ER lumen and the Golgi, Dol-P and special transporters are used to deliver monosaccharides in the continuing synthesis of glycan.

Monosaccharides mostly originate from dietary sources. Man is synthesized from fructose-6-phosphate with enzymes mannose-6-phosphate isomerase (MPI) and phospohomannomutase 2 (PMM2, Figure 4). Activated nucleotid-

linked sugar GDP-Man is synthesized from mannose-1-phosphate and guanosine triphosphate by guanosine diphosphate mannose pyrophosphorylase A and B (GMPPA and GMPPB respectively). Two important types of CDG are caused by Man deficiency, PMM2-CDG and MPI-CDG (Marquardt and Denecke 2003; Sparks 2012; Jaeken and Peanne 2017).

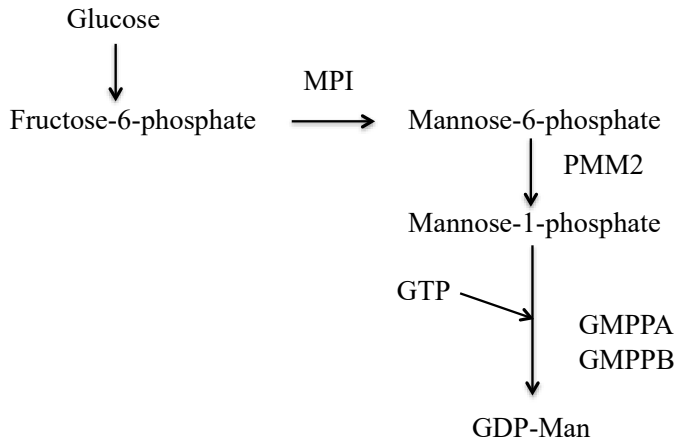

Figure 4. GDP-mannose synthesis.

For UDP-Glu synthesis, normal activity of phosphoglucomutase 1 (PGM1) is important, since its deficit causes lack of glucose-1-phosphate, the precursor of UDP-Glu. PGM1 is also an important enzyme in glycolysis and glyconeogenesis, and its deficiency causes different phenotypes: glycogen storage disease XIV with muscle involvement, and hypoglycosylation of proteins with multisystem presentation (Morava 2014).

2.1.1.3. Dolichol-linked oligosaccharide biosynthesis

Biosynthesis of LLO starts in the cytoplasmic ER membrane where Dol-P acts as a lipid carrier (Figure 1). In the cytoplasmic side, the process uses activated nucleotide linked sugars. First, GlcNAc-1-phosphate is linked to Dol-P by dolichyl-phosphate N-acetylglucoseamine phosphotransferase (DPAGT1). UDP-GlcNAc transferase (ALG13/ALG14) adds the second GlcNAc. $GlcNAc_2$-PP-Dol is elongated by different mannosyltransferases (ALG1, ALG2 and ALG11), and altogether five molecules of Man are added. After the attachment of first Man, oligosaccharide branches into two arms (α1,3 and α1,6) and α1,3 arm is extended by two additional Man.

Biantennary structure Man_5-$GlcNAc_2$-PP-Dol is translocated from the cytoplasmic side of ER to the luminal side by flippase (including its accessory

unit RNA polymerase-associated protein, RFT1). Now, only Dol-P-Man and Dol-P-Glu can be used as monosaccharide carriers for the further elongation of translocated oligosaccharide. Four additional mannoses are added by ER-localized mannosyltransferases (ALG3, ALG9, ALG12) and additional α1,3/α1,6 branch is generated to a branch α1,6. Finally, three glycoses are added to the first α1,3 arm by glycosyltransferases (ALG6, ALG8) and the final structure of oligosaccharide Glu_3-Man_9-$GlcNAc_2$-PP-Dol is ready. In the assembly stage, twelve different types of CDG have been described (Chapter 2.1.3.3.) (Marquardt and Denecke 2003; Jaeken 2010; Stanley et al 2015).

2.1.1.4. Transfer of oligosaccharide

At the luminal side of ER, an assembled LLO (Glu_3-Man_9-$GlcNAc_2$-PP-Dol) is removed from its lipid carrier and attached to the protein by oligosaccharyl transferase complex (OST, Figure 1). The transfer occurs during or after the translocation of the protein. N-glycosylation uses the amide group of amino acid asparagine (Asn) for binding to the protein. It is important that Asn should be located within Asn-Xxx-serine (Ser)/threonine (Thr) sequence and Xxx cannot be proline (Pro). OST has high substrate affinity and carries mostly fully assembled oligosaccharides. Incomplete oligosaccharides reduce the transfer efficiency and cause hypoglycosylation with empty N-glycan sites. Four types of CDG are known to have been caused by defective OST subunits (DDOST, STT3A, STT3B, TUSC3) (Marquardt and Denecke 2003; Stanley et al 2015).

2.1.1.5. Processing of protein-bound oligosaccharide

After oligosaccharide transfer, three Glu and one Man (branch α1,6) are trimmed by glucosidases (MOGS, GANAB, PRKCSH) and mannosyl-oligosaccharide alpha-1,2-mannosidase (MAN1B1). The process takes place in the luminal side of ER and in the Golgi (Figure 1 and 2). During trimming, protein quality control is performed, which determines whether glycoprotein will be degraded or processing will continue.

The Golgi has cisternal organization, which ensures the sequential process on N-glycosylation. Each enzyme and transporter localizes in cisternae where it needs to be active either by catalyzing the reaction or transporting the monosaccharides. Cisternae also help to create appropriate optimal microenvironment for enzymes (Reynders et al 2011). The essential substrates CMP-Sia, UDP-Gal, UDP-GlcNAc and GDP-Fuc are provided by the respective transporters (SLC35A1, SLC35A2, SLC35A3 and SLC35C1, respectively), which are important also in other glycosylation pathways.

In the *cis*-Golgi, additional three Man are removed from protein-bound Man_8-$GlcNAc_2$ by mannosidases, creating Man_5-$GlcNAc_2$ intermediate, which is a key intermediate. In the medial Golgi, GlcNAc is added by GlcNAc-transferase I to the branch α1,3. Then two other Man are removed leaving three Man

core. The second GlcNAc is added to the other arm by beta-1,2-N-acetylglucosaminyltransferase II (MGAT2) and biantennary glycan is formed. Next, in the *trans*-Golgi, two Gal are added to both antenna of $GlcNAc_2$-Man_3-$GlcNAc_2$ by beta-1,4-galactosyltransferase 1 (B4GALT1). Finally, two Sia residues are added by sialyltransferase and the final glycan Sia_2-Gal_2-$GlcNAc_2$-Man_3-$GlcNAc_2$ is ready. Glycoprotein is secreted, moved to the plasma membrane or lysosomes.

In addition to the described formation of biantennary glycans, some synthesized glycoproteins are also fucosylated (e.g. ABO blood group antigens) or have more than two branches in their glycan structure as a result of the activity of different GlcNAc-transferases (e.g. α-1-antitrypsin) (Marquardt and Denecke 2003; Caramelo and Parodi 2007; Rymen et al 2013; Stanley et al 2015).

2.1.1.6. Other proteins affecting N-glycosylation

As mentioned at the beginning of the chapter, many other proteins are involved in the process of N-glycosylation. They are important in multiple glycosylation pathways and some of them were previously described (e.g. Dol synthesis, monosaccharide transporters). In addition, vacuolar H^+-ATPase complex subunits ATP6AP1 (localized in ER and ER-Golgi Intermediate Compartment, ERGIC) and ATP6V0A2 (localized in Golgi) are important for acidification of intracellular compartments and regulate Golgi pH, which is essential for the activity of glycosyltransferases. Conserved oligomeric Golgi (COG) complex is important in retrograde trafficking of vesicles from Golgi to ER as well as intra-Golgi to maintain the proper localization of different resident proteins, including enzymes needed for glycosylation in the Golgi. COG complex includes eight subunits and defects are described in all, except in subunit COG3. Subunits COG1-4 are organized in lobe A and subunits GOG5-8 in lobe B. Subunits COG1 and COG8 interact two lobes. Defect in one subunit destabilizes other subunits, reduces their expression and association with the Golgi. GOG defects lead to mislocalization and decreased activity of glycosyltransferases such as mannosidase II and B4GALT1 as well as Golgi transporters, which disturb the normal process of glycosylation (Reynders et al 2011).

Coiled-coil domain containing protein 115 (CCDC115) and transmembrane protein 199 (TMEM199) locate in ERGIC and play a role in Golgi homeostasis (Jansen et al 2016a; Jansen et al 2016b). Manganese and zinc transporter (SLC39A8) is a membrane protein important for uptake of manganese, zinc and other cations into cell, and probably thereby affects the affinity of galactosyltransferase to UDP-Gal. In conclusion, all these proteins help to provide a proper environment and trafficking in Golgi, which is important for the function of different enzymes (Smith and Lupashin 2008; Park et al 2015; Jaeken and Peanne 2017).

2.1.2. Screening of N-glycosylation defects

The standard screening test for N-glygosylation disorders is serum Tf IEF (Jaeken and Peanne 2017). Serum Tf is rather simple N-glycosylated glycoprotein, which has only two Asn with appropriate motif. Therefore, Tf carries two biantennary glycans with four Sia, and normally, the most prevalent band is tetrasialotransferrin. Small amounts of mono-, di-, tri-, penta- and hexasialotransferrins are present.

Tf IEF separates Tf isoforms on the basis of their charge. As Sia is located on the top of biantennary glycan, and is negatively charged, its partial deficiency causes the loss of negative charge, which leads to the change of Tf isoelectric point, and a cathodal shift in IEF. In case of type 1 pattern, decreased tetrasialotransferrin together with increased disialo- and asialotransferrin are seen. It refers to CDG-I defects (Table 1, Figure 5), where the supply of substrates, the synthesis of lipid carrier Dol-P, the assembly of the LLO or its transfer to the peptide chain is disturbed, and only one or none of the glycans are built. Thirty types of CDG show type 1 pattern in screening (Peanne et al 2017).

In type 2 pattern, increased trisialo- and monosialotransferrin are also present in addition to disialo- and asialotransferrin bands. Tetrasialotransferrin can be normal or reduced. This refers to CDG-II defects (Table 1, Figure 5), where the processing of protein-bound glycans is disturbed and they are structurally abnormal. At least 18 types of CDG show type 2 pattern in the screening (Peanne et al 2017).

Normal Tf IEF does not exclude CDG. First, some types of CDG always show normal sialotransferrins (e.g. MOGS-CDG, GANAB-CDG, PRKCSH-CDG, SLC35A3-CDG, SLC35C1-CDG), and second, some types may show both, normal and abnormal pattern in Tf IEF (e.g. SRD35A3-CDG, ALG13-CDG, SLC35A2-CDG). Third, the results can be influenced by age. For instance, false-negative results have been described in adolescents and adults. On the contrary, up to 6 weeks of age, mild type 2 pattern, caused by hyposialylated Tf, might be present, and all the Tf bands appear slightly more intense than usual (false-positive). One possibility is to do in parallel an α-1-antitrypsin IEF in early infancy to evaluate the possible diagnosis of CDG (Thiel et al 2013). α-1-antitrypsin has three N-glycosylation sites to which bi-, tri- and tetraantennary glycans are attached (Barone et al 2008). Another possibility is to repeat Tf IEF in later infancy.

Table 1. Different types of CDG with positive Tf IEF (according to Jaeken and Peanne 2017, Peanne et al 2017 and Ferreira et al 2018).

	Tf IEF		
CDG	**Type 1**	**Type 2**	**Comments**
DDOST-CDG	+		
DHDDS-CDG	+		Normal Tf IEF has been reported
DPM1-CDG	+		
DPM2-CDG	+		
DPM3-CDG	+		
DOLK-CDG	+		
DPAGT1-CDG	+		
ALG1-CDG	+		
ALG2-CDG	+		
ALG3-CDG	+		
ALG6-CDG	+		
ALG8-CDG	+		
ALG9-CDG	+		
ALG11-CDG	+		
ALG12-CDG	+		
ALG13-CDG	+		Normal Tf IEF has been reported
ALG14-CDG	+		Normal Tf IEF has been reported
MPDU1-CDG	+		
MPI-CDG	+		
NUS1-CDG	+		
PMM2-CDG	+		Normal Tf IEF has been reported
RFT1-CDG	+		
SRD5A3-CDG	+		Normal Tf IEF has been reported
STT3A-CDG	+		
STT3B-CDG	+		
TUSC3-CDG	+		
PGM1-CDG	+	+	Mixed type 1 and 2 profile
ATP6AP1-CDG		+	
ATP6V0A2-CDG		+	
BGALT1-CDG		+	
CCDC115-CDG		+	
COG1-CDG		+	
COG2-CDG		+	
COG4-CDG		+	
COG5-CDG		+	
COG6-CDG		+	
COG7-CDG		+	
COG8-CDG		+	
MAN1B1-CDG		+	
MGAT2-CDG		+	
SLC35A1-CDG		+	Normal Tf IEF has been reported
SLC35A2-CDG		+	Normal Tf IEF has been reported
SLC39A8-CDG		+	
TMEM199-CDG		+	

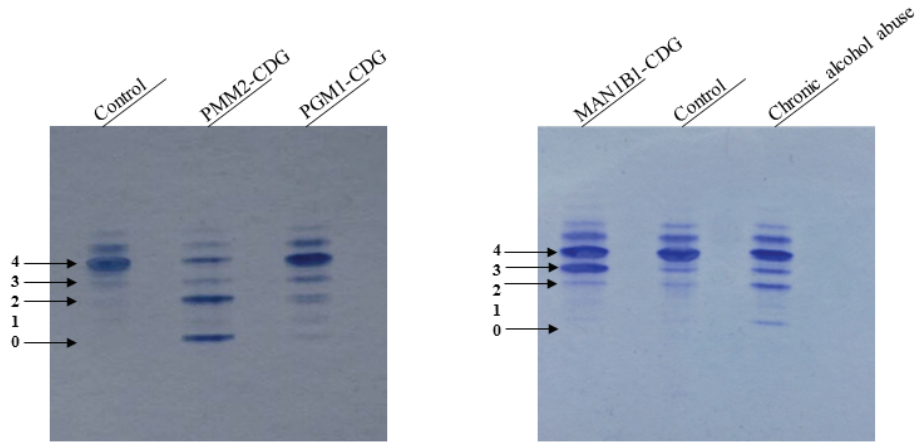

Figure 5. Different profiles in Tf IEF. The arrows are indicating the number of sialic acid residues. The samples originate from ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism) Quality Control scheme for CDG screening 2018 (with the permission of Dirk Lefeber).

False-positive results can be also seen with diseases where secondary hypo-glycosylation might be present (galactosemia, hereditary fructose intolerance, alcohol abuse, infections with neuraminidase-producing organism, and liver pathology). Tf polymorphisms can also cause a change of Tf isoelectric point and show abnormal pattern in IEF. Polymorphism should be excluded with neuraminidase digestion, which reduces all glycoforms into two asialotrans-ferrins. Still, if Tf polymorphism is caused by a variant in the Asn-Xxx-Ser/Thr sequence leading to Tf with a single glycan, the treatment with neuraminidase can be misleading. Comparative Tf IEF from the serum of the parents or alternative glycoprotein IEF can be helpful then (Guillard et al 2011).

In addition to Tf and α-1-antitrypsin IEF, other serum proteins like thyroxine-binding globuline can be screened for CDG. In case of patients with type 2 Tf IEF, diagnostics should proceed with mass spectrometry (MS, Chapter 2.1.4.2.) and serum apolipoprotein C-III (ApoC-III) IEF after inconclusive MS result. ApoC-III is a marker of mucin type core 1 O-glycan synthesis and abnormalities detected with IEF refer to combined N- and O-glycosylation defect (e.g. COG-associated CDG). ApoC-III shows three isoforms in IEF depending on the number of Sia residues attached to only one core 1 mucin-type O-glygan: ApoC-III_0 (nonglycosylated), ApoC-III_1 and ApoC-III_2 (normally, a major isoform) (Wopereis et al 2003; Lefeber et al 2011).

Alternative approaches for screening of N-glycosylation defects are capillary zone electrophoresis and high-pressure liquid chromatography, which also separate Tf isoforms based on their charge. These methods are more rapid and results are easily quantified (Quintana et al 2009; Parente et al 2010; Jaeken and

Peanne 2017). Still, serum Tf IEF is advised, when abnormal results are seen (Quintana et al 2009; Parente et al 2010; Jaeken and Peanne 2017).

A new potential biomarker for detecting hypoglycosylation in CDG type 1 is a plasma membrane glycoprotein called intercellular cell adhesion molecule 1 (ICAM-1). Its expression is shown to be reduced or absent in plasma membranes of glycosylation-deficient cells. In addition, ICAM-1 can be used to follow therapeutic response in different types of CDG where potential therapeutics are possible. In case of MPI-CDG, Man supplementation to MPI-deficient cells increased ICAM-1 expression (He et al 2012).

2.1.3. Overview of clinical features of different CDG

In this chapter an overview of the clinical phenotypes of selected CDG with or without positive screening caused by impaired N-glycan synthesis is given (Table 1). In many types of CDG, very few patients have been described. The symptoms are often variable, overlapping and unspecific, and may not lead to clinical suspicion of CDG. The severity of a phenotype might differ between different types of CDG as well as in patients with the same CDG type. The nervous system is almost always affected. Currently, the majority of CDG do not have effective treatment.

Recently, a comprehensive scheme based on sialotransferrin profile and clinical symptoms was published to narrow the selection of the possible candidate CDG, which could help shorten the time period from clinical suspicion to genetic confirmation of CDG (Perez-Cerda et al 2017). Once the diagnosis is confirmed, the disease course can be objectively monitored with Nijmegen CDG rating scale. Its sections include parental or a caretaker's evaluation for current function, a clinician's evaluation of systems involved, and present clinical assessment (Achouitar et al 2011).

2.1.3.1. Defects in dolichol synthesis and metabolism

Variants in genes *DHDDS*, *NUS1*, *SRD5A3* and *DOLK* cause DHDDS-CDG, NUS1-CDG (described only in one family), SRD5A3-CDG, and DOLK-CDG respectively. DHDDS-CDG (OMIM 613861) has been related to retinal involvement (retinitis pigmentosa) but at least one patient with multisystem presentation and protein hypoglycosylation has been reported (Zuchner et al 2011; Sabry et al 2016). SRD5A3-CDG (CDG-Iq, OMIM 612379) symptoms include developmental delay/intellectual disability (DD/ID), hypotonia, visual impairment (optic nerve atrophy, cataract, glaucoma), cerebellar atrophy, dilated cardiomyopathy, ichthyosiform dermatitis, hepatic dysfunction, and coagulation abnormalities (Cantagrel et al 2010). DOLK-CDG (CDG-Im, OMIM 610768) causes DD/ID, epilepsy, hypsarrhythmia in EEG, hypotonia, nystagmus, dilated cardiomyopathy, and ichthyosis (Kranz et al 2007). In all four types, serum Tf

IEF shows type 1 pattern, although in adult with SRD5A3-CDG, a normal pattern was described (Cantagrel and Lefeber 2011; Kahrizi et al 2011; Mohamed et al 2011; Jaeken and Peanne 2017; Peanne et al 2017).

Variants in genes *MPDU1, DPM1, DPM2* and *DPM3* cause MPDU1-CDG, DPM1-CDG, DPM2-CDG, and DPM3-CDG. MPDU1-CDG (CDG-If, OMIM 609180), DPM1-CDG (CDG-Ie, OMIM 608799) and DPM2-CDG (CDG-Iu, OMIM 615042) symptoms include DD/ID, epilepsy, ataxia, and ophthalmological symptoms including visual loss. In addition, MPDU1-CDG causes dwarfism, ichthyosiform skin, and DPM1- and DPM2-CDG muscular dystrophy with elevated creatine kinase level (Imbach et al 2000; Kranz et al 2001; Schenk et al 2001; Barone et al 2012). In DPM3-CDG (CDG-Io, OMIM 612937) elevated creatine kinase level, muscle dystrophy and dilated cardiomyopathy are seen (Lefeber et al 2009). Serum Tf IEF shows type 1 pattern in all four CDG (Cantagrel and Lefeber 2011; Peanne et al 2017).

2.1.3.2. Defects in monosaccharide synthesis

MPI-CDG (CDG-Ib, OMIM 602579) is caused by the deficiency of mannose-6-phosphate isomerase (Niehues et al 1998). It used to be the third most common CDG but now few other CDG types are more frequently diagnosed. Symptoms arise mainly from gastrointestinal system and include congenital hepatic fibrosis, steatosis, cirrhosis, secretory diarrhea and protein-losing enteropathy, gastrointestinal bleeding, vomiting, failure to thrive, bleeding, thrombosis, and hyperinsulinemic hypoglycemia. The nervous system is usually spared unless complications like hypoglycemia occur. Serum Tf IEF shows type 1 pattern. MPI-CDG is treatable with oral mannose, as Man-6-phosphate can be alternatively synthesized directly from Man by enzyme hexokinase. The treatment improves gastrointestinal symptoms, hypoglycemia, coagulopathy and growth. Alternatively, heparin can be used. Man does not prevent the progression of liver disease and sometimes liver transplantation is needed. After transplantation, normal glycosylation of transferrin has been described as well as the improvement of coagulopathy and gastrointestinal symptoms. The glycosylation of non-liver-glycosylated proteins remained unchanged (de Koning et al 1998; de Lonlay et al 2001; de Lonlay and Seta 2009; Janssen et al 2014).

PGM1-CDG (CDG-It, OMIM 614921) symptoms include muscle weakness, dilated cardiomyopathy, chronic hepatitis, endocrine anomalies (hypogonadotropic hypogonadism), rhabdomyolysis, and dysmorphic features (Pierre-Robin sequence). Patients have normal intelligence, as PGM1 is not expressed in the nervous system (Stojkovic et al 2009; Timal et al 2012). PGM1 deficiency also affects Gal metabolism. UDP-Glu and UDP-Gal are in metabolic balance, which is supported by the epimerization of UDP-Gal to UDP-Glu. PGM1 deficiency leads to the reduced amounts of UDP-Gal, which causes decreased galactosylation of glycans. Treatment with oral galactose is possible and improves glycosylation as well as symptoms, except dilated cardiomyopathy. Serum Tf IEF shows mixed type 1 and type 2 pattern (Timal et al 2012; Morava 2014).

PMM2-CDG (CDG-Ia, OMIM 212065) will be thoroughly discussed in Chapter 2.2. GMPPA-CDG (OMIM 615510) and GMPPB-CDG (OMIM 615350) affect multiple glycosylation pathways (Carss et al 2013; Koehler et al 2013). They do not show abnormal Tf IEF pattern and will not be described.

2.1.3.3. Defects in the assembly of lipid-linked oligosaccharide

DPAGT1-CDG (CDG-Ij, OMIM 608093) symptoms include progressive microcephaly, epilepsy, hypotonia, cerebral atrophy, ophthalmological symptoms, jaundice, dysmorphic features, joint contractures, and myasthenia (Wu et al 2003). In adolescents, normal Tf IEF pattern is possible. Improvement of myasthenia with cholinesterase inhibitor pyridostigmine has been described (Klein et al 2015; Peanne et al 2017).

ALG13-CDG (CDG-Is, OMIM 300884) causes early infantile epileptic encephalopathy and ALG14-CDG (OMIM 616227) congenital myasthenia (Timal et al 2012; Cossins et al 2013). ALG1-CDG (CDG-Ik, OMIM 608540) symptoms include DD/ID, refractory epilepsy, hypotonia, cerebral atrophy, hepatosplenomegaly, dysmorphic features and hypogonadism (Schwarz et al 2004). ALG2-CDG (CDG-Ii, OMIM 607906) symptoms are DD/ID, epilepsy, cataract, and hepatomegaly (Thiel et al 2013). ALG11-CDG (CDG-Ip, OMIM 613661) causes DD/ID, epilepsy, hypotonia, deafness, ophthalmological symptoms, opisthotonus, dysmorphic features, feeding difficulties, and vomiting (Rind et al 2010).

RFT1-CDG (CDG-In, OMIM 612015) symptoms include DD/ID, hypotonia, hyperreflexia, early-onset epilepsy, decreased visual acuity, dysmorphic features, sensorineural deafness, and short stature (Haeuptle et al 2008).

ALG3-CDG (CDG-Id, OMIM 601110) symptoms are DD/ID, axial hypotonia, hyperreflexia, epilepsy, cerebellar and corpus callosum hypoplasia, optic atrophy, cortical blindness, arthrogryposis, clubfeet, dysmorphic features, failure to thrive, vomiting, and diarrhea (Korner et al 1999). ALG9-CDG (CDG-IL, OMIM 608776) patients have DD/ID, hypotonia, epilepsy, microcephaly, diffuse brain atrophy with delayed myelination, pericardial effusion, hepatosplenomegaly, cystic renal disease, and failure to thrive (Frank et al 2004). ALG12-CDG (CDG-Ig, OMIM 607143) symptoms include DD/ID, hypotonia, microcephaly, widening of the lateral ventricles, blindness, cardiomyopathy, skeletal anomalies and delayed ossification, dysmorphic features, immunodeficiency with recurrent infections, and deafness (Chantret et al 2002).

ALG6-CDG (CDG-Ic, OMIM 603147) is the second most common CDG. Symptoms include DD/ID, epilepsy, cortical blindness, ataxia, intention tremor, agenesis of corpus callosum, ophthalmological symptoms, distal limb defects, brachydactyly, endocrine as well as gastrointestinal disturbances (Burda et al 1998; Imbach et al 1999). ALG8-CDG (CDG-Ih, OMIM 608104) causes ID, structural heart defects, hepatomegaly, cystic bile ducts, cholestasis, diffuse renal microcysts, dysmorphic features, protein-losing enteropathy, vomiting, brachydactyly, and skeletal anomalies (Chantret et al 2003).

All twelve CDG show type 1 pattern in Tf IEF and are autosomal recessive diseases except ALG13-CDG, which is X-linked disorder (Peanne et al 2017).

2.1.3.4. Defects related to oligosaccharide transfer

TUSC3-CDG (OMIM 611093), DDOST-CDG (CDG-Ir, OMIM 614507), STT3A-CDG (CDG-Iw, OMIM 615596) and STT3B-CDG (CDG-Ix, 615597) are OST subunits and cause similar symptoms like DD/ID, hypotonia, epilepsy, ophthalmological symptoms, and failure to thrive. Serum Tf IEF shows type 1 pattern in all (Peanne et al 2017).

2.1.3.5. Defects related to protein-bound oligosaccharide processing

MOGS-CDG (CDG-IIb, OMIM 606065) symptoms include DD/ID, epilepsy, hypotonia, cerebral atrophy, small corpus callosum, optic nerve atrophy, hepatomegaly, recurrent bone fractures, dysmorphic features, sensorineural hearing loss, and hypogammaglobulinemia (shortened immunoglobulin G half-life) (De Praeter et al 2000). Despite hypogammaglobulinemia, there seems to be increased resistance to certain viral infections. Serum Tf IEF shows normal pattern. Nevertheless, the N-glycan profile of immunoglobulin G was found to be abnormal in MALDI-TOF (matrix-assisted laser detection desorption ionization–time-of-flight) MS (Sadat et al 2014; Peanne et al 2017).

GANAB-CDG (OMIM 600666) and PRKCSH-CDG (OMIM 174050) cause autosomal dominant polycystic kidney disease and polycystic liver disease respectively. Serum Tf IEF shows normal pattern in both (Peanne et al 2017).

MAN1B1-CDG (OMIM 614202) is characterized by DD/ID, hypotonia, dysmorphism, and truncal obesity (Rafiq et al 2011; Rymen et al 2013). MGAT2-CDG (CDG-IIa, OMIM 212066) symptoms include DD/ID, epilepsy, stereotypic movements, muscle atrophy, dysmorphic features, feeding problems, diarrhea, recurrent respiratory infections, and sensorineural hearing loss (Tan et al 1996; Ramaekers et al 1991). B4GALT1-CDG (CDG IId, OMIM 607091) causes transient axial hypotonia, myopia, dysmorphic features, coagulation abnormalities, hepatopathy, and diarrhea. Normal psychomotor development is described as enzyme B4GALT1 has low expression in the brain, where other galactosyltransferases are more prevalent. Serum Tf IEF shows type 2 pattern in all three (Hansske et al 2002; Peters et al 2002; Guillard et al 2011; Rymen et al 2013; Peanne et al 2017).

SLC35A1-CDG (CDG-IIf, OMIM 603585), SLC35A3-CDG (OMIM 615553) and SLC35C1-CDG (CDG-IIc, OMIM 266265; also known as leukocytes adhesion deficiency II) cause all DD/ID, epilepsy, hypotonia or ataxia. SLC35A1-CDG also shows proteinuria, bleeding; SLC35C1-CDG dwarfism, dysmorphic features, and recurrent infections with neutrophilia. Patients have a rare Bombay blood group (Lubke et al 1999; Martinez-Duncker et al 2005; Edvardson et al 2013). Serum Tf IEF shows type 2 pattern in

SLC35A1-CDG, but normal pattern in SLC35A3-CDG and SLC35C1-CDG. Oral Fuc has shown to reduce the infections in SLC35C1-CDG in some patients, but as some patients do not show benefit from Fuc treatment, more studies are needed in this matter (Marquardt et al 1999; Peanne et al 2017).

SLC35A2-CDG (CDG-IIm, OMIM 300896) will be thoroughly discussed in Chapter 2.3.

2.1.3.6. Defects in other proteins affecting N-glycosylation

ATP6AP1-CDG (OMIM 300972) causes immunodeficiency (hypogammaglobulinemia, recurrent bacterial infections), neonatal jaundice and hepatosplenomegaly. It is X-linked disorder (Jansen et al 2016). ATP6V0A2-CDG (OMIM 219200, and 282250) symptoms are DD/ID, epilepsy, hypotonia, cobblestone-like brain malformation, myopia, skeletal anomalies and characteristic cutis laxa (Kornak et al 2008). CCDC115-CDG (CDG-IIo, OMIM 616828) and TMEM199-CDG (CDG-IIp, OMIM 616829) cause liver disease, which can mimic Wilson disease due to low levels of serum ceruloplasmin. Contrary to CCDC115-CDG, patients with TMEM199-CDG show normal development (Jansen et al 2016a; Jansen et al 2016b). SLC39A8-CDG (CDG-IIn, OMIM 616721) causes DD/ID, epilepsy, hypotonia, cerebellar atrophy, cranisynostosis, dwarfism, recurrent infections and hearing impairment as well as low manganese levels in blood. As an enzyme B4GALT1 is manganese-dependent, defect in SLC39A8 leads to hypogalactosylation of glycans. Treatment with manganese and Gal is recommended (Park et al 2015).

The mutual symptoms of CDG caused by COG1, COG2, COG4, COG5, COG6, COG7 and COG8 defects include DD/ID, microcephaly, and hepatic dysfunction. In some, epilepsy, cerebral or cerebellar atrophy, recurrent infections, failure to thrive and skeletal anomalies are seen (Peanne et al 2017). More patients are described with lobe B COG defects and hypothesis is that the clinical phenotype is less severe compared to lobe A defects (Haijes et al 2018). The difference in the phenotype severity between different COG-CDG subtypes might depend on how variants affect the whole COG complex integrity and function (Laufman et al 2013).

Serum Tf IEF shows type 2 pattern in all (Park et al 2015; Jaeken and Peanne 2017; Peanne et al 2017).

2.1.4. Other diagnostic methods of N-glycosylation defects

2.1.4.1. Enzymatic testing and lipid-linked oligosaccharide analysis

In case of type 1 Tf IEF profile and suspected MPI-CDG or PMM2-CDG, enzymatic assays from cultured fibroblasts or peripheral blood leukocytes are available to measure MPI or PMM activity. In case of PMM2-CDG, it is possible that PMM residual activity in fibroblasts is within normal limits, but

profoundly low in leukocytes. Therefore, enzymatic tests are neither confirmative nor exclusive. Now, after detecting type 1 Tf IEF, first, the sequencing of *PMM2* is suggested. A negative result should be followed by targeted CDG panel analysis or whole genome/exome sequencing (Grunewald et al 2001; Jaeken 2011; Cylwik et al 2013; Peanne et al 2017).

If the deficiency of MPI and PMM2 is excluded, LLO analysis in fibroblasts helps to evaluate the accumulation of glycan intermediates, which might give a hint to the possible molecular cause. LLO analysis was used to identify novel types of CDG-I caused by defects located in cytosol or ER. As N-glycosylation pathway in ER is highly conserved between humans and yeast, an accumulation of the substrate of affected reaction helped to identify the human ortholog to a culprit gene in yeast with similar finding. With LLO analysis, the patients' cells are labeled with radioactive [2-(^{3}H)]-Man, LLOs are extracted, and oligosaccharides are released from the lipid carrier, followed by high performance liquid chromatography analysis (Burda et al 1998; Peanne et al 2017). Nevertheless, LLO analysis might not be reliable in detecting the accumulation of short LLOs caused by defects in early LLO synthesis (Schwarz et al 2004).

2.1.4.2. Glycoprofiling with mass spectrometry

Different MS methods analyse the N-glycans of intact Tf or whole plasma glycoproteins. They give quantitative structural information about the glycans, and they can be used in diagnosing of CDG and monitoring the effect of therapy. MS can be used as the first step in CDG screening as MS analysis of intact Tf can quickly detect both, type 1 and type 2 CDG. In case of type 1 defects, a loss of one or two complete N-glycans is detected. Otherwise, glycoprofiling is currently indicated for patients with type 2 pattern in screening, in order to detect the presence of specific truncated glycan structures, which might hint at the diagnosis. For instance, characteristic diagnostic profiles for intact Tf are seen with high-resolution MS in MGAT2-CDG (peak at two monoantennary complex N-glycans), B4GALT1-CDG (peak at two truncated biantennary glycans lacking Gal and Sia), SLC35A2-CDG (truncated glycans lacking one to four Gal-Sia disaccharides), MAN1B1-CDG (hybrid glycans), and PGM1-CDG (truncated glycans and loss of complete glycans). COG1-CDG and ATP6V0A2-CDG patients show increase of truncated structures with the reduction of Sia and Gal (van Scherpenzeel et al 2015). As previously mentioned, SLC35C1-CDG and SLC35A1-CDG can both show normal Tf IEF pattern and the first step in diagnostics should be MS. For SLC35C1-CDG, the whole plasma N-glycan profiling with MS shows decreased fucosylation. It is preferred over high-resolution MS of intact Tf, which shows non-specific decrease of fucosylation that is also evident in normal population. Inversely, in SLC35A1-CDG, the MS of whole plasma N-glycans is comparable with normal population, but with high-resolution MS of intact Tf, a loss of Sia

and increased peaks of di- and trisialylated Tf are seen (Guillard et al 2011; van Scherpenzeel et al 2015).

In addition, high-resolution MS detects sensitively a loss of intact glycans in analyses where Tf IEF shows mild or near-normal type 1 profiles, and mild Tf IEF type 2 profile might turn out to be normal result or type 1 CDG with MS. The secondary causes of abnormal glycosylation with type 1 (galactosemia, fructosemia, alcohol abuse) or 2 (increased sialidase activity, liver disease) profile, and Tf polymorphisms can also be excluded with MS (van Scherpenzeel et al 2015).

In conclusion, the detection of a specific diagnostic profile may lead to direct variant analysis and significantly shorten the time of reaching the diagnosis. MS of intact Tf is preferred as glycan levels are standardized to one protein instead of many. Still, MS is implemented in few laboratories at the moment (Guillard et al 2011; van Scherpenzeel et al 2015; Van Scherpenzeel et al 2016; Peanne et al 2017).

2.1.4.3. Molecular diagnostics

Molecular genetic analyses confirm the diagnosis of suspected CDG and are important in prenatal diagnosis for families with history of CDG. Since these analyses have become more available, their role has increased in the diagnostics of CDG. Different NGS techniques have helped to solve many screening-positive (e.g. MAN1B1-CDG) as well as screening-negative (e.g. SLC35A2-CDG) patients, and discover new disorders. Nowadays it is not unusual that NGS precedes biochemical studies and some patients have been diagnosed with CDG without previous suspicion of the disorder.

One way to approach molecular diagnosis is sequencing of single genes, especially if a certain type of CDG is suspected, based on clinical symptoms and biochemical studies. In case of CDG type I, first, sequencing of *PMM2* is proposed (Peanne et al 2017). As the phenotype of CDG is often unspecific, variable, and overlapping, and may not indicate to a certain type of CDG, different gene panels (including CDG gene panel) are available as an alternative to single gene approach. They are more cost effective (Jones et al 2013).

If gene panel results are negative, whole exome sequencing (WES) is another option for identifying the genetic cause of Mendelian disorders. Many variants of unknown significance, some of which are not disease causing, can be detected with gene panels or WES and must be evaluated carefully. In single cases it might be difficult to decide whether the findings are pathogenic or not, and diagnosis requires confirmation with functional assays, which may not be available for many gene defects yet (Ng and Freeze 2018). In addition, a previously not described variant in gene, which is assumed to cause a novel type of CDG, needs to be confirmed with functional studies. Complementation studies are often used. Alternatively, the availability of multiple unrelated individuals with similar phenotypes and variants in the same gene support the pathogenicity of the variants. Nevertheless, in some patients with suspicion of CDG due to clinical picture, positive Tf IEF or peculiar glycan profile in MS, the causative gene

remains unknown (Timal et al 2012; Jones et al 2013; Calvo et al 2018). In conclusion, the choice of first line molecular studies is determined primarily by the availability of specific NGS techniques in different institutions and countries.

2.2. PMM2-CDG

PMM2-CDG (former names carbohydrate-deficient glycoprotein syndrome, Jaeken syndrome, and CDG-Ia; OMIM 212065) is the most common CDG diagnosed worldwide. It was first reported in identical twin sisters by Jaeken and colleagues., followed by the discovery of the cause, decreased activity of the enzyme PMM2 fifteen years later (Jaeken et al 1980; Van Schaftingen and Jaeken 1995). According to the data from 12 European laboratories, PMM2-CDG constituted 62% of all molecularly confirmed CDG (Peanne et al 2017). Interestingly, in Saudi Arabia, only two patients have been reported and other types of CDG seem to be more prevalent (Alsubhi et al 2017). The estimated frequency of PMM2-CDG might be as high as 1/20,000 (Schollen et al 2000) and in Sweden, the incidence has been reported to be 1/80,000 live births (Kristiansson et al 1998).

PMM2-CDG is caused by variants in *PMM2*, which is located in chromosomal region 16p13 (Martinsson et al 1994; Matthijs et al 1997). Although inherited autosomal recessively, Schollen and colleagues have observed preferential transmission of *PMM2* variants and increased recurrence risk in PMM2-CDG families (close to 1 in 3), which is possibly due to reproductive advantage (Schollen et al 2004). *PMM2* encodes an enzyme PMM2, which converts Man-6-phosphate into Man-1-phosphate (Van Schaftingen and Jaeken 1995). Subsequently, Man-1-phosphate is synthesized into GDP-Man, which is an essential substrate in N-glycan synthesis by being direct donor for LLO synthesis in cytoplasmic side of ER and precursor of Dol-P-Man. As Man is not only needed in N-glycosylation, PMM deficiency can affect multiple glycosylation pathways. However, PMM2-CDG is classified as protein N-glycosylation disorder (Freeze et al 2015; Jaeken and Peanne 2017).

As PMM2 influences the assembly part of LLO, Tf IEF shows type 1 profile. Still, false-negative results are described in adolescents and adults. In cultured fibroblasts and blood leukocytes, reduced activity of PMM2 is detected, although in fibroblasts, enzyme activity within normal limits is possible. Therefore, sometimes the enzyme assay should be performed on freshly isolated leukocytes. PMM2 activity is also lower in *PMM2* variant carriers without clinical symptoms (Matthijs et al 2000; Grunewald et al 2001). If PMM2-CDG is suspected based on the clinical symptoms and screening results, it would be reasonable to continue with *PMM2* sequencing to confirm the diagnosis.

Unfortunately, there is no specific treatment for the patients with PMM2-CDG. Although effective *in vitro*, several interventions have not given expected effect *in vivo*. Alimentary Man does not improve the symptoms or protein hypoglycosylation, possibly due to Man-6-phosphate conversion to fructose-6-

phosphate by MPI. Membrane permeable Man-1-phosphate proved to be toxic and unstable, enzyme replacement has not been tried, and increasing the Man-6-phosphate flux into glycosylation pathway by combining the Man administration with the inhibition of the MPI activity is challenging (Freeze 2009). Pharmacological chaperones are potential novel therapeutic tool that might rescue protein folding for some *PMM2* destabilizing variants where encoded protein has some residual activity (Yuste-Checa et al 2015).

Nevertheless, symptomatic and interdisciplinary management is essential, and recently, international clinical guideline for the management of patients with PMM2-CDG was published (Altassan et al 2018). Patients may require tube and/or enhanced feeding, physical, speech and occupational therapy, medications like diazoxide, thyroxin, and antiepileptic drugs. Adequate hydration and avoidance of tissue damage are important for reducing the risk of thrombotic complications.

2.2.1. Phenotype of PMM2-CDG

The clinical phenotype of PMM2-CDG and its severity are variable (Table 2). First described patients with PMM2-CDG showed marked DD, cerebral and cerebellar hypotrophy, delayed nerve conduction velocity, altered levels of different glycoproteins such as serum prolactin, growth hormone (GH), follicle-stimulating hormone (FSH), and thyroxine-binding globulin (TBG) (Jaeken et al 1980). *PMM2* is highly expressed in the liver but weakly in the brain. However, clinical picture always includes a moderate to severe neurological disease. With the classical phenotype, typical dysmorphic signs (inverted nipples, abnormal fat pads) and involvement of other organ systems are also additionally present (Matthijs et al 1997; Matthijs et al 1999). Three stages are differentiated: the infantile multisystem stage, the late-infantile and childhood ataxia-intellectual disability stage, and the adult stable disability stage. As the phenotype is more characteristic in children (especially with multiorgan involvement), PMM2-CDG is mainly diagnosed in early ages.

Prenatal period is usually uneventful, although non-immune fetal hydrops and hypertrophic cardiomyopathy might be present (Garcia Silva et al 1996; van de Kamp et al 2007). In infants, neurovisceral presentation is common, but isolated neurologic form is possible. In neonatal period, multisystem symptoms may include cardiac involvement (pericardial effusion, hypertrophic cardiomyopathy), hepatopathy, enteropathy, coagulopathy, hyperinsulinemic hypoglycemia and feeding difficulties. In childhood, multiorgan involvement subsides and patients show hypotonia, ataxia and DD. They continue slowly to gain skills but in the majority of cases, ID ranges from moderate to severe.

Table 2. Clinical symptoms of PMM2-CDG (Altassan et al 2018).

Dysmorphology	Inverted nipples, fat pads, large protruding ears, prominent forehead, thin upper lip, prominent jaw, long and slender fingers and toes
Neurology	Axial hypotonia, hyporeflexia, delayed motor development, intellectual disability in variable degrees, non-progressive ataxia, dysarthria, seizures, stroke-like episodes, peripheral neuropathy, myopathy, microcephaly
MRI findings	Cerebellar atrophy, delayed myelination
Opthalmology	Strabismus, myopia, cataract, pigmentary retinopathy, nystagmus, transient cortical blindness
Gastroenterology	Failure to thrive, feeding difficulties, vomiting, protein-losing enteropathy, diarrhea, hepatomegaly and increased transaminases, cholestasis in infants
Cardiology	Pericardial effusion, hypertrophic cardiomyopathy, cardiac failure, conotruncal heart defects
Nephrology	Multicystic kidneys, enlarged kidneys, tubulopathy (proteinuria)
Hematology	Coagulopathy (bleeding, venous and arterial thrombosis), rarely thrombocytopenia and neutropenia
Endocrinology	Hyperinsulinemic hypoglycemia, hypothyroidism, short stature, hypergonadotropic hypogonadism, delayed or absent puberty
Orthopaedics	Kyphoscoliosis, osteopenia, joint contractures, thoracic deformities
Biochemistry	Elevated transaminases, FSH, luteinizing hormone (LH), prolactin, GH, thyroid-stimulating hormone (TSH, might be increased in newborn screening), insulin, ferritin Decreased factors IX and XI, ATIII, protein S, protein C, albumin, cholesterol, triglycerides, TBG, insulin-like growth factor 1 (IGF-1), insulin-like growth factor-binding protein 3, immunoglobulins, haptoglobulin, α-1-antitrypsin
Other	Recurrent infections, non-immune fetal hydrops

Stroke-like episodes may be present up to half of the patients. These are often triggered by infection or head trauma, and present as an acute transient neurological episode with possible somnolence, stupor, seizures and/or hemiparesis. Full recovery may take hours to months. The brain MRI shows localized oedema not corresponding to any territory of arteries. In EEG, abnormal background and sometimes, focal seizures are seen. Vascular emergencies can also mimic stroke-like episodes (Schiff et al 2017, Altassan et al 2018).

In adults, mild-to-severe ID, cerebellar ataxia, dysarthria, peripheral neuropathy, kyphoscoliosis, osteopenia, thrombosis, retinitis pigmentosa, short

stature, cerebellar atrophy on MRI are described. They are often wheelchair dependent. Females have primary ovarian insufficiency. Subcutaneous fat pads are present in up to a third of adult patients (Krasnewich et al 2007; Monin et al 2014; Wolthuis et al 2014).

Despite the age, one of the most important symptoms in PMM2-CDG is cerebellar atrophy (both, vermis and hemispheres) in various degrees. It might not be present at birth but it usually develops later in almost all patients. The progression of atrophy has been reported in the first decade of life. Contrary to that, cerebellar symptoms do not show progression but they show stabilization or even mild improvement (de Diego et al 2017; Serrano et al 2017).

According to recent studies in France and Spain, the mortality of PMM2-CDG ranges from 9 to 15%, and occurs mainly in early childhood among patients with multiorgan involvement (Perez-Cerda et al 2017; Schiff et al 2017).

2.2.2. Variants and genotypes of PMM2-CDG

PMM2 (MIM 601785) has eight exons and encodes a protein of 246 amino acids (Matthijs et al 1997). According to the data from 2018, *PMM2* has at least 129 pathogenic variants listed in the Human Gene Mutation Database (HGMD® Professional) from BIOBASE Corporation (Stenson et al 2009), and 105 of them are missense variants. They affect the residual activity, affinity, structure and the stability of the PMM2 (Pirard et al 1999). Most of the variants are located in exons 5 and 8 (Vuillaumier-Barrot et al 2000).

Some variants are considered severe, as they lead to very low, almost absent residual activity of PMM2 (i.e. p.Arg123Gln, p.Arg141His, p.Phe157Ser, p.Thr237Arg), and some mild (p.Leu32Arg, p.Thr237Met, p.Cys241Ser), where residual activity of PMM2 ranges from 16 to 54% (Vega et al 2011). Variants in C-terminal part are associated, but not always, with a milder phenotype (Matthijs et al 2000).

The majority of the patients are compound heterozygotes and have a combination of severe and mild variant (Vega et al 2011; Yuste-Checa et al 2015). A homozygous state of some variants is possible (Matthijs et al 2000; Neumann et al 2003; Najmabadi et al 2011; Perez et al 2011). The most common variant among Caucasian patients is p.Arg141His representing 21–50% of all variants in PMM2-CDG patients from different populations. Its homozygous state is considered lethal, as the PMM2 has no residual activity (Matthijs et al 1997; Matthijs et al 1998; Vuillaumier-Barrot et al 2000). Among PMM2-CDG patients, the second most common variants after p.Arg141His are p.Phe119Leu, p.Leu32Arg, p.Glu139Lys and p.Asp65Tyr in Scandinavia, Italy, France and Iberian Peninsula respectively (Matthijs et al 1997; Bjursell et al 1998; Le Bizec et al 2005; Perez et al 2011; Barone et al 2015; Schiff et al 2017). p.Val231Met is the third most common variant in Europe. Protein shows residual activity of 38.5%, but is very unstable (Matthijs et al 1997; Pirard et al 1999).

2.2.3. Genotype-phenotype correlation of PMM2-CDG

Observations about the genotype-phenotype correlations might help to consult PMM2-CDG patients and their parents about the possible course and prognosis of the disease. In case of PMM2-CDG, a clear correlation does not exist, especially between measured PMM2 residual activity and the severity of the disease. Patients with very low PMM2 activity can show a relatively mild phenotype and vice versa. Also, between the patients with the same genotype, differences in the disease severity can be seen, although interfamilial clinical homogeneity is generally observed (Grunewald et al 2001; Barone et al 2008; Schiff et al 2017). There is a possibility that the severity is also determined by other, environmental or genetic factors. For instance, concurrent polymorphism p.Phe304Ser in *ALG6* causes more severe PMM2-CDG phenotype (Westphal et al 2002; Bortot et al 2013). In adult sisters with genotype p.Leu32Arg/ p.Thr237Met the phenotype difference was explained by *ALG6* polymorphism present in one sibling (Bortot et al 2013). In adult males with genotype p.Arg141His/p.Val129Met the difference of severity correlated with different degree of Tf glycosylation which in turn correlated with PMM2 activity (Barone et al 2008). However, it remains unknown why the degree of hypoglycosylation differs between the patients with the same genotype.

Milder phenotypes might be associated with variants that affect the folding and/or stability but not catalytic activity (Altassan et al 2018). Usually, in compound heterozygotes, severe variant is accompanied by milder one, which determines the phenotype. Genotypes p.Arg141His/p.Phe119Leu, p.Arg141His/ p.Val231Met and p.Val129Met/p.Arg141His usually cause a severe phenotype. The patients have severe DD, are bedridden or need supported walk, have visual defects, severe cerebellar atrophy, microcephaly and the presence of systemic involvement. A mild-moderate phenotype can be seen with p.Arg141His/ p.Cys241Ser, p. Leu32Arg/ p.Arg141His. The patients have mild-to moderate ID, cerebellar atrophy and they can walk independently. Also, it is not impossible that the patients are capable of living an independent life and acquire higher education. p.Arg141His/p.Thr237Met can cause a mild as well as severe phenotype (Grunewald et al 2001; Barone et al 2015).

In Iberian Peninsula, lower prevalence of severe variants and therefore milder phenotypes are noted, whereas in Scandinavia, p.Arg141His/ p.Phe119Leu is common and patients show severe end of PMM2-CDG phenotype (Erlandson et al 2001; Perez et al 2011).

2.3. SLC35A2-CDG

SLC35A2-CDG (CDG-IIm, OMIM 300896) is a relatively new type of CDG. It is inherited by X-linked dominant manner and caused usually by *de novo* variants in *SLC35A2* that locates on chromosome Xp11.23, and encodes the UDP-galactose/UDP-*N*-acetyl-galactosamine transporter (UGT) (Ng et al

2013). UGT is a transmembrane protein and delivers UDP-Gal and UDP-*N*-acetyl-galactosamine in the Golgi. The N-terminal region of UGT plays the most important role in galactosylation (Sosicka et al 2014). Defects of UGT affect the galactosylation of N-glycosylated proteins. In addition, its defects also influence O-glycosylation of proteins, and glycosylation of lipids.

In humans, UGT has two splice variants, UGT1 (transcript NM_ 001042498.2) and UGT2 (transcript NM_005660.1), with slightly different C-terminal amino acid sequence. Compared to UGT1, UGT2 has five different terminal amino acids and is three amino acids longer. UGT1 and UGT2 localize in the Golgi, and UGT2 also in ER. If UGT is defective, hypogalactosylation and subsequent hyposialylation occurs. Still, experimental studies in UGT-deficient cell lines have shown an overexpression of UDP-*N*-acetylglucosamine transporter as well as its complexes with UGT that restore partial galactosylation, and it is possible that UDP-*N*-acetylglucosamine transporter can partially replace UGT function (Olczak et al 2013).

SLC35A2-CDG patients are mostly females and their phenotype includes rather non-specific severe symptoms as DD/ID, hypotonia, epilepsy and even epileptic encephalopathy, cerebral and cerebellar atrophy, dysmorphic features, and shortened extremities. Visceral symptoms are rare (Kodera et al 2013; Ng et al 2013). *SLC35A2* has been proposed as one of the candidate genes for early-onset epileptic encephalopathy and cerebral visual impairment (Kodera et al 2013; Euro et al 2014; Bosch et al 2016). As there are still relatively few patients who usually present with different variants, the genotype-phenotype correlations are not possible.

The data from twelve European laboratories show that SLC35A2-CDG accounts 7% of the type II CDG (Peanne et al 2017). The number of patients keeps rising and many of them are discovered by NGS, which sometimes precedes the investigations of serum glycoproteins. The diagnosis of SLC35A2-CDG can be challenging. Tf IEF and other screening methods show type 2 pattern, but not in all patients. In addition, pattern normalization in the early childhood is possible. The improvement of glycosylation does not correlate with the improvement of clinical symptoms (Ng et al 2013). With Tf MS, characteristic glycoprofile with truncated glycans and lack of Gal and Sia is seen (van Scherpenzeel et al 2015). Diagnosis is confirmed either by gene sequencing, gene panels or WES. According to HGMD® Professional, missense and frameshift variants from small deletions are described so far (Stenson et al 2009). In addition, somatic mosaicism in both, males and females, and skewed X-inactivation of mutant alleles in females have been reported. The positive selection for the wild type allele in hepatocytes might be the reason why some patients with SLC35A2-CDG show normal Tf glycosylation (Kodera et al 2013; Ng et al 2013).

SLC35A2-CDG is one of the few CDG, where promising results are seen with oral Gal treatment as it improves the galactosylation, and therefore N-glycosylation of glycoproteins by increasing UDP-Gal in cytosol. In addition to glycosylation, an improvement of the clinical condition and reduced seizure activity was reported in one patient (Dorre et al 2015).

2.4. Syntaxin 5 gene

In Chapter 2.1.1.6., we shortly described the importance of COG complex in the retrograde trafficking of vesicles carrying resident Golgi proteins, and how the defects in COG subunits affect N-glycosylation. In addition to COG complex, other trafficking machineries such as Soluble *N*-ethylmaleimide-sensitive factor Attachment protein Receptors (SNAREs) are required for vesicular transport. They catalyze the vesicle docking and membrane fusion with the target membrane. SNAREs include many proteins that form different complexes with each other, and also with COG complex. Defects in COG complex destabilize SNAREs, cause impaired assembly of SNAREs complexes, lead to disturbed interactions between GOG and SNAREs complexes, and ultimately affect intra-Golgi retrograde transport. Additionally, defects in SNAREs also cause disintegration of the Golgi (Climer et al 2015).

Syntaxine 5 (STX5) is a member of SNAREs family. *STX5* gene locates on chromosome 11q12.3. STX5 regulates the vesicle-mediated protein transport, docking and fusion in every cell. In vitro, it has been shown to regulate ER-to-Golgi transport in yeast (Ravichandran and Roche 1997). This transmembrane protein localizes in the *cis*-Golgi region and forms together with other SNAREs complexes with subunits COG4, COG6 and COG8 (Laufman et al 2013). Presently, no pathogenic variants in *STX5* gene have been described in humans.

2.5. Summary of the literature review

CDG has become a rapidly expanding diverse group of metabolic diseases. Many patients all over the world are diagnosed with different types of CDG, and the number keeps rising. In spite of that CDG seemed to be underdiagnosed in Estonia. It has been partly due to its unfamiliar, variable, and nonspecific symptoms, and also because of the limited availability of diagnostic tests. Nevertheless, serum Tf IEF has remained the main method of choice of CDG screening since its introduction in the early eighties. To determine the occurrence of CDG in patients in Estonia, first, our aim was to implement serum Tf IEF in routine clinical practice. To our knowledge, similar CDG pilot screening has not been previously reported in other populations. In addition, the results of CDG screening have led to many discoveries, which have helped to add new clinical and epidemiological data about different known types of CDG, but also to expand the group of CDG by the discovery of the new type of CDG.

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To establish and evaluate the effectiveness of Tf IEF in screening of CDG in Estonia;
2. To evaluate the genotype and phenotype of Estonian PMM2-CDG patients;
3. To study the expected frequency of PMM2-CDG based on Estonian population data, and the prevalence of PMM2-CDG based on diagnosed patients in Estonia;
4. To characterize and compare the clinical phenotype and the genotype of Estonian SLC35A2-CDG patient with a cohort of international patients with SLC35A2-CDG;
5. To present a patient with a novel type II CDG likely caused by homozygous variant in *STX5* gene.

4. MATERIAL AND METHODS

4.1. Study subjects

4.1.1. Cohort screened with transferrin isoelectric focusing

The pilot screening period lasted from June 1, 2012 to June 30, 2015. To improve the coverage of the patients with possible CDG, Tf IEF was performed to all patients whose serum samples were sent to Department of Clinical Genetics in Tartu University Hospital for any metabolic analyses. Therefore, the whole cohort was formed from two groups of patients – the ones whom the clinicians requested intentionally Tf IEF alone or among other metabolic analyses, and the patients whose samples were analyzed primary for other metabolic diseases. The samples were sent from Tartu University Hospital (Department of Clinical Genetics and its subunits from Tartu and Tallinn, Children's Clinic, Psychiatry Clinic, Internal Medicine Clinic, Surgery Clinic, and Neurology Clinic), Tallinn Children's Hospital, West Tallinn Central Hospital (Multiple Sclerosis Centre), East Tallinn Central Hospital, and the North Estonia Medical Centre.

In a 37-month screening period, Tf IEF was performed to 1230 patients. In addition, in 52 patients, the screening was repeated once or twice in different time periods based on the results of the first screening or by the request of the treating clinician. The age distribution of screened patients (N=1229, the age of one patient remained unknown) is shown in Figure 6.

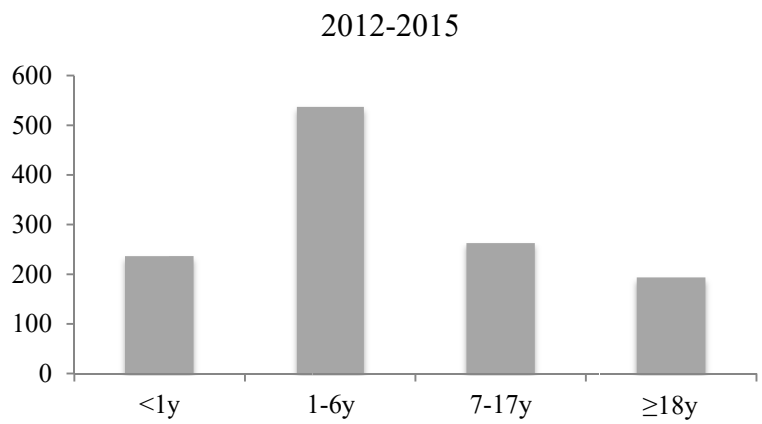

Figure 6. The age distribution of screened patients from June 1, 2012 – June 30, 2015.

4.1.2. Subjects in PMM2-CDG prevalence study

4.1.2.1. Population cohort

At the time of the study period, the population-based biobank of the Estonian Genome Center at the University of Tartu (EGCUT) represented 5% of Estonian adults, and it has been a unique database for research (Leitsalu et al 2015). In 2016, the query for the whole genome sequencing (WGS) data of 2,244 individuals from EGCUT was submitted to assess the presence and frequency of disease-causing variants in *PMM2* among biobank participants. WGS samples were sequenced at the Broad Institute (Cambridge, Massachusetts, USA). Libraries were sequenced on the Illumina HiSeq X Ten (Illumina, San Diego, California, USA).

4.1.2.2. Patients cohort

Altogether, four Estonian patients with PMM2-CDG from two families were alive and were included in prevalence calculations. All patients were diagnosed during the pilot screening period and were also included in the screening cohort (Chapter 4.1.1.). They all had type 1 pattern in Tf IEF and PMM2-CDG was molecularly confirmed.

4.1.3. Subjects in SLC35A2-CDG study

4.1.3.1. Estonian patient with SLC35A2-CDG

A male patient with type 2 Tf IEF was molecularly confirmed with SLC35A2-CDG. His clinical, biochemical, neuroradiological (including the data of the brain magnetic resonance imaging, MRI) and molecular data were collected and compared with international patients' cohort data.

4.1.3.2. International patients with SLC35A2-CDG

The clinical, biochemical, neuroradiological and molecular data of 14 patients (11 females and 3 males) from nine different countries and thirteen clinical centers were collected. Three patients in the cohort were previously shortly described in other papers (Bosch et al 2016; Lopes et al 2016; Demos et al 2017). In eight patients, the brain MRI data were available for re-evaluation of neuroradiological features.

4.1.4. Subjects with new type of CDG

A male patient (proband 1) with multisystem presentation was screened for suspected CDG in the neonatal period, and showed type 2 Tf IEF. His clinical, biochemical, radiological and molecular data were collected and compared with

the clinical, radiological and molecular data of the fetus from his mother's previous aborted pregnancy (proband 2).

4.2. Methods

4.2.1. Screening of N-glycosylation disorders

4.2.1.1. Serum transferrin isoelectric focusing

The protocol of Tf IEF from Radboud University Medical Center, Nijmegen was used as the primary source for this method (unpublished).

Patient's serum was centrifuged at 11 000 RPM for 10 minutes. Ten µl of serum was mixed with 10 µl solution of Fe(III)citrate/0.5M sodium bicarbonate. The sample was incubated for 30 minutes at room temperature and then diluted with 100 µl ultrapure water (Milli-Q).

To exclude Tf polymorphism and remove Sia, 3 µl of serum was mixed with 3 µl solution of Fe(III)citrate/0.5M sodium bicarbonate. The sample was incubated for 30 minutes at room temperature. Then, 1.5 µl of neuraminidase 10U/ml from *Clostridium perfringens* (Roche) was added and the sample was incubated overnight at room temperature. The next day, the sample was diluted with 90 µl ultrapure water.

Gel (PhastGel Dry IEF, GE Healthcare) was hydrated in 125 µl Servalyt^{TM} 5–7 carrier ampholyte (SERVA) solution with 4875 µl ultrapure water, and incubated for at least 90 minutes at room temperature.

For IEF, GE Healthcare Phast System was used, which allows to run IEF on two gels at a time. Hydrated gel was layed on a cooling plate (15 C°). The sample amount of 1.3 µl was pippeted on the paraffin film, and then applied on the gel using a special sample comb. On both gels, up to eight samples could be applied, with one used as positive control sample. Gel was run with the following program:

Step 1	2000 V	2.0 mA	3.5 W	15 C°	485 Vh	Prefocusing
Step 2	200 V	2.0 mA	3.5 W	15 C°	15 Vh	The application of samples
Step 3	2000 V	5.0 mA	3.5 W	15 C°	130 Vh	Separation
Step 4	50 V	0.5 mA	0.5 W	15 C°	50 Vh	

After IEF, gel was immunoprecipitated for 30 minutes with 60 µl of polyclonal rabbit anti-human transferrin (DAKO), and subsequently repeatedly washed in NaCl 0.9% solution overnight.

The next day gel was fixated in 20% trichloroacetic acid solution for 10 minutes. Then, gel was coloured with Coomassie blue solution (GE Healthcare PhastGel Blue R-350) for 30 minutes and subsequently washed repeatedly with

the solution of 30% ethanol/10% trichloroacetic acid. Finally, the gel was dried in the room air.

Tf IEF pattern and the intensity of sialotransferrin bands were evaluated only qualitatively.

4.2.1.2. Subjects with positive screening

In patients with type 1 Tf IEF pattern NGS of a large gene panel (TruSight One, Illumina Inc., San Diego, CA) with confirmation of *PMM2* variants with Sanger sequencing were performed (Tartu University Hospital). The PMM2 activity in skin fibroblasts was studied only in one patient (Radboud University Medical Center, Nijmegen).

To the patients with type 2 Tf IEF pattern, quantitative Tf IEF, ApoC-III IEF, serum Tf quadrupole time-of-flight MS, Illumina chromosomal microarray analysis, NGS and/or WES were done (Radboud University Medical Center and Tartu University Hospital).

In addition, the samples of two subjects from the screening cohort with indicative CDG pattern in Tf IEF were sent to Radboud University Medical Center either for quantitative Tf IEF and/or ApoC-III IEF.

The clinical data were collected from the electronic health record databases.

4.2.2. Statistical analysis of PMM2-CDG prevalence study

A paper by Schollen *et al.* (Schollen et al 2000) was taken as a basis in calculating the expected PMM2-CDG frequency in Estonia. We used the assumption that only p.R141H homozygosity is embryonically lethal (Matthijs et al 1998). We modified Hardy-Weinberg equilibrium as follows: the expected disease frequency in the Estonian population equals r^2+2qr where q is the allele frequency of p.R141H and r the combined allele frequency for other identified variants in the cohort. All statistical analyses were conducted in R version 3.2.3 (R Core Team 2016).

4.2.3. Investigations in the SLC35A2-CDG study

4.2.3.1. Estonian patient with SLC35A2-CDG

Tf IEF was performed according to the protocol. The quantitative Tf IEF, ApoC-III IEF, serum Tf quadrupole time-of-flight MS were done in Radboud University Medical Center. For molecular diagnostics HumanCytoSNP-12 BeadChips (Illumina Inc., San Diego, California, USA), NGS of a large gene panel (TruSight One, Illumina Inc., San Diego, California, USA) and subsequent WES were performed.

A questionnaire was created for the clinical and biochemical data to standardize information collection from the international collaborators. The clinical and the brain MRI data were collected from the electronic health record

database. The 1.5 or 3T brain MRI data were re-evaluated by two neuroradiologists. Age specific MRI reference data for the biometry of the corpus callosum in children was used for the assessment of corpus callosum length (Garel et al 2011). Short stature was defined as height or length less than 5 percentiles. Z-scores were calculated by using the CDC Height for Age Percentiles for Girls or Boys (2– 20 years) and CDC/NCHS Infant Length for Age Percentiles (< 36 months) (https://reference.medscape.com/guide/medical-calculators).

4.2.3.2. International patients with SLC35A2-CDG

Molecular genetic studies and Tf isoform analyses were performed in different institutions. All variants in *SLC35A2* were discovered using WES. Conformational studies and familial segregation analysis were commonly performed using Sanger sequencing.

A questionnaire about the clinical and biochemical data was sent to corresponding clinicians. In eight patients, the brain MRI was available for re-evaluation by two neuroradiologists. Age specific MRI reference data for the biometry of the corpus callosum in children was used for the assessment of corpus callosum length (Garel et al 2011). Short stature was defined as height or length less than 5 percentiles. Z-scores were calculated by using the CDC Height for Age Percentiles for Girls or Boys (2–20 years) and CDC/NCHS Infant Length for Age Percentiles (< 36 months) (https://reference.medscape.com/guide/medical-calculators).

4.2.4. Investigations of the subjects with new type of CDG

Tf IEF was done to proband 1 according to the protocol. In addition, quantitative Tf IEF, ApoC-III IEF, serum Tf quadrupole time-of-flight MS were done in Radboud University Medical Center.

DNA of proband 1 was extracted either from peripheral blood according to the standard salting out protocol, and in proband 2, from amnionic fluid cell culture. Screening for chromosomal abnormalities was performed using HumanCytoSNP-12 BeadChips (Illumina Inc., San Diego, California, USA). Genotypes were called by GenomeStudio v2011.1 software and the data were analyzed by using GenomeStudio Genome Viewer tool (Illumina Inc.). The minimum threshold for the regions of long contiguous stretches of homozygosity (LCSH) was set at 5 Mb.

WES was done in Radboud University Medical Center. The probable candidate gene for the phenotype of probands is *STX5*, which is located in one LCSH and is not previously associated to CDG. The functional studies are in work in order to find out how this gene affects glycosylation pathways (the data are inconclusive).

The clinical data of the probands was collected from the electronic health record databases.

4.2.5. Ethical approval

This study was approved by the Research Ethics Committee of the University of Tartu (181/T-16, 20.04.2009 and 235/M-13, 17.03.2014). Informed consent was obtained from the parents or legal guardians of the patients to whom additional studies were indicated due to positive screening of N-glycosylation.

5. RESULTS AND DISCUSSION

5.1. Screening of N-glycosylation disorders in Estonia

Altogether, 1230 patients were screened for N-gycosylation disorders in the study period. In addition, we repeated Tf IEF in 52 new samples taken from the patients whose first Tf IEF pattern was possibly indicative to N-glycosylation disorders, or when clinicians decided to repeat the analysis.

The analyses were mainly performed to children aged 1–6 years (44%). Infants constituted 19%, children aged 7–17 years 21%, and adults 16% of all the screened patients. As expected, Tf IEF was mainly investigated in children, and most of the time, the screening was ordered among many other metabolic analyses. Out of six patients with a positive screening result and confirmed CDG diagnosis, CDG was suspected and studied in five, and only one patient reached the diagnosis due to the expanded screening strategy, as Tf IEF was not ordered in the first choice. Department of Clinical Genetics in Tartu University Hospital is the only center in Estonia that consults the patients suffering from inborn errors of metabolism, and offers screening of N-glycosylation disorders. Therefore, it is highly likely that we are aware of all molecularly confirmed CDG patients in Estonia. As CDG is relatively new but also a rare group of metabolic diseases, the overall awareness of the disease among clinicians has been consequently low. All our positive screening results were seen among children in different ages. Adult age group was modestly represented and often the samples were sent for other metabolic analyses. One cause for more extensive use of different metabolic studies among children is the involvement of clinical geneticists in the consulting process. Nevertheless, the presence of undiagnosed patients, especially among adults, is possible. Some of the patients might be deceased, have milder disease course or have undergone diagnostic work-up many years ago when the awareness of CDG and the availability of different diagnostic procedures (Tf IEF, NGS panels, WES) were insufficient.

During the study period, six patients with three different N-glycosylation disorders were detected. There seems to be no reports of such expanded CDG pilot screenings in other populations, but reports about the experiences with N-glycosylation disorders in single or multiple centers have been published in different countries. In a single institution in Dubai, ten patients with seven different type of CDG were reported, and similarly in Canada, 15 patients with seven different CDG were diagnosed (Al Teneiji et al 2017; Bastaki et al 2018). Recently, the results of a 10-year period of CDG screening from Argentina were published, where seven patients out of 554 studied patients showed abnormal Tf IEF profile and had molecularly confirmed CDG diagnosis (Asteggiano et al 2018). Multi-center experiences reports come from Saudi Arabia and Spain. In Saudi Arabia, 27 patients with six types of CDG were reported (Alsubhi et al 2017). During a 20-year period, 97 patients with 18 different CDG caused by N- or combined N- and O-glycosylation disorders were diagnosed in different

hospitals in Spain, where all the patients with multisystem disease of unknown etiology were studied. Interestingly, different screening methods (including Tf IEF) showed positive results in all their molecularly confirmed CDG patients (Perez-Cerda et al 2017).

Tf IEF can be positive in up to 48 different CDG (Table 1) and it covers most of the N-glycosylation disorders and disorders of combined or other glycosylation pathways affecting N-glycosylation pathway (Jaeken and Peanne 2017). Nevertheless, there are some limitations in this study. First, the evaluation of screening was subjective and we could not quantify the intensity of different sialotransferrin bands. This could lead to false positive or false negative interpretations. If the pattern seemed to refer to CDG, the clinicians were asked to repeat the analysis after one or two months. The analyses of the newborns were always asked to be repeated as in newborns, the results can be either false negative or refer to mild type 2 profile. It is possible that in some cases, analyses were not repeated. There is also a possibility that the same patient was diagnosed with another disease, which eliminated the need for repeated CDG screening. Altogether, the serum samples of two patients from the whole cohort were sent to Nijmegen University Hospital for quantification and additional glycosylation studies if indicated (Chapter 5.1.2.).

Opposite, some patients might show very mild positive pattern that is qualitatively very difficult to evaluate as disease referring and leads to false negative interpretation. Age-dependent false negative results are also possible and cannot be excluded. At the same time, two CDG-patients with mild phenotype showed strong type 1 pattern despite their older age.

Second, as seen in some types of CDG (e.g. ALG13-CDG, SLC35A2-CDG) both normal and abnormal Tf IEF pattern is described, which could lead to premature exclusion of CDG. In addition, some N-glycosylation defects like MOGS-CDG or SLC35A3-CDG always show normal screening results. It is important for clinicians to think about these aspects and even if, regardless the negative screening, the suspicion of CDG remains, another diagnostic analyses like whole plasma glycoprotein or intact Tf MS, gene sequencing, gene panels or WES should follow Tf IEF.

Third, with extended use of NGS, it is important to carefully interpretate its results. Not all identified variants are deleterious and cause CDG (Chapter 5.1.4.).

Next, more detailed information of positive screening results and false positive screening results are given. In addition, two patients with variants in glycosylation-related genes but negative screening are presented.

5.1.1. Positive screening results

Clearly abnormal Tf profile was found in six patients. Positive type 1 Tf IEF pattern was present in four patients from two families. All the patients had molecular confirmation for the most common PMM2-CDG. In family 1, Tf IEF

was first ordered to a 10-month old girl, and showed classical increase of di- and asialotransferrin. As her older siblings had also shown DD, Tf IEF was performed with their serum samples and the results showed similar pattern to their sister.

In family 2, Tf IEF was ordered to a 3-month old girl with neurovisceral symptoms. The clinical phenotype of all PMM2-CDG patients is thoroughly described in Chapter 5.2.

Positive type 2 Tf IEF pattern indicating either N-glycosylation or multiple pathway disorder was seen in two patients. The patient 1 was almost 2 years old male, when in search of a possible cause of mitochondrial Leigh syndrome, other metabolic analyses were ordered and Tf IEF was first done as expanded screening. Positive screening led to additional glycosylation studies and finally, SLC35A2-CDG was molecularly confirmed by WES. At the age of 4-years the patient still showed type 2 Tf IEF pattern. In SLC35A2-CDG, the isoforms of Tf have been reported to normalize already in the early childhood, and this might cause a delay in diagnosis (Ng et al 2013).

The second male patient with type 2 Tf IEF pattern died at the age of 25 days. CDG screening was ordered from the serum taken on the 5th day of life. The patient had a new, type 2 CDG, which affects multiple pathways of glycosylation.

The clinical phenotype of these two patients is thoroughly described in Chapters 5.4. and 5.5.

5.1.2. False positive screening results

Two samples were sent to Radboud University Medical Center for quantitative measurements of sialotransferrin and other glycosylation studies when indicated. In a 16-year old female patient with severe DD/ID, microcephaly, brain atrophy and epilepsy, an increased trisialotransferrin was present, which indicated possible type 2 CDG. For instance, the patients with MAN1B1-CDG show an increase of trisialotransferrin (Rymen et al 2013). Quantification of Tf isoforms showed isolated increase of trisialotransferrin (16.9%, normal limits 4.9–10.6%). ApoC-III IEF was also abnormal (increased ApoC-III$_2$ and decreased ApoC-III$_1$) indicating an increased level of Sia on mucin type O-glycans. The MS was not performed due to the limited amount of sample. As the WES did not reveal CDG-associated changes, we can probably exclude CDG. The patient is deceased, and further analyses are not possible.

Another patient with false-positive screening result was a 5-month old boy with DD, hypotonia and normal brain MRI. Repeated Tf IEF profile was suggestive to mild type 2 pattern. Quantification revealed that the sialotransferrins were within normal limits.

5.1.3. Patient with a variant in *ALG13* and negative screening result

A 4-year old patient was diagnosed with Lennox-Gastaut syndrome at the age of 3 years. In addition, he had slightly delayed speech development, but no visceral symptoms. Brain MRI showed focal cortical dysplasia in mesial temporo-occipital area. A targeted NGS panel of epilepsy-associated genes revealed missense variant c.1641A>T, p.Gln547His in *ALG13* (Moller et al 2016). Tf IEF was done after molecular analysis and showed normal result.

ALG13 encodes ALG13 protein, which is a subunit of GlcNAc-transferase. ALG13 forms complex with ALG14 and adds the second GlcNAc to GlcNAc-PP-Dol in the early steps of N-glycan synthesis. Variants in *ALG13* are considered to be associated with epileptic encephalopathy, seen both in males and females (Epi et al 2013). Variants are inherited in a X-linked manner. In female patients, random X-inactivation pattern is described (Hamici et al 2017). The most frequently reported *de novo* variant p.Asn107Ser is confirmed mainly in female patients with severe phenotype including epileptic encephalopathy. A hypothesis was, that this variant is lethal in males. In 2017, first male patient with p.Asn107Ser was reported (Galama et al 2018).

As ALG13 is important enzyme in N-glycosylation, variants in *ALG13* could presumably cause ALG13-CDG. The first patient with ALG13-CDG was described by Timal and colleagues in 2012 (Timal et al 2012). The deceased 1-year old male patient had visceral symptoms and refractory epilepsy. Abnormal type 1 Tf IEF was seen, and compared to the control sample, reduced GlcNAc-transferase activity was measured in fibroblasts. Thereafter, patients with *ALG13* variants but normal screening have been described. In few reported females, glycosylation studies were limited to CDG screening with negative results, whereas in some reported males other glycosylation studies were performed in addition.

Recently, a boy with infantile spasms and the commonest *ALG13* variant had normal Tf IEF but MS of Tf revealed minor glycosylation abnormalities (Galama et al 2018). Another male patient with slightly delayed motor but normal cognitive development and seizures was reported. He showed normal Tf IEF but cellular glycosylation assay confirmed abnormal glycosylation with reduced expression of ICAM-1. The mother of the patient carried the variant but was asymptomatic (Gadomski et al 2017). The mother and grandmother of Estonian patient are also unaffected carriers of p.Gln547His.

Unfortunately, due to limited accessibility, similar glycosylation assays have not been done to Estonian patient (e.g. enzyme assay is not available at the moment) and it remains unknown whether despite the negative screening the glycosylation is affected. The c.1641A>T variant detected in the patient is absent from the Genome Aggregation Database (gnomAD) and in-silico pathogenicity prediction algorithms show inconsistent results, thus according to American College of Medical Genetics and Genomics (ACMG) variant classification criteria (Richards et al 2015) this variant is of uncertain significance.

However, pathogenicity could not be ruled out at this moment. Therefore, to further investigate the pathogenicity of this variant, functional studies are needed in order to diagnose ALG13-CDG. In addition, as only the epilepsy-associated genes have been studied, one cannot exclude that there is another, unstudied cause for his symptoms.

5.1.4. Patient with homozygous variant in *ALG6*

A 13-year old male patient was consulted due to asymmetric progressive spasticity in legs. Perinatal anamnesis was uneventful. In late infancy, delayed gross motor development was noticed. The patient had a speech and language delay. In spite of that he has been able to attend in regular school program. His growth was normal. He showed brisk reflexes, spasticity and ankle clonus. In brain MRI a large arachnoidal cyst behind the medulla and narrow isthmus of corpus callosum was seen. Tf IEF was normal. WES revealed homozygous missense variant c.391T>C, p.Tyr131His in exon 6 of *ALG6*, which was confirmed with Sanger sequencing. His parents were heterozygous for p.Tyr131His. In addition, reanalysis of WES data detected *de novo* missense variant c.20G>A, p.Gly7Glu in *CBX8*. Encoded protein CBX8 plays role in the repair of DNA lesions (Oza et al 2016). The functional studies are presently at work for proving the pathogenicity of *CBX8* variant.

An enzyme ALG6 adds the first Glu to LLO. The prevalence of ALG6-CDG is 8% of all CDG in the Europe cohort (Peanne et al 2017). It causes DD/ID, hypotonia, ataxia, proximal muscle weakness, epilepsy and failure to thrive. Cerebellar hypoplasia has not been observed. Tf IEF shows type 1 pattern and LLO analysis accumulation of $Man_9GlcNAc_2$ intermediate. The most common variant is p.Ala333Val (Grunewald et al 2000; Morava et al 2016).

In North American population, variant p.Tyr131His is also frequent and estimated frequency of its homozygotes is 1/2200, whereas in Croatia, 1/1000 (Westphal et al 2003; Goreta et al 2012). In EGCUT cohort, the homozygosity of p.Tyr131His is present in two subjects (2/2244). At the same time, p.Tyr131His homozygosity was reported in an adult female with diagnosis of ALG6-CDG. She showed early hypotonia, enteropathy, strabismus, cortical blindness, partial agenesis of corpus callosum, myoclonus and cerebellar syndrome. Tf IEF was normal. Enzymatic analysis in skin fibroblasts showed normal LLO size, but the complementation analysis of ALG6-deficient yeast indicated that variant p.Tyr131His was severe and did not rescue defective glycosylation (Westphal et al 2003; Miller et al 2011). Although the variant is located in conserved sequence, it remains controversial whether p.Tyr131His is just a polymorphism or its homozygosity can cause ALG6-CDG. Contrary to the patient described here, a reported female patient had symptoms consistent with ALG6-CDG. In a recent review of 41 patients with ALG6-CDG, p.Tyr131His was considered as a polymorphism that was present in three

patients in addition to two other *ALG6* variants, and it did not seem to affect the severity of their phenotype (Morava et al 2016).

We conclude that the patient does not have ALG6-CDG, and it is more likely that the disease is caused by other factors, for example, the studies clarifying the role of *CBX8* gene variant are ongoing.

5.2. Patients with PMM2-CDG (Paper I)

Type 1 pattern in Tf IEF led to PMM2-CDG diagnosis in two infants and two school-aged children from two families. In family 1, two older patients with mild clinical presentation got their diagnosis in adolescence after the diagnosis of their youngest sibling (Vals et al 2017). The older sibs had been consulted and investigated by the clinical geneticist in their early childhood. At that time, however, Tf IEF was not widely accessible in Estonia and CDG was not studied. In family 2, the child presented with classical phenotype of PMM2-CDG.

5.2.1. Phenotype

In family 1 and 2, the parents of the sibling(s) are non-consanguineous and all children were born at term after an uncomplicated pregnancy and delivery. In family 1, both parents are Estonians and in family 2, one parent is French and the other is Estonian.

In family 1, PMM2-CDG was first diagnosed in the youngest sibling at the age of 10 months. She presented with delayed motor skills, muscular hypotonia, truncal ataxia, and strabismus. At the time of diagnosis she was not crawling and sat insecurely. Her cognitive development and growth parameters were appropriate for her age. She started to walk independently at the age of 21 months and she has had a delayed speech development. The brain MRI has not been done.

Her siblings also had problems with gross motor development and ataxia in infancy and early childhood. Older brother (16 years old) started to walk at the age of 19 months and his speech development was delayed. Older sister (8 years old) started to walk at 21 months of age but had normal speech development. The brain MRI of both probands showed cerebellar atrophy (Figure 7). Interestingly, progression of atrophy was not evident in the brother although the MRIs were done at the ages of nine and seventeen. According to the Wechsler Intelligence Scale for Children (WISC) done at the age of diagnosis, the full scale IQ scores were 72 (borderline) and 81 (low average) for brother and older sister respectively, which are normal. Older sister studies in basic school and brother has graduated the high school and continues his education at the vocational education center.

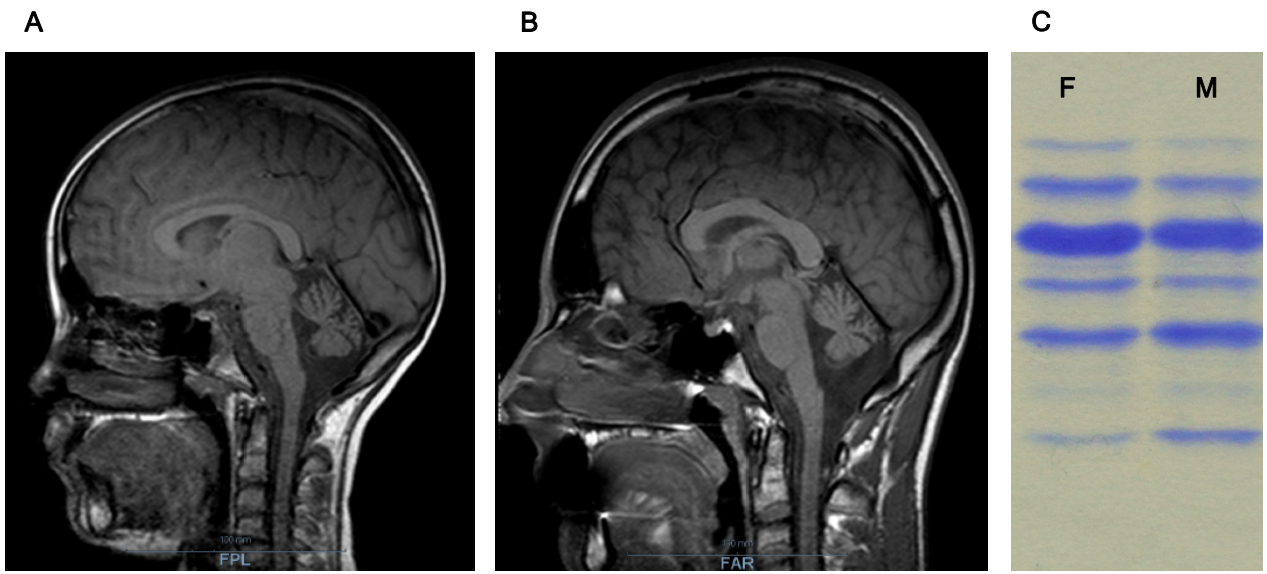

Figure 7. The brain MRI of a female patient (A) and a male patient (B) showing cerebellar atrophy, and Tf IEF of the same patients (C) showing type 1 profile with increased di- and asialotransferrin (F-female, M-male) in family 1.

All sibs have needed orthodontic treatment because of occlusion abnormalities. All have shown some coagulation abnormalities (including low ATIII), and females increased FSH and LH.

All children showed a clear type 1 pattern on serum Tf IEF. In the male patient, enzymatic analysis showed a residual activity of PMM2 in skin fibroblasts 1.6 mU/mg (normal limits 4.7–9.2, i.e. 34% of the normal lower limit).

In family 2, the female patient was hospitalized at the age of 3 months due to failure to thrive, and vomiting. She showed abnormal fat pads and inverted nipples, strabismus, DD, hypotonia, pericardial effusion and myocardial hypertrophy, relatively high transaminases, hypoalbuminemia, and low levels of ATIII. In addition, she is diagnosed with bilateral sensorineural deafness and hypothyreosis. Central blindness is suspected. The brain MRI showed hypoplasia of the vermis, cerebellar atrophy and hypomyelination. Tf IEF showed type 1 profile. Enzymatic analyses were not done.

The development has been severely delayed and she is fed through gastrostomy. At the age of one year, the symptoms worsened due to adenoviral infection, dehydration and possible sepsis. During decompensation, pericardial effusion worsened and was accompanied by ascites. Coagulopathy was evident. Biochemical analyses showed even more decreased albumin and ATIII, thrombocytopenia and increased transaminases. This illustrates that in patients with PMM2-CDG, stressful conditions, such as infections, quickly destabilize the multisystem symptoms of the disease, which can be possibly life threatening.

5.2.2. Genotype

In family 1, NGS of a large gene panel (TruSight One, Illumina Inc., San Diego, California, USA) in the youngest sibling revealed compound heterozygosity for two previously reported variants in exon 8: c.691G>A (p.Val231Met) and c.715A>T (p.Arg239Trp). Sanger sequencing confirmed *PMM2* variants in all sibs and their parents.

In family 2, *PMM2* sequencing confirmed compound heterozygosity for c.368G>A (p.Arg123Gln) in exon 5 and c.619G>A (p.Val231Met) in exon 8.

5.2.3. Genotype-phenotype correlation

As seen from these two families, the severity of PMM2-CDG can be variable. If the patient shows characteristic phenotype, the diagnosis is often made in infancy. In addition to severe phenotype, more patients with mild or moderate phenotypes are reported, which makes the diagnosis challenging. These patients have presented either with failure to thrive and increased level of transaminases, or with the symptoms of the nervous system like gross motor DD, ataxia, hypotonia and/or cerebellar hypoplasia with preserved ability to ambulate independently and participate in regular school programs. Hence, it is important to screen CDG in patients even with subtle neurological symptoms and normal

cognitive development. According to recent PMM2-CDG guidelines, only 12% of 179 patients with PMM2-CDG showed normal or borderline IQ (Altassan et al 2018). Unfortunately, in milder cases, Tf IEF has shown to be mildly abnormal or normal also in older infants, and therefore diagnosis might be missed with screening (Vermeer et al 2007; Casado et al 2012). Still, despite the age and the clinical severity of four Estonian patients, in all, Tf IEF was strongly suggestive to CDG type I.

In family 1, the genotype p.Val231Met/p.Arg239Trp seems to relate to mild/moderate phenotype without relevant multisystem involvement in all siblings. In two older siblings, WISC showed normal cognitive development ranging from borderline (score 72) to low average (score 81). Despite that, motor development has been delayed in all three, and cerebellar hypoplasia in two older siblings. Central nervous system involvement and cerebellar hypoplasia are characteristic even in mild PMM2-CDG (Grunewald et al., 2001; Scott et al., 2014; Barone et al., 2015).

Unfortunately, this genotype has not been reported in other patients to compare the phenotypes. p.Val231Met is frequently found among patients with PMM2-CDG, and it is most frequently detected in combination with p.Arg141His. As mentioned earlier, this genotype causes a severe clinical phenotype (Matthijs et al., 1997; Matthijs et al., 2000; Grunewald et al., 2001; Barone et al., 2015). p.Arg239Trp has been described only once in combination with p.Phe157Ser, in which case the patients had either a mild-moderate or a moderate phenotype (Grunewald et al 2001). As p.Phe157Ser is considered to be severe variant with no residual activity (Vega et al 2011), p.Arg239Trp must be the milder variant with higher residual activity, which explains mild-moderate phenotype of patients in family 1. Also, it might compensate the variant p.Val231Met, where encoded protein shows the 38% activity of wild-type but is unstable (Pirard et al 1999).

In family 2, the genotype p.Arg123Gln/p.Val231Met relates to severe neurovisceral phenotype. p.Arg123Gln is severe variant with null residual activity (Vega et al 2011). The patients with this variant have often severe clinical picture, but mild phenotypes in combination with more functional *PMM2* variant (e.g. p.Cys241Ser) have been reported (Westphal et al 2001; Casado et al 2012). It is possible that p.Arg123Gln/p.Val231Met cause similar phenotype as p.Arg123Gln/p.Arg141His as p.Val231Met is not mild enough to increase the activity of PMM2.

The summary of all four patients (patients 2, 3, 4 from family 1, and patient 5 from family 2) detected in screening, and in comparison, also first PMM2-CDG patient (patient 1) in Estonia are presented in Table 3. In conclusion, although the correlation between the genotype and the clinical phenotype may be controversial, based on the current data of Estonian patients it seems possible to predict the phenotype according to the variants present. Although not reported before, it seems likely that the genotype p.Val231Met/p.Arg239Trp locates in the milder, and p.Arg123Gln/p.Val231Met in the severe end of PMM2-CDG phenotype.

Table 3. Genotype and phenotype of five Estonian PMM2-CDG patients.

	Patient 1 (M)*	**Patient 2 (F)** Family 1	**Patient 3 (M)** Family 1	**Patient 4 (F)** Family 1	**Patient 5 (F)** Family 2
Age at diagnosis	3d	10m	16y	8y	3m
Year of birth	2007	2014	1998	2006	2015
Genotype	p.Arg141His/ p.Val231Met	p.Val231Met/ p.Arg239Trp	p.Val231Met/ p.Arg239Trp	p.Val231Met/ p.Arg239Trp	p.Arg123Gln/ p.Val231Met
PMM activity in fibroblasts (mU/mg)	0.43**		1.6 (4.7–9.2)		
Inverted nipples, fat pads	+				+
Delayed motor development		+	+	+	+ (plus ID)
Ambulatory		+	+	+	-
Hypotonia		+	+	+	+
Ataxia		+	+	+	
Cerebellar anomaly	+		+	+	+
Pericardial effusion	+				+
Strabismus		+		+	+
Failure to thrive					+
Hypothyreosis					+
FSH/LH (U/L)		34.7 (0.5–4.5)/ 2.75 (<0.45)		18.1 (0.5–4.5)/ 0.54 (<3.36)	
ATIII (%)	25	63 (101–131)	46 (96–126)	80 (95–134)	27
Increased transaminases	+				+

*Died on the sixth day of life
**Normal limits absent

5.3. Prevalence of PMM2-CDG in Estonia (Paper II)

5.3.1. Prevalence of PMM2-CDG according to population cohort data

Similarly to other European countries, PMM2-CDG is the most common type of CDG diagnosed in Estonia. At the end of the screening period, PMM2-CDG was altogether diagnosed in five patients from three families. Interestingly, they all had variant p.Val231Met in one allele, and only one patient carried the most commonly reported p.Arg141His in *PMM2*. This urged us to find out whether the most common variant among Estonians was p.Val231Met. Also, we wanted to evaluate the presence of other *PMM2* variants in our population and to calculate the expected frequency of PMM2-CDG in Estonia based on the data from population-based biopank (EGCUT). ECCUT includes the data and samples of almost 52,000 individuals. It represents 5% of the Estonian adult population reflecting closely the age, sex and geographical distribution of the Estonian population (Leitsalu et al 2015).

From WGS data of 2,244 individuals from EGCUT, all variants identified in the *PMM2* were extracted. Possible novel disease-causing variants were not found. Altogether, 19 alleles listed as pathogenic according to HGMD were detected. Five different variants were identified: p.Arg141His (10 alleles), p.Val231Met (5 alleles), p.Arg239Trp (2 alleles), p.Val67Met (1 allele) and p.Thr237Arg (1 allele). Although the data from EGCUT did not allow us to fully exclude the possibility of the presence of biobank participants with variants in both alleles, it is highly unlikely. Observed carrier and allele frequencies of *PMM2* variants are shown in Table 4.

Table 4. Observed carrier and allele frequencies of *PMM2* variants of 2,244 individuals from EGCUT.

***PMM2* variant**	**N of alleles**	**Carrier frequency**	**Allele frequency**
p.Arg141His	10	1/224	1/449
p.Val231Met	5	1/449	1/898
p.Arg239Trp	2	1/1122	1/2244
p.Val67Met	1	1/2244	1/4488
p.Thr237Arg	1	1/2244	1/4488
Overall	**19**	**1/118**	**1/236**

Homozygosity of p.Arg141His is considered lethal and has never been described in any patient (Matthijs et al 1998). In most cases, PMM2-CDG

patients are compound heterozygotes. Still, homozygosity of p.Leu32Arg, p.Lys51Arg, p.Tyr64Cys, p.Asp65Tyr, p.Pro113Leu, p.Phe119Leu, p.Val129Met, p.Asn216Ile, p.Tyr106Phe, p.Phe183Ser and p.Thr237Met has been reported (Matthijs et al 2000; Neumann et al 2003; Vermeer et al 2007; Najmabadi et al 2011; Perez et al 2011; Kasapkara et al 2017; Schiff et al 2017). As some of the possible genotype recombinants have not been reported in any patient, we cannot exclude that the homozygosity of variants other than p.Arg141His, or recombinants between the variants found in our population cohort are incompatible with life. Still, to calculate the expected disease frequency in Estonian population, we assumed that only homozygosity of p.Arg141His was impossible. Based on modified Hardy-Weinberg equilibrium (r^2+2qr), where q is the allele frequency of p.R141H (1/449) and r the combined allele frequency for other four identified variants (1/499), the expected frequency of the disease in Estonian population is 1/77,000.

Only one report has previously estimated the PMM2-CDG frequency based on allele frequencies among healthy individuals (Schollen et al 2000). The frequencies of the p.Arg141His and p.Phe119Leu were studied among two normal populations consisting of 950 Dutch neonates and 420 Danish blood donors. Altogether, in Dutch, Flemish and Danish populations, the reported carrier frequency for p.Arg141His was 1/72 and expected disease frequency was 1/20,000. These numbers are much higher compared to our results. Even our combined carrier frequency for all variants (1/118) could not reach the carrier frequency of the most common variant in their study population. In the same paper, carrier frequency for other variants was estimated to be 1/300 to 1/400, which in our population ranges from 1/448 to 1/2,244.

There are reports about the prevalence of different *PMM2* variants found in patients as well as overviews about genotypes in different populations. Although, p.Arg141His has always been the most commonly identified variant among Caucasian PMM2-CDG patients, other variants differ between countries. The selection of variants is wide, but in general their prevalence is rather low. Also, the genotypes are very variable. Variant p.Arg123Gln, which was not present in our population cohort, constitutes 1.9% of French, 3.9% of Spanish and 10% of Portuguese *PMM2* variants present in patients. P.Val231Met is rather common among PMM2-CDG patients in United Kingdom and Portugal, but less prevalent in France, Italy and Spain (Imtiaz et al 2000; Le Bizec et al 2005; Perez et al 2011; Perez-Cerda et al 2017). According to HGMD, p.Arg239Trp and p.Val67Met have been reported in few patients (Bjursell et al 2000; Matthijs et al 2000; Grunewald et al 2001). Patients heterozygous for p.Arg239Trp have shown mild or moderate phenotype (Grunewald et al 2001; Vals et al 2017). p.Val67Met/p.Arg141His causes severe multisystem presentation with involvement of the nervous system (Erlandson et al 2001).

p.Thr237Arg has been reported in French, Italians and Dutch. Genotype p.Val231Met/p.Thr237Arg caused severe neurovisceral syndrome and early death in an infant (Aronica et al 2005). On the contrary, p.Thr237Arg/p.Cys241Ser caused multisystem disorder but borderline mental development

(Barone et al 2007). Similarly to p.Arg141His, p.Thr237Arg shows very low residual activity (Kjaergaard et al 1999). Theoretically it is possible, that the recombinant with p.Arg141His or homozygosity of p.Thr237Arg are lethal as one allele must retain its residual activity and this might influence our estimated frequency by making it a little bit lower (1/84,000). Still, at least one patient with genotype p. Arg141His /p.Thr237Arg has been described in French cohort, although the data about the phenotype was not presented.

It is interesting that p.Phe119Leu was not presented in Estonian population cohort. This is the second most common variant among South-Scandinavian population (Kjaergaard et al 1998; Bjursell et al 2000).

5.3.2. Prevalence of PMM2-CDG according to patient cohort data

Several reports from different European countries describe allele frequencies of different *PMM2* variants present in PMM2-CDG patients. In Estonia, differences between allele frequencies based on population and patient cohorts were seen. One reason for this is probably a small number of molecularly confirmed patients, and it is possible that patients carrying p.Arg141His have been missed. Interestingly, in patient cohort, the p.Arg141His was only confirmed in one subject, whereas the second most common variant p.Val231Met in population cohort was present in all three families. Therefore, based on population data, our hypothesis about p.Val231Met being the most common variant in Estonia, was wrong. Another difference between two cohorts was the presence of p.Arg123Gln in one patient. This variant was inherited from French parent, which explains its absence in the Estonian population cohort.

The five patients were born from 1997 to 2015. One of them is deceased and one has reached adulthood. During 2011 census, 1.29 million enumerated residents were counted in Estonia and 18.4% (approximately 237,000) of them were children, defined as age less than 18 years. This makes the current prevalence of PMM2-CDG for the whole population (four alive patients) 1/322,000 (95% CI 1/117,000–1/1,007,000) and for the children less than 18 years age group (three patients), 1/79,000 (95% CI 1/25,000–1/306,000).

If we compare the estimated frequency 1/77,000 with Estonian population size and its age-specific distribution, approximately 16 people, including three children, should have PMM2-CDG. The calculated prevalence in the less than 18 years age group is 1/79,000, which is similar to the expected frequency of PMM2-CDG in Estonia based on population allele frequencies. On the other hand, the current prevalence based on diagnosed patients is much lower (1/322,000). It is possible that the actual prevalence is higher since the disease has not been diagnosed for instance in any adult patient. PMM2-CDG might be under-diagnosed among adults as well as older children as some of them either might be deceased, or present mild or stable phenotype. In addition, although the availability of different diagnostic tests is significantly increased in Estonia,

congenital metabolic diseases, including CDG, are less likely considered and investigated among the adult patients. It is possible that adults are not under regular follow-up (e.g. people in special care institutions) or their families are either not aware or interested in the new possibilities of metabolic and molecular studies.

5.4. Clinical phenotype of SLC35A2-CDG (Paper III)

5.4.1. Estonian patient with SLC35A2-CDG

5.4.1.1. Clinical description

The male patient was born at term (38+6 gestational weeks) from uneventful pregnancy. He needed tactile stimulation and additional oxygen after birth due to perinatal hypoxia (Apgar scores 4/8/8). His birth weight was 2986 gr (<50 ct), length 45.5 cm (3 ct) and head circumference 34 cm (50 ct). During the second half of the first year of life he needed physiotherapy due to spastic diplegia. At the age of 18 months he was investigated because of craniosynostosis. At that time, his motor and cognitive development was in accordance with the age of 12 months. He preferred to crawl but also made some steps. His muscular tone was decreased. He showed turribrachycephaly, small umbilical hernia, tongue-tie, labial frenulum, hypertelorism, small contact nose, long philtrum, and small mandible. On growth chart both, weight and height were less than 3 ct but head circumference was within normal limits. 3D computed tomography showed isolated synostosis of sagittal suture (Figure 8J). At the age of 22 months, the brain MRI showed short corpus callosum and delayed myelination of the white matter with patchy high signal intensity lesions in the periventricular white matter, in medial globus pallidus, in posterior mesencephalon, in medial cerebellar peduncule, and in dentate nucleus (Figure 8A-I). Leigh disease was suspected. This led to expanded metabolic investigations, including Tf IEF. Biochemical studies showed increase of aspartate aminotransferase (AST) and thyreoglobulin, and decreased ATIII. His EEG was normal.

At the age of 3 years, adenoidectomy was performed and after the procedure the bleeding was more pronounced than usual. His craniosynostosis did not need the surgical correction. At the moment he is ambulatory and shows mild developmental and speech delay. He is attending in special day care. At the age of 5 years and 1 month his height was 94.9 cm (0.1 ct, -3 SD) and weight 14.2 kg (1.02 ct, -2.32 SD). The patient is under regular follow-up by the clinical geneticist, ophthalmologist, endocrinologist, psychologist and speech therapist.

The patient has been treated with alimentary oral Gal since the age of 27 months. The starting dose was 0.5 g/kg/day in addition to dietary Gal 1 g/kg/day, and increased to 1–1.5 g/kg/day. After six months of therapy, the activity and development of the patient showed improvement, the child was more alert. Also, the level of ATIII increased, and thyreoglobulin normalized,

whereas AST remained slightly elevated. Alternating levels of ATIII and thyreoglobulin have reflected the compliance to the treatment.

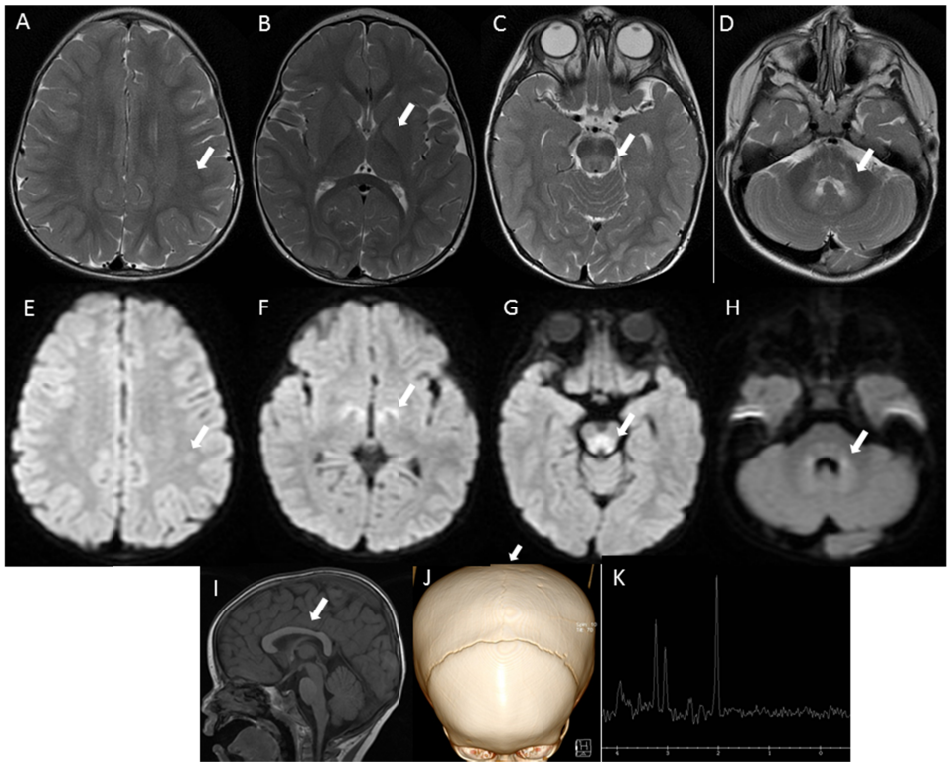

Figure 8. The brain MRI of Estonian patient with SLC35A2-CDG. T2 weighted image and diffusion weighted image show no significant atrophy but delayed myelination with patchy high signal intensities in the periventricular white matter (A and E), in medial globus pallidus (B and F), in posterior mesencephalon (C and G) and in medial cerebellar pedunculi (D and H), short corpus callosum (I), craniosynostosis of sagittal suture (J), and normal spectroscopy (K) (Vals et al. 2018).

5.4.1.2. Glycosylation and molecular studies

The hypothesis of mitochondrial Leigh disease was based on a brain MRI finding, whereas magnetic resonance spectroscopy as well as blood lactate, pyruvate, and ketones were within normal values. Tf IEF type 2 profile, first done at the age of 23 months, suggested glycosylation disorder. Quantification of sialotransferrins revealed increase of di- and trisialotransferrin (10.9%, normal limits 3.3–7.6%, and 19.3, normal limits 4.9–10.6%, respectively), and slight decrease of tetrasialotransferrin (44.5%, normal limits 47.6–62.7%). Asialotransferrin was normal (2%, normal limits 0–3.2%) and monosialo-

transferrin was on the upper normal limit (4.1%, normal limits 0–5%). Normal ApoC-III isoforms excluded combined N- and O-glycosylation defect. Glycoprofiling of intact Tf with quadrupole time-of-flight MS showed the increased loss of Sia (21%, normally <5–6%) but also several minor glycan peaks showing the lack of Gal and Sia residues (Figure 9). NGS of a large gene panel revealed defects neither in genes related to CDG nor mitochondrial diseases. Trio WES revealed a novel *de novo* hemizygous variant c.670C>T, p.Leu224Phe in *SLC35A2* located on X-chromosome. This variant was confirmed by Sanger sequencing. *SLC35A2* was not included in the previously performed gene panel.

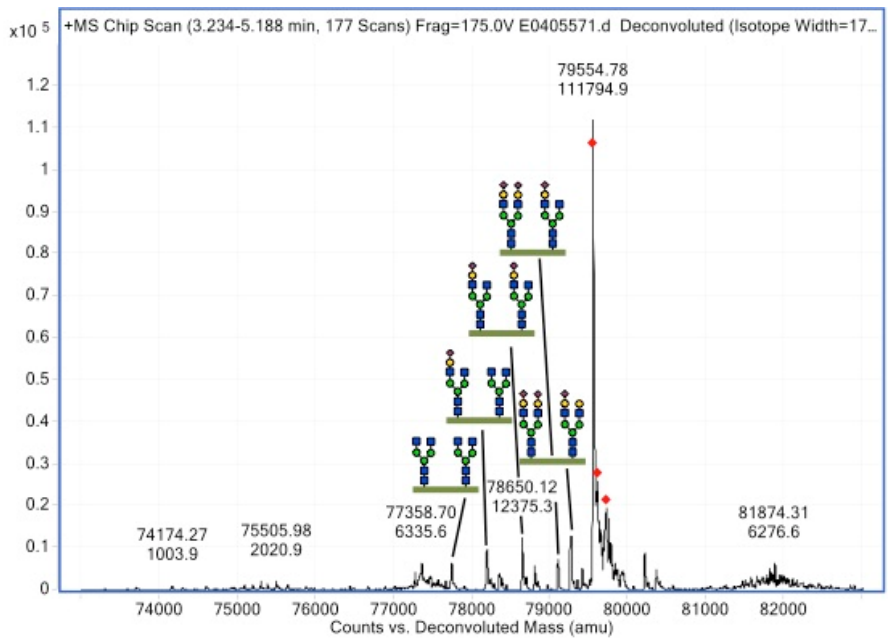

Figure 9. Serum Tf quadrupole time-of-flight MS of the Estonian patient with SLC35A2-CDG (with the permission of Dirk Lefeber).

5.4.2. International patients with SLC35A2-CDG

The comparison of the phenotype between Estonian patient and patients in the international cohort is shown in Table 5. International cohort included three males and eleven females with current ages 4 to 28 years. One of these patients was deceased. All showed global DD. Only three were able to ambulate. Muscular hypotonia was present in all. Epilepsy was also frequent (12/14), and often hypsarrhythmia was described in EEG. Many patients had feeding difficulties and required tube feeding. Ophthalmologic findings were frequent: 9/14 had either strabismus and/or refractive error, and 3/14 had cerebral visual

impairment. Ten out of thirteen had short stature. Unspecific dysmorphic features were described in 10/14. Musculoskeletal anomalies were common (e.g. contractures, deformities of the lower limbs, scoliosis).

The brain MRI was available in 8/14, and was performed at the ages of 10 months to 18 years. In two patients, repeated MRIs were available, which did not show the progression of the radiological findings. Unlike Estonian patient, many patients showed cerebral, cerebellar, pons and/or brainstem atrophy. All patients had short corpus callosum. Delayed myelination was seen in 6/8. White matter atrophy was present in 6 patients. In all, patchy hyperintensities in supra- and subtentorial white matter were seen.

Table 5. The phenotype of the patients in SLC35A2-CDG cohort.

	Estonian patient	International cohort	Summary
Clinical findings			
Global developmental delay	+	14/14	15/15
Muscular hypotonia	+	14/14	15/15
Seizures	-	12/14	12/15
Hypsarrhythmia or findings consistent with epileptic encephalopathy in EEG		9/11	9/11
Opthalmologic findings	-	13/14	13/15
Short stature	+	10/13	11/14
Musculoskeletal abnormalities	+	9/14	10/15
Abnormal N-glycosylation of Tf	+	8/14	9/15
MRI findings			
Cerebral atrophy	-	7/8	7/9
Cerebellar	-	5/8	5/9
Brain stem and/or pons atrophy	-	4/8	4/9
Thin corpus callosum	-	7/8	7/9
Short corpus callosum	+	8/8	9/9
White matter atrophy	-	6/8	6/9
Delayed/hypomyelination	+	6/8	7/9
High signal intensity lesions of white matter	+	8/8	9/9

Similarly to Estonian patient, isolated mild increase of AST was seen in half, and some patients showed changes in coagulation markers. CDG screening with Tf isoform analyses or Tf MS was abnormal in 8/14. Tf IEF showed type 2 profile and Tf MS characteristic loss of Gal. Normalization of Tf IEF in early infancy was reported in two patients, whereas one patient showed the worsening of glycosylation studies.

Overall, fourteen different variants in *SLC35A2* were presented. All variants presented in the cohort affected both transcripts, UGT1 and UGT2. Eleven patients had missense variant in *SLC35A2* gene. One had in-frame deletion, and two had frameshift deletions. The majority of variants appeared *de novo*. However, one patient had inherited the variant from the mother. Mosaicism was only reported in one male and one female patient, but often, mosaicism was not studied in other tissues. X-inactivation was studied in three females. One patient showed skewed X-inactivation in blood leukocytes, whereas in the second female, skewed pattern was only seen in buccal cells and skin fibroblasts. In the third female, X-inactivation was random in blood leukocytes.

5.4.3. Challenges in the diagnosis of SLC35A2-CDG

The phenotype of fifteen patients with *SLC35A2* variant shows that SLC35A2-CDG affects mainly and often severely the nervous system, and the reported symptoms are similar to previous reports (Kodera et al 2013; Ng et al 2013; Dorre et al 2015; Kimizu et al 2017; Westenfield et al 2018; Yates et al 2018). All the patients in the cohort had global DD and hypotonia, the majority was non-ambulatory, and epilepsy was frequently present.

As it was seen also in our international cohort, the majority of reported patients so far are females. No clear correlation between the gender and severity of symptoms, including abnormal glycosylation studies, was seen. SLC35A2-CDG is an X-linked disorder and therefore, a more severe phenotype might be expected in males. One hypothesis is that in males, mosaicism would be necessary for survival. The findings in our cohort do not support that, as only one male presented with mosaicism. In addition, non-mosaic Estonian male patient seemed to have the mildest phenotype in the cohort. Nevertheless, he has presented continuously abnormal glycosylation, which also makes difficult to associate the degree of clinical severity with the presence of abnormal glycosylation.

The clinical phenotype of SLC35A2-CDG is rather unspecific, and the diagnosis based on clinical symptoms, is difficult. In addition, the main challenge in diagnosing SLC35A2-CDG is the absence of abnormal Tf screening in some patients. Tf isoform analyses may have diagnostic value in early childhood (Ng et al 2013). As in other reports, the collected data in our cohort showed that glycosylation can improve, but also worsen over time, or be normal at the time of the first study. In case of abnormal screening, the diagnosis of SLC35A2-CDG is not challenging. Tf MS shows characteristic glycan profile

and disorder can be confirmed either with *SLC35A2* sequencing or gene panel. In many reported patients, including seven in our cohort, *SLC35A2* variants were first detected by WES, which was followed by glycosylation studies that were normal in six. This raises the question whether all patients with *SLC35A2* variant have CDG. Indeed, some CDG genes can cause divergent disorders (Ng and Freeze 2018), but all the reported patients with *SLC35A2* variant show a similar clinical phenotype. The hypothesis is that normalization of glycosylation might be associated with somatic mosaicism in boys, or skewed X-inactivation of mutated alleles in girls, which might lead to negative selection of mutated cells in visceral organs such as liver. Another possibility is that such patients have shown or will show abnormal glycosylation in different ages, but at the time of screening glycosylation has been normal. In future, functional studies may become helpful in confirming the pathogenicity of *SLC35A2* in the case of negative screening. Functional assays of UGT in the fibroblasts of SLC35A2-CDG patients, and UGT-deficient cell lines showed increased binding of glycans to the lectins indicating incomplete galactosylation (Ng et al 2013; Dorre et al 2015). At the moment these studies are not routinely available.

Therefore, presently the diagnostic approach to patients with new *SLC35A2* variant and normal glycosylation (including normal Tf MS) includes careful pathogenicity classification that should follow internationally acclaimed variant interpretation guidelines like those published by ACMG (Richards et al 2015). As previously mentioned, among six patients, *SLC35A2* variants were not supported by abnormal glycosylation profiles at the time of screening. All *de novo* variants were absent in population databases, and according to ACMG classification, they were considered likely pathogenic. One patient had inherited the variant from his unaffected mother, and it was considered as the most probable cause for the symptoms. All six patients have similar severe neurological symptoms, and many are treated with dietary galactose, which has previously been shown to improve seizure activity as well as Tf glycosylation in a female patient with SLC35A2-CDG (Dorre et al 2015).

Since a growing number of patients is reported with *SLC35A2* variants, similar genotypes have appeared. New patients with similar genotypes could add valuable information of the glycosylation status, and also about possible genotype-phenotype correlations in the future. Together with the subject in our cohort, there are at least two females and one male with the variant p.Val331Ile, and interestingly, they all did not have seizures. In a male and female with a variant p.Val331Ile, glycosylation showed improvement over time (Ng et al 2013; Westenfield et al 2018). Additionally, two females with p.Ala88Pro, one male and one female with p.Ser308Phe have been described (Bruneel et al 2018; Vals et al 2018; Yates et al 2018). In our cohort, the patients with previously mentioned three genotypes showed abnormal glycosylation. Unfortunately so far, there are no reports presenting the patients with similar genotypes to the six patients with normal glycosylation in our cohort.

It is also important to remember that in addition to diagnostic pitfall due to false-negative screening, gene sequencing studies might also miss the variants,

and sometimes, abnormal glycosylation profile helps to identify the variant as was also seen among two patients in our cohort (Kodera et al 2013; Ng et al 2013). Additional studies are needed to clarify the different aspects of SLC35A2-CDG such as causes for variable glycosylation status and possible positive effect of dietary galactose.

5.5. Novel type II CDG caused by homozygous variant in *STX5*

5.5.1. Clinical description of two siblings with CDG-IIx

The male proband 1 (IV:9 in Figure 10) was born prematurely (premature rupture of membranes and acute chorioamnionitis) to a 26-year-old gravida 9 para 3 woman on 29th gestational week. His birth weight was 1380 gr (0 SD), length 36.2 cm (-2 SD) and head circumference 29.5 cm (+2 SD). Apgar scores were 3, 6, and 7. During the pregnancy, fetal anatomical ultrasound scan showed shortening of long bones, bilateral clubfeet and flection contracture of both knees.

He was admitted to neonatal intensive care unit due to prematurity and respiratory distress. The following dysmorphic features were observed: macrocephalic abnormal skull configuration with asynclitism, prominent forehead, mild exophthalm, hypertelorism, short contact nose, high nasal bridge, long philtrum, sublingual cyst, dysmorphic ears, edematous skin, excessive skin on neck, bilateral simian lines, short extremities (proximal>distal), narrow thorax and bilateral clubfeet. An echocardiography revealed ventricular septal defect. The ultrasound scan showed hepatomegaly, agenesis of the left kidney, and hyperechogenicity in periventricular and thalamic areas of the brain, which might refer to hypoxic-ischemic brain injury.

After birth, the main clinical problem was progressive liver failure with coagulation abnormalities (ATIII 6% and protein C 20%), hypercholesterolemia (8.5 mmol/L), hyperbilirubinaemia (480 μmol/L, direct bilirubin 180 μmol/L) and – hyperammonaemia (180 μmol/L), high AST (360 U/L) and alkaline phosphatase (1611 U/L). In addition, he had hyperinsulinemic (33.5 mU/L) hypoglycemia, lower level of TSH (1.36 mU/L) and free thyroxine (8,1 pmol/L), very low IGF-I (<25 μg/L) and anemia. Very long chain fatty acid analysis showed slightly higher hexacosanoate (C26:0, 1.58 μmol/l, range 0.45–1.32 μmol/l), and hexacosanoate and docosanoic acid ratio (C26:0/C22:0) 0.02 μmol/l (0.01–0.02 μmol/l) possibly due to secondary changes.

The child died on 25th day of life due to multiorgan failure. Autopsy showed hyperemia of internal organs, hepatomegaly with stage 3 to 4 liver fibrosis, agenesis of the left kidney, ventricular septal defect, ischemic necrosis and perforation of small intestine with mild peritonitis, infant respiratory distress syndrome stage III and pulmonary edema, and suggestive pathohistological features of chondrodysplasia.

Female proband 2 was the elder sibling of proband 1 (IV:8 in Figure 10). The pregnancy was medically aborted on the 21st week of pregnancy due to clinical suspicion of achondroplasia, as the shortening of long bones was seen in the fetal ultrasound scan. Fetus had short extremities, which was more evident in legs (proximal>distal), bilateral clubfeet, ulnar deviation of wrists, clinodactyly of fifth finger, and excessive skin on neck. She had a rather similar facial phenotype to patient 1 – high forehead, mild exopthalm, high nasal bridge, short nose, long philtrum, and dysmorphic right ear. The weight was 332 grams (in accordance with 20–21 gestational weeks) and length 20.5 cm (in accordance with 17–18 gestational weeks). In autopsy, bilateral hydronephrosis and sacral lordosis were seen. Otherwise, the macroscopic and histological findings of the organs were in accordance with gestational age.

In the family history, the mother has one healthy son (IV:2 in Figure 10). Three pregnancies were medically interrupted (IV:1, IV:4, and IV:8), two of them on the 20th to 21st week of pregnancy due to abnormal ultrasound scan findings of fetuses (IV:4 and described IV:8). In addition, the mother had three spontaneous abortions (IV:5, IV:6, and IV:7). Male sibling IV:3 died immediately after birth. As he was born abroad, we do not have any clinical information about him.

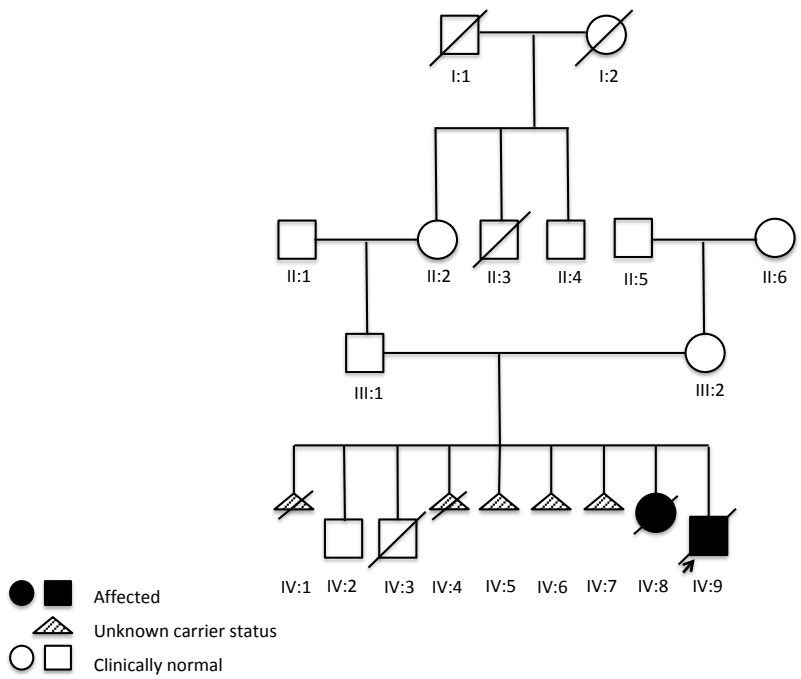

Figure 10. The pedigree of siblings with CDG-IIx.

5.5.2. Glycosylation and molecular studies

Tf IEF of proband 1 serum showed abnormal type 2 profile with increased asialo- and trisialotransferrin and decreased tetrasialotransferrin. Quadrupole time-of-flight MS of intact Tf revealed a loss of Sia and many additional abnormal glycan structures, with a peculiar transferrin having a short mannose containing glycan which has not been seen in any other known type of CDG (Figure 11). IEF of ApoC-III revealed a strongly abnormal profile with marked increase of ApoC-III_0 (56%, normal range 0.2–4.5%), and decreased ApoC-III_1 and ApoC-III_2 suggesting combined N- and O-glycosylation defect.

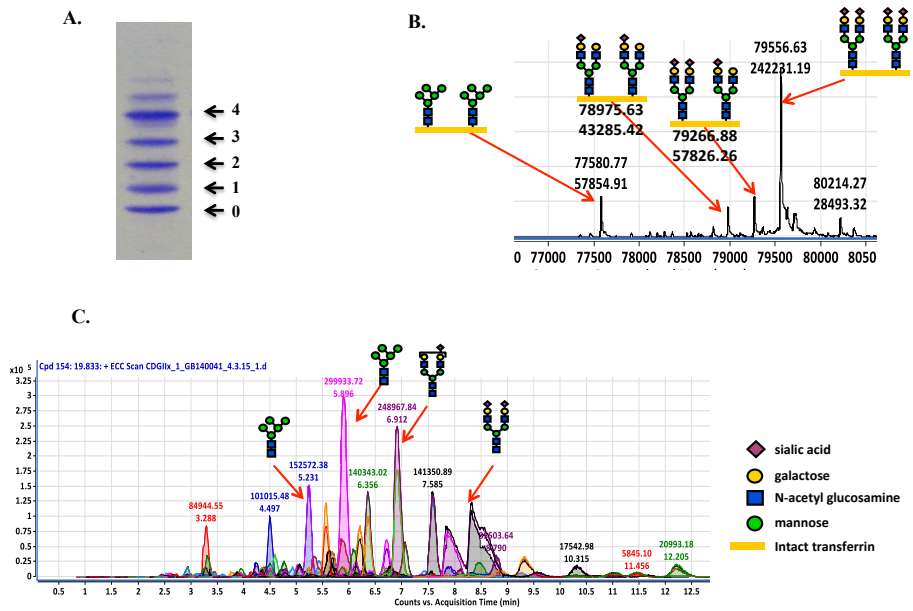

Figure 11. Glycosylation studies of proband 1. A. Abnormal type 2 Tf IEF. B. Quadrupole time-of-flight MS of intact Tf (with the permission of Dirk Lefeber). C. Quadrupole time-of-flight MS of total serum N-glycans (with the permission of Dirk Lefeber).

In proband 2, fetal karyotype was 46,XX. Variant analysis of *FGFR3* gene and other skeletal dysplasias was normal.

Chromosomal microarray analysis revealed multiple LCSH (>5 Mb) distributed across the entire genome of probands 1 and 2 (Figure 12). The percentage of homozygosity was ~8.3% and ~5.3% for probands 1 and 2, respectively, which points to a probable parental consanguinity. However, the parents of the described probands do not know that they are related and according to the recommendations of ACMG, speculations on a specific relationship should be avoided (Rehder et al 2013).

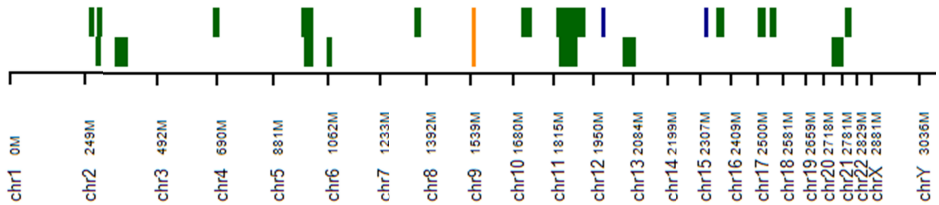

Figure 12. Multiple LCSH distributed across the entire genome in proband 1 (with the permission of Olga Fjodorova, previous name Žilina).

Taking into account the similar clinical phenotype of proband 1 and 2, LCSH were further compared to find overlapping regions (Table 6). The candidate gene for this novel type of CDG was already found in 2014, when a homozygous c.163A>G (p.Met55Val) variant in *STX5,* located on chromosome 11, was detected in one LCSH region by WES. Sanger sequencing analysis showed that identified variant was in homozygous state in both affected probands and heterozygous in the parents. The variant is absent from gnomAD.

Table 6. Overlapping chromosomal regions of proband 1 and 2. *STX5* gene locates on chromosome 11q12.3 (with the permission of Olga Fjodorova, previous name Žilina).

Chromosome	Start Position (bp)	End Position (bp)	Length (bp)
2p22.1-p21	41 051 123	47 273 511	6 222 389
5q22.3-q23.2	113 940 187	123 909 500	9 969 314
11p15.2-p13	15 745 640	32 583 695	16 838 055
11p13-p11.12	33 423 590	51 530 241	18 106 651
11q11-q22.1	55 091 268	102 091 632	47 000 364

STX5 encodes a protein STX5, which is a member of SNAREs family, and important in the intra-Golgi vesicle transport, docking and fusion. Vesicles carry Golgi resident proteins, some of which are important in glycosylation process. As defects in COG complex subunits affect additionally the normal functioning of other components in the vesicle trafficking machineries such as SNAREs, one might assume that similarly, the defects in any family member of SNAREs have the same effect on the normal function of COG complex. Therefore, it is possible that defective STX5 protein causes Golgi disorganization, and leads to disrupted intra-Golgi trafficking and abnormal protein glycosylation. Notably, some overlapping of the symptoms (liver and skeletal involvement, and dysmorphism) is seen between described two siblings and patients with COG-CDG, which also supports the assumption that complexes regulating

Golgi trafficking and homeostasis complete each other. Although multiple lines of computational evidence support a deleterious effect of the p.Met55Val variant in *STX5*, functional studies are needed to prove whether this variant is the definite cause of the new type of CDG. These studies could also demonstrate the exact function of *STX5* in the glycosylation pathway.

The introduction of Tf IEF in our practice led to unexpected discovery of novel type II CDG, which might be caused by homozygous variant in *STX5*, and which affects multiple glycosylation pathways. Although the functional confirmation studies are still ongoing to prove the pathogenicity of the variant, it is possible that it impairs severely the function of encoded protein. Both probands shared common clinical characteristics such has distinct facial features, shortened extremities and anomalies of the limbs. How this defect would impact the development, and whether this type of CDG is lethal, remains unknown, but progressive neonatal liver failure alone poses a great challenge to clinicians.

Unfortunately, we have not found any other patients in the world with a similar phenotype and *STX5* defects, although we have presented this case in the conference of The European Society of Human Genetics, as well as at the meeting of European Metabolic Group. Moreover, we added this *STX5* variant also to GeneMatcher database (Sobreira et al 2015), but did not find a match. The clinical cases of our patients highlight the difficulties in defining a new type of CDG that is based on a single family. Still, we plan to publish this case after finishing this PhD study.

6. CONCLUSIONS

1. The results of CDG screening among Estonian patients during a three-year screening period were reported (Paper I and IV).
   1.1 Transferrin isoelectric focusing is simple and efficient method to detect most defects affecting N-glycosylation pathway.
   1.2 We confirmed CDG in six patients among 1230 subjects screened.
   1.3 The most frequent CDG in Estonia is PMM2-CDG, which is similar to other populations.
   1.4 We improved markedly the awareness of CDG among Estonian clinicians.
2. The clinical phenotype and molecular findings of four patients with PMM2-CDG from two families were evaluated (Paper I).
   2.1 The phenotypic features of Estonian PMM2-CDG patients are similar to previously described patients.
   2.2 We detected a family with three siblings who show a mild neurological phenotype with normal-borderline cognitive development, which has previously been seldom described.
   2.3 Among PMM2-CDG patients, the most common variant in *PMM2* was p.Val131Met, and not p.Arg141His as seen in other populations.
3. The expected frequency of PMM2-CDG based on Estonian population data, and the prevalence of PMM2-CDG based on diagnosed PMM2-CDG patients in Estonia were reported.
   3.1 Five different heterozygous variants in *PMM2* were identified in Estonian normal population. The most frequent variant is p.Arg141His with carrier frequency 1/224; carrier frequency for p.Val131Met is 1/449.
   3.2 The expected frequency of PMM2-CDG based on Estonian normal population data is 1/77,000. We demonstrated that the population cohort data give useful new information about the epidemiology of the PMM2-CDG.
   3.3 Based on the patient population data, the prevalence of PMM2-CDG for the age group 0 to 17 years is 1/79,000.
4. The clinical phenotype and molecular findings of 15 international patients with SLC35A2-CDG were characterized and compared (Paper III).
   4.1 The diagnosis of SLC35A2-CDG is very challenging. It presents as the non-specific neurological syndrome with global developmental delay, hypotonia, seizures, and the brain MRI changes such as cerebral and cerebellar atrophy, delayed myelination of white matter and short corpus callosum, together with dysmorphic features and short stature.
   4.2 A negative transferrin isoform screening does not exclude SLC35A2-CDG.
   4.3 A diagnostic approach to patients with new *SLC35A2* variant but normal glycosylation screening is proposed. First, the glycosylation profile should be assessed with high-resolution mass spectrometry of

intact transferrin. If the profile is diagnostic then the rare variant found could be considered as pathogenic. If the profile is normal, a careful assessment of patients' phenotype should be combined with a careful pathogenicity classification that follows internationally acclaimed variant interpretation guidelines like those published by American College of Medical Genetics and Genomics classification.

5. The clinical phenotype and molecular findings of a patient with a novel type II CDG likely caused by homozygous variant in *STX5* gene are presented.
   5.1 Index patient presented multisystem clinical CDG features including dysmorphy, hepatomegaly, liver fibrosis and failure, skeletal anomalies (short extremities and clubfeet), renal anomaly, coagulopathy, and endocrine disturbances.
   5.2 Transferrin isoelectric focusing performed on the fifth day of life revealed markedly abnormal type 2 profile with increased asialo- and trisialotransferrin and decreased tetrasialotransferrin. This led us to continue studies towards CDG diagnosis, which was the main cause of the symptoms in the patient.
   5.3 The combination of different genomic analyses revealed the homozygous variant in *STX5* gene, which impairs Golgi trafficking and normal glycosylation.
   5.4 It is very likely that the identified variant in *STX5* gene is pathogenic. The functional studies are presently at work.

The introduction of CDG screening in clinical practice in Estonia showed that the isoelectric focusing of serum transferrin is an effective method to detect CDG among patients. It offers relatively fast primary information to clinicians, and if negative, CDG is likely to be excluded. The isoelectric focusing of serum transferrin is now routinely used in our clinical practice, and we have diagnosed two new patients with CDG.

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8. SUMMARY IN ESTONIAN

Kaasasündinud N-glükosüülimise haigused Eestis

Sissejuhatus

Kaasasündinud glükosüülimise haigused (KGH) moodustavad kiirelt areneva ainevahetushaiguste grupi ning on põhjustatud valkude ja lipiidide primaarsest hüpoglükosüülimisest. Normaalne glükosüülimisprotsess tagab paljude sekretoorsete, membraansete ja rakusiseste valkude häireteta funktsioneerimise.

Alates haiguse esmakirjeldusest 1980. aastal on kirjeldatud vähemalt 125 erinevat KGH (Ng and Freeze 2018). KGH alatüüpe tähistatakse põhjusliku geeni sümboliga, millele järgneb lühend CDG (*congenital disorders of glycosylation*). Kõige sagedamini esineb valkude N-glükosüülimise defekte, mis päranduvad peamiselt autosoom-retsessiivsel teel. Sünteesi on haaratud paljud ensüümid ja teised vajalikud valgud, mille funktsiooni ja seeläbi N-glükosüülimist mõjutavad neid kodeerivate geenide mutatsioonid. Lisaks vajab glükosüülimisprotsess normaalseks kulgemiseks rakuorganellide õiget struktuuri ja sobivat mikrokeskkonda.

N-glükaanide sünteesis eristatakse kolme põhietappi: nukleotiididega seotud monosahhariidide süntees, lipiidiga seotud oligosahhariidi kokkupanek ja glükaani töötlemine. Süntees toimub kindlas järjekorras tsütosoolis, endoplasmaatilises retiikulumis (ER) ning Golgi kompleksis (GK). Tsütoplasmas liidetakse lipiidse kandja, fosforüleeritud dolihhooli külge kaks N-atsetüülglükoosamiini (GlcNAc) molekuli ja viis mannoosijääki (Man). Struktuur pööratakse flipaasi toimel ER-i valendikku, kus seda pikendatakse veel nelja Man-i ning kolme glükoosimolekuliga. Seejärel kantakse oligosahhariid lipiidselt kandjalt üle valgu koostises olevale aminohappele asparagiin ning algab glükaani töötlemine. Protsess algab ER-is ja lõppeb GK-s. Oligosahhariidilt eemaldatakse ükshaaval monosahhariidide jäägid, kuni järele jääb kaheharuline kolmest Man-ist koosnev struktuur. Kummagi haru külge lisatakse üks GlcNAc, üks galaktoosi (Gal) ning üks siaalhappe molekul ja seejärel on glükoproteiin valmis. Olenevalt glükoproteiinist võib glükaanil olla ka rohkem harusid.

N-glükosüülimise defektide peamiseks skriiningmeetodiks on seerumi transferriini isoelektriline fokuseerimine (IEF), millega hinnatakse skriinitava patsiendi transferriini isovorme ja mis võib anda viite KGH esinemisele kuni 48 alatüübi puhul, mis otseselt või kaudselt mõjutavad N-glükosüülimist (Jaeken and Peanne 2017). Eristatakse kahte tüüpi profiile sõltuvalt sellest, milline N-glükosüülimise sünteesi etapp on häirunud. Skriiningu puuduseks on see, et esineda võivad näiteks patsiendi vanusest või KGH alatüübist tingitud vale-negatiivsed või sekundaarsest hüpoglükosüülimist põhjustavatest seisunditest (näiteks galaktoseemia, infektsioon) tingitud vale-positiivsed tulemused. Eestis on IEF interpreteerimine kvalitatiivne ning subjektiivne.

Olenevalt skriiningul leitud profiilist ja patsiendil esinevatest sümptomitest järgneb positiivsele skriiningule kas kindla geeni sekveneerimine, apolipoproteiin C-III IEF, glükaani struktuuride hindamine massispektromeetria meetodil ja/või ensümaatilised uuringud. Diagnoosi kinnitavad molekulaarsed uuringud (kindla geeni sekveneerimine, ülegenoomsed uuringud). Võimalik on, et KGH diagnoositakse ülegenoomsete uuringute tulemusel ilma biokeemiliste kõrvalekallete toetuseta, mis teeb uute mutatsioonide patogeensuse hindamise keeruliseks. Seetõttu on oluline funktsionaalsete lisauuringute olemasolu KGH kinnitamiseks.

Sõltuvalt KGH alatüübist võivad kliinilised sümptomid olla tihti kattuvad ja mittespetsiifilised ning sama alatüübi puhul võib nende raskusaste olla patsientide lõikes erinev. Sümptomid haaravad peaaegu alati närvisüsteemi (üldine arengu hilistumine erinevas raskusastmes, lihashüpotoonia, epilepsia, ataksia ja aju väärarendid), kuid kaasuda võivad ka teiste organite funktsioonihäiretest põhjustatud sümptomid (maksapuudulikkus, kardiaalsed ja oftalmoloogilised probleemid, skeletianomaaliad, hüübimishäired ja sagedased infektsioonid). Paljude alatüüpide puhul on kirjeldatud vaid üksikuid patsiente. Põhjuslik ravi on olemas vaid mõnele KGH alatüübile (näiteks MPI-CDG) ning peamiselt on ravi sümptomaatiline ning toetav.

Kõige sagedasem KGH alatüüp on PMM2-CDG, mille hinnanguline esinemissagedus mutatsioonikandluse põhjal on 1/20,000 (Schollen et al 2000). Seda alatüüpi põhjustavad erinevad mutatsioonid *PMM2* geenis, mis kodeerib ensüüm fosfomannomutaasi (PMM2). PMM2 osaleb Man-i sünteesis, mis on glükosüülimisprotsessis väga oluline substraat. Ensüümi aktiivsuse vähenemise tagajärjel on valkude küljes tavapärasest vähem glükaane või puuduvad need üldse. Klassikalise PMM2-CDG korral esinevad haigetel nii düsmorfsed tunnused (rinnanibude sissetõmme, omapärased rasvapadjandid), mitme organsüsteemi haaratus (näiteks seedetrakt ja hüübimissüsteem) kui ka neuroloogilised sümptomid (üldine arengu hilistumine, lihashüpotoonia ja ataksia). Klassikaline multisüsteemne fenotüüp on iseloomulik eeskätt varajases imikueas. Edaspidi domineerib närvisüsteemi haaratus, kuid haigus ei progresseeru. Vaatamata haiguse raskusastmele on kõikidele haigetele iseloomulik närvisüsteemi haaratus ning väikeaju atroofia. PMM2-CDG puhul on suremus 9–15% (Perez-Cerda et al 2017; Schiff et al 2017). Enamik patsiente on *PMM2* mutatsioonide suhtes liitheterosügoodid. Euroopa rahvastiku hulgas on kõige sagedasem mutatsioon p.Arg141His, mille puhul PMM2 aktiivsus on nullilähedane. Fenotüübi raskusastme määrab sageli kergem mutatsioon. Siiski ei pruugi sarnase genotüübiga patsientide fenotüüp sarnaneda ja oletatakse, et haiguse raskusastmes mängivad rolli ka teised faktorid.

SLC35A2-CDG on KGH alatüüp, mis pärandub X-liitelisel teel. Geen *SLC35A2* kodeerib Gal-i transporterit, mis viib Gal GK-sse ja mille puudumisel kannavad valgud enda küljes ehituslikult puudulikke glükaanistruktuure. SLC35A2-CDG puhul võib skriining olla negatiivne, mistõttu paljud haiged on diagnoositud ülegenoomsete uuringute abil. Haigetele on iseloomulik peamiselt

raskeloomuline üldise arengu hilistumine ja epileptiline entsefalopaatia. Haigete sümptomeid on leevendanud ravi Gal-iga (Dorre et al 2015).

STX5 geeni pole siiani KGH-ga seostatud. STX5 reguleerib vesiikulite vahendatud valkude transporti ja liitumist sihtmembraaniga. STX5 lokaliseerub trans-Golgis ja moodustab komplekse teiste glükosüülimises oluliste valkudega, mis aitavad luua sobivat GK keskkonda ja ehitust.

Töö eesmärgid

1. Juurutada Eestis KGH diagnostikaks transferriini isoelektriline fokuseerimine ja hinnata skriiningu efetkiivsust.
2. Hinnata Eesti PMM2-CDG diagnoosiga patsientide genotüüpi ja fenotüüpi.
3. Hinnata populatsiooni ja diagnoositud patsientide andmete põhjal PMM2-CDG eeldatavat sagedust ning levimust Eestis.
4. Iseloomustada Eesti SLC35A2-CDG patsiendi feno- ja genotüüpi ja võrrelda seda 14 rahvusvahelise patsiendi feno- ja genotüübiga.
5. Esitleda uudse glükosüülimisdefektiga patsienti, kelle puhul põhjustab defekti tõenäoliselt *STX5* homosügootne mutatsioon.

Uuringugruppide, patsientide ja meetodite lühikirjeldus

KGH skriiningu periood kestis ajavahemikul 01.06.2012–30.06.2015. Skriiningu meetodina kasutati transferriini IEF-i. Et suurendada võimalike KGH-ga patsientide hõlmatust, teostati skriining kõigile ainevahetushaiguste kahtlusega patsientidele, kelle seerum saadeti SA Tartu Ülikooli Kliinikumi ühendlabori kliinilise geneetika keskusesse erinevate ainevahetushaiguste uuringuteks nii Tallinna kui ka Tartu haiglatest. Skriiningut teostati 1230 patsiendile. Positiivse skriiningu korral tehti diagnoosi täpsustamiseks ka muid biokeemilisi ja/või molekulaarseid analüüse.

Et välja selgitada KGH sagedaseima alatüübi PMM2-CDG eeldatav sagedus Eesti populatsioonis, analüüsiti Tartu Ülikooli Eesti Geenivaramu 2244 geenidoonori *PMM2* geeni andmeid. Arvutustes kasutati veidi muudetud Hardy-Weinbergi seaduse matemaatilist väljundit.

Uurimustöö osana kirjeldati kahe patsiendi erinevaid KGH alatüüpe, mis diagnoositi positiivse skriiningu tulemusel. SLC35A2-CDG on küllaltki harva esinev KGH ning Eesti patsienti võrreldi 14 rahvusvahelise patsiendiga. Teisel patsiendil on tegemist uue, varem kirjeldamata KGH alatüübiga, mille põhjuseks on tõenäoliselt *STX5* geeni homosügootne mutatsioon.

Uuringu peamised tulemused ja järeldused

1. Esitasime kolme aasta jooksul teostatud KGH pilootskriiningu tulemused Eesti patsientide kohta.
   1.1 Transferriini IEF on lihtne ja tulemuslik meetod, et leida patsientidel N-glükosüülimist mõjutavaid defekte.
   1.2 KGH kinnitus kuuel patsiendil 1230 skriinitust.
   1.3 Kõige sagedasem KGH alatüüp Eestis on sarnaselt teise riikidega PMM2-CDG.
   1.4 Parandasime oluliselt Eesti arstide hulgas teadlikkust KGH osas.
2. Hindasime kahest perekonnast pärit nelja Eesti PMM2-CDG diagnoosiga patsiendi fenotüüpi ja *PMM2* geeni molekulaarseid muutusi.
   2.1 Eesti PMM2-CDG diagnoosiga patsientide fenotüüp sarnanes eelnevalt kirjeldatud patsientide fenotüübiga.
   2.2 Diagnoosisime PMM2-CDG ühe pere kolmel lapsel, kellel esineb haiguse kerge neuroloogiline fenotüüp ning normaalne-piiripealne kognitiivne areng, mida varasemalt on PMM2-CDG diagnoosiga patsientidel harva kirjeldatud.
   2.3 PMM2-CDG diagnoosiga patsientide hulgas on kõige sagedasem *PMM2* geenivariant p.Val131Met, mitte p.Arg141His nagu teistes populatsioonides.
3. Esitasime andmed PMM2-CDG eeldatava sageduse ning levimuse kohta.
   3.1 Leidsime Tartu Ülikooli Eesti Geenivaramu geenidoonorite kogu genoomi sekveneerimise tulemustes viis erinevat *PMM2* heterosügootset mutatsiooni. Kõige sagedasem geenivariant on p.Arg141His kandlussagedusega 1/224; kandlussagedus geenivariandi p.Val131Met puhul on 1/449.
   3.2 Eeldatav PMM2-CDG sagedus Eestis on 1/77,000. Tartu Ülikooli Eesti Geenivaramu andmeid saab kasutada erinevate haiguste eeldatava sageduse hindamiseks.
   3.3 Eesti patsientide andmete põhjal on PMM2-CDG levimus laste hulgas 1/79,000.
4. Iseloomustasime SLC35A2-CDG fenotüüpi Eesti ja 14 rahvusvahelise patsiendi andmete põhjal.
   4.1 SLC35A2-CDG diagnoosimine on keeruline. Patsientidele on iseloomulik mittespetsiifiline neuroloogiline haigus üldise arengu hilistumise, lihashüpotoonia, krampide ning epileptilise entsefalopaatiaga, aju MRT muutused (suur- ja väikeaju atroofia, hilinenud valgeaine müelinisatsioon ja lühike mõhnkeha), düsmorfsed ilmingud ja lühike kasv.
   4.2 Negatiivne KGH skriining ei välista SLC35A2-CDG diagnoosi.
   4.3 Esitasime diagnostilise algoritmi hindamaks patsiente varasemalt kirjeldamata *SLC35A2* geenivariantidega ning negatiivse KGH skriininguga. Esmalt tuleks hinnata glükaane massispektromeetria meetodil. Kui glükaanide struktuur viitab glükosüülimishäirele, tuleks geenivariant lugeda haiguspõhjuslikuks. Kui glükaanide struktuur on normaalne,

tuleks patsientide kliinilisi tunnuseid kombineerida geenivariandi kahjulikkuse hindamisega, kasutades *in silico* arvutuslikke meetodeid.

5. Kirjeldasime uue, II tüüpi KGH-ga patsienti, kellel esinevat KGH alatüüpi põhjustab tõenäoliselt homosügootne geenivariatsioon *STX5* geenis.
   5.1 Patsiendil olid multisüsteemsed KGH-le iseloomulikud sümptomid nagu düsmorfsus, maksafibroos ja -puudulikkus, skeletianomaaliad (lühikesed jäsemed ja komppöiad), neeruanomaalia ning hüübimis- ja endokrinoloogilised häired.
   5.2 Viiendal elupäeval teostatud transferriini IEF viitas II tüüpi KGH defektile, mis arvati patsiendil esinenud sümptomite peamiseks põhjuseks ning see võimaldas jätkata suunitletult täpsustavaid uuringud kinnitamaks KGH diagnoosi.
   5.3 Erinevate ülegenoomsete analüüside tulemusel leiti *STX5* geenis homosügootne geenivariant, mis tõenäoliselt häirib Golgi kompleksi struktuuri ja funktsiooni ning seeläbi normaalse glükosüülimisprotsessi kulgemist.
   5.4 On väga tõenäoline, et leitud *STX5* geenivariant on patogeenne. Funktsionaalsed uuringud jätkuvad.

Antud uuring näitas, et KGH skriining on tulemuslik ning enamjaolt abistav vahend haiguste diagnostikas ja diferentsiaaldiagnostikas. Kahjuks ei saa välistada vale-negatiivseid ja –positiivseid vastuseid, näiteks tingituna patsiendi vanusest, KGH alatüübist, aga ka subjektiivsest hindamisest. Kuna osa KGH alatüüpe ei ole transferriini IEF-iga skriinitavad või tulemused võivad ajas muutuda, on oluline, et klinitsistid seda nüanssi silmas peaksid ning KGH kahtluse püsimisel uuringuid jätkaksid.

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10. PUBLICATIONS

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Member of the Estonian Society of Perinatology
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Publikatsioonide nimekiri

1. **Vals, MA**.; Ashikov, A.; Ilves, P.; Loorits, D.; Zeng, Q.; Barone, R.; Huijben, K.; Sykut-Cegielska, J.; Diogo, L.; Elias, AF.; Greenwood, RS.; Grünewald, S.; van Hasselt, PM.; van de Kamp, JM.; Mancini, G., Okninska, A.; Pajusalu, S.; Rudd, PM.; Rustad, CF.; Salvarinova, R.; de Vries, BBA.; Wolf, NI.; EPGEN Study; Ng, BG.; Freeze, HH.; Lefeber, DJ.; Õunap, K. (2018). Clinical, neuroradiological and biochemical features of SLC35A2-CDG patients. J Inher Metab Dis (accepted 2018).
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