Charles University in Prague
First Faculty of Medicine

Academic program: Biochemistry and pathobiochemistry

Mgr. Nina Ondrušková

Studium dědičných poruch glykosylace na biochemické a molekulární úrovni

Biochemical and molecular studies of the congenital disorders of glycosylation

PhD thesis

Supervisor: RNDr. Hana Hansíková, CSc.
Consultant: Ing. Kateřina Veselá, Ph.D.

Prague, 2016

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 2016

Mgr. Nina Ondrušková

Podpis

Identifikační záznam:

ONDRUŠKOVÁ, Nina. *Studium dědičných poruch glykosylace na biochemické a molekulární úrovni. [Biochemical and molecular studies of the congenital disorders of glycosylation]*. Praha, 2016. 92 stran. Disertační práce. Univerzita Karlova v Praze, 1. lékařská fakulta, Klinika dětského a dorostového lékařství. Vedoucí závěrečné práce: RNDr. Hana Hansíková, CSc.

Identification record:

ONDRUŠKOVÁ, Nina. *Biochemical and molecular studies of the congenital disorders of glycosylation. [Studium dědičných poruch glykosylace na biochemické a molekulární úrovni]*. Prague, 2016. 92 pages. PhD thesis. Charles University in Prague, First Faculty of Medicine, Department of Pediatrics and Adolescent Medicine. Supervisor: RNDr. Hana Hansíková, CSc.

ABSTRAKT

Dědičné poruchy glykosylace (Congenital disorders of glycosylation, CDG) jsou rychle rostoucí skupinou vzácných dědičných metabolických poruch s prevalencí až 1:20 000, které jsou zapříčiněny genetickými defekty narušujícími proces glykosylace, tj. enzymatického připojení specifické sacharidové struktury na kostru proteinů nebo lipidů. Kvůli nespecifitě a variabilitě klinických příznaků u pacientů je určení diagnózy CDG velmi složité, a významně se spoléhá na správnou biochemickou a genetickou analýzu.

Záměrem předložené dizertační práce bylo studium CDG na biochemické a molekulárně-genetické úrovni v rámci České a Slovenské republiky, které zahrnovalo tři specifické cíle: A.) zavést a optimalizovat laboratorní screeningové metody pro detekci CDG u klinicky suspektních pacientů, B.) určit odpovídající genetický defekt u pozitivních pacientů zachycených pomocí screeningu a studovat patobiochemické aspekty jednotlivých typů CDG na buněčné úrovni, a C.) analyzovat poruchy glykosylace s jinou (non-CDG) etiologií.

Mezi přínosy této práce patří optimalizace isoelektrické fokusace apolipoproteinu C-III (ApoC-III) jako metody pro detekci abnormální O-glykosylace, a také popis praktických implikací metod pro screening CDG (např. detekce specifického polymorfizmu transferinu, u kterého nelze screening CDG použít, nebo nález hyposialovaného ApoC-III u glykogenóz). Kromě toho při charakterizaci subcelulární struktury a různých patobiochemických aspektů ve fibroblastech pacientů s vybranými typy CDG byly popsány dosud nepublikované jevy (např. buněčná akumulace reaktivních forem kyslíku u CDG). Díky aplikaci nových metod a studiu průběhu onemocnění, identifikace biochemické a genetické podstaty onemocnění byla provedena u více než 20 pacientů. Unikátní případy (RFT1-CDG, PGM1-CDG, MAN1B1-CDG, defekt NgBR) byly publikovány a přinesly zcela nové poznatky vztahující se k fenotypu nebo genotypu dané poruchy.

Klíčová slova: dědičné poruchy glykosylace, CDG, screening, apolipoprotein C-III, transferin

ABSTRACT

Congenital disorders of glycosylation (CDG) represent a rapidly growing group of rare inherited metabolic diseases with estimated prevalence as high as 1:20 000, which are caused by genetic defects that impair the process of glycosylation, i.e. the enzymatic addition of a specific saccharide structure onto a protein or lipid backbone. Due to non-specificity and variability of clinical symptoms in the patients, the medical diagnosis of CDG remains extremely challenging and significantly relies on accurate biochemical and genetic analyses.

The overall goal of the present dissertation thesis was to study CDG at the biochemical and molecular genetic level in the context of the Czech and Slovak Republic, which involved three specific aims: A.) to introduce and optimize laboratory screening methods for CDG detection in a group of clinically suspected patients, B.) to determine the corresponding genetic defect in the positive patients selected via CDG screening and to study the pathobiochemical aspects of specific CDG types at the cellular level, and C.) to analyze glycosylation disturbances of non-CDG etiology.

Contributions of this work include optimization of isoelectric focusing of apolipoprotein C-III (ApoC-III) as a screening method for O-glycosylation abnormalities, as well as the description of practical implications for using CDG screening methods (e.g., the detection of a specific transferrin polymorphism that hampers N-glycosylation screening, or the finding of hyposialylated ApoC-III in glycogen storage diseases). Moreover, while studying the subcellular structure and various pathobiochemical aspects in fibroblasts from CDG patients, we made observations that had not been previously reported (e.g., the cellular accumulation of reactive oxygen species in CDG). We accomplished biochemical characterization and genetic diagnosis in more than 20 patients, and selected cases (RFT1-CDG, PGM1-CDG, MAN1B1-CDG, NgBR defect) were published, bringing novel phenotype and genotype findings.

Keywords: congenital disorders of glycosylation, CDG, screening, apolipoprotein C-III, transferrin

ACKNOWLEDGEMENTS

This PhD thesis was completed during the author's doctoral studies in biomedical sciences at the First Faculty of Medicine, Charles University in Prague, between years 2010-2016. The studies involved in this thesis were elaborated within projects supported by the following grants: Charles University institutional programs GAUK 638512, PRVOUK-P24/LF1/3, UNCE 204011, and SVV - UK 260256/2016; the project EXAM from European Regional Program Research and Development Fund – for Innovation Ministry of Education, Youth and Sports ExAM CZ.1.05./ 2.1.00/ 03.0124 (MSMT). Specific support was provided by grant NT12166-5/2011 from the Ministry of Health of the Czech Republic.

In the first place, I would like to thank my supervisor RNDr. Hana Hansíková, CSc. and Prof. MUDr. Jiří Zeman, DrSc. for the opportunity to participate in the research in Laboratory for Study of Mitochondrial Disorders, as well as for their guidance and advice throughout my studies. I would also like to express gratitude to our collaborating clinicians, especially to doc. MUDr. Tomáš Honzík, Ph.D., who often provided helpful critical insights. Last but not least, I would like to thank my colleagues from the laboratory for the friendly and encouraging working atmosphere, and my family and friends for their constant support.

TABLE OF CONTENTS

ABBREVATIONS 9
1 INTRODUCTION 14
1.1 Glycosylation 14
1.1.1 The biological importance of glycosylation 14
1.1.2 Regulation of glycosylation 19
1.1.3 Unfolded protein response 19
1.2 Congenital disorders of glycosylation: a general overview 21
1.2.1 Disorders of protein N-glycosylation 22
1.2.2 Disorders of protein O-glycosylation 23
1.2.3 Disorders of lipid and glycosylphosphatidylinositol anchor glycosylation 24
1.2.4 Disorders of (potentially) multiple glycosylation pathways 25
1.3 Diagnostic approach for CDG 28
1.3.1 Biochemical screening of congenital disorders of glycosylation 28
1.3.2 Further biochemical analyses 31
1.3.3 Genetic analyses 32
1.4 Therapy in CDG 33
1.5 Secondary glycosylation disturbances (of non-CDG etiology) 34
1.6 Glycosylation disorders: future perspectives 36
2 AIMS OF THE THESIS 37
3 MATERIAL AND METHODS 38
3.1 Material 38
3.1.1 Patients 38
3.2 Methods 39
3.2.1 Golgi labeling 40
3.2.2 PNA staining 40
3.2.3 In vivo ROS (reactive oxygen species) detection 40
3.2.4 Catalase staining 41
3.2.5 Transmission electron microscopy (TEM) 41
4 RESULTS AND DISCUSSION 42
4.1 Results and discussion related to the aim A.) 42
Screening methods for the detection of congenital disorders of glycosylation (CDG). ... 42
4.1.1 A rare transferrin mutation at the glycosylation site hampers the screening of CDG 43
4.1.2 A case of S-adenosylhomocysteine hydrolase deficiency clinically resembles PMM2-CDG. 44

4.1.3 A novel rapid diagnostic test for the diagnosis of MAN1B1-CDG from serum. 45
4.1.4 ApoC-III isoelectric focusing is a sensitive method for detection of O-glycosylation defects. 46
4.2 Results and discussion related to the aim B.) 48
Further biochemical and molecular characterization of CDG patients. 48
4.2.1 Siblings with novel mutations in RFT1 gene show a milder phenotype of RFT1-CDG. 49
4.2.2 A boy diagnosed with PGM1-CDG presents with a novel feature of neurological impairment. 50
4.2.3 A novel CDG type due to dolichol biosynthesis defect is caused by mutations in NGBR gene. 51
4.2.4 Altered subcellular structure and pathophysiology found in fibroblasts from CDG patients. 53
4.3 Results and discussion related to the aim C.) 61
Alterations in protein glycosylation of non-CDG etiology. 61
4.3.1 Prader-Willi patients with elevated triacylglycerols show ApoC-III hypoglycosylation. 62
4.3.2 Novel observation of ApoC-III hypoglycosylation in various glycogen storage diseases. 62
5 CONCLUSION 64
REFERENCES 66
SUPPLEMENT 84
Supplementary tables. 85
List of original articles (in chronological order) 91

ABBREVATIONS

A: adenine

ApoC-III: apolipoprotein C-III

ApoC-III$_0$: asialoapolipoprotein C-III

ApoC-III$_1$: monosialoapolipoprotein C-III

ApoC-III$_2$: disialoapolipoprotein C-III

ApoE: apolipoprotein E

AD: autosomal dominant

AR: autosomal recessive

Arg: arginine

Asn: asparagine

ATF4: activating transcription factor 4

ATF6: activating transcription factor 6

ATP: adenosine triphosphate

ATP6V0A2: component 2 of a V(0) domain in the vacuolar ATP-ase

BiP: binding immunoglobulin protein (also known as GRP78)

C: cytosine

Ca^{2+}: calcium ions

CCDC115: coiled-coil domain containing 115

CDG: congenital disorder(s) of glycosylation

CDP: cytidine diphosphate

CDT: carbohydrate-deficient transferrin

CHOP: C/EBP homologous protein

cis-PT: cis-prenyltransferase

CMP: cytidine monophosphate

CMP-NeuAc: cytidine-5′-monophospho-N-acetylneuraminic acid

CNX: calnexin

CO_2: carbon dioxide

COG: conserved oligomeric Golgi (subunits 1-8)

COPII: coat protein complex II

CRT: calreticulin

CTP: cytidine triphosphate

Cys: cysteine

DAPI: 4’,6-diamidino-2-phenylindole dihydrochloride (nucleic acid stain)

α-DG: α-dystroglycan

DHDDS: dehydrodolichyl diphosphate synthase subunit (also known as hCIT)

DHE: dihydroethidium

DMEM: Dulbecco's modified Eagles's medium

Dol-P: dolichol-phosphate

Dol-P-Man: dolichol-phosphate-mannose

eIF2α: eukaryotic translation initiation factor 2α

EOGT: epidermal growth factor domain-specific O-linked N-acetylglucosamine transferase

ER: endoplasmic reticulum

ERAD: endoplasmic reticulum-associated degradation pathway

ERp57: endoplasmic reticulum resident protein 57

FBS: fetal bovine serum

Fe^{3+}: ferric ions

Fru-1-P: fructose-1-phosphate

Fru(c)-6-P: fructose-6-phosphate

Fuc: fucose

G: guanine

GAG: glycosaminoglycans

Gal: galactose

Gal-1-P: galactose-1-phosphate

GalNAc: N-acetylgalactose

GalNAc-Ts: N-acetylgalactose transferases

GDP: guanosine diphosphate

GDP-Fuc: guanosine-5′-diphospho-fucose

GDP-Man: guanosine-5′-diphospho-mannose

Glc: glucose

Glc-6-P: glucose-6-phosphate

GlcA: glucuronic acid

GlcN: glucosamine

GlcNAc: N-acetylglucosamine

GlcNAc-1-P: N-acetylglucosamine-1-phosphate

Gln: glutamine

Gly: glycine

GM3: monosialodihexosylganglioside

GPI: glycosylphosphatidylinositol

GRP78: 78 kDa glucose-regulated protein (also known as BiP)

GSD: glycogen storage disease(s)

GTP: guanosine triphosphate

GWAS: genome-wide association study

H_2O_2: hydrogen peroxide

HCC: hepatocellular carcinoma

hCIT: human cis-isoprenyltransferase (also known as DHDDS)

HDL: high-density lipoproteins

HeLa: immortal cell line derived from cervical cancer (named after the donor Henrietta Lacks)

HFI: hereditary fructose intolerance

His: histidine

HPLC: high-performance liquid chromatography

Hyl: hydroxylysine

ICAM-1: intercellular adhesion molecule 1

IdoA: iduronic acid

IEF: isoelectric focusing

IF: impact factor

IgA(1): immunoglobulin A(1)

IgG: immunoglobulin G

IRE1: inositol-requiring protein 1

IQ: intelligence quotient

JNK: c-Jun N-terminal protein kinase

LAMP1: lysosomal-associated membrane protein 1

LDL: low-density lipoproteins

LLO: lipid linked oligosaccharide(s)

Lys: lysine

MALDI: matrix-assisted laser desorption ionization

Man: mannose

ManAc: N-acetylmannosamine

MAN1B1: α-1,2-mannosidase

Man-1-P: mannose-1-phosphate

Man-6-P: mannose-6-phosphate

Met: methionine

MPI: phosphomannose isomerase

mRNA: messenger ribonucleid acid

MS: mass spectrometry

M_w: molecular weight

nano-LC: miniaturized technique of liquid chromatography

NeuAc/Neu5Ac: N-acetylneuraminic acid (sialic acid)

NgBR: Nogo-B receptor

NGS: next generation sequencing

NGLY1: N-glycanase 1

NPC2: Niemann-Pick disease, type C2 protein

NRF2: nuclear respiratory factor 2

OGT: O-linked N-acetylglucosamine transferase

OST: oligosaccharyltransferase

P: phosphate

$p58^{IPK}$: protein kinase inhibitor of 58 kDa (also known as DNAJC3)

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PDI: protein disulfide isomerase

PERK: protein kinase RNA-like ER kinase

PFA: paraformaldehyde

PGM(1): phosphoglucomutase (1)

pI: isoelectric point

PMM(2): phosphomannomutase(2)

PNA: peanut agglutinin

PP_i: pyrophosphate

Pro: proline

PWS: Prader-Willi syndrome

QTOF: quadrupole time-of-flight

RA: rheumatoid arthritis

RFLP: restriction fragment length polymorphism

RFT1: protein RFT1 (requiring fifty three 1) homolog

RNA: ribonucleid acid

ROS: reactive oxygen species

RT: room temperature

SAA4: serum amyloid A4

SAHH: S-adenosylhomocysteine hydrolase

SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis

SEC23A, **SEC23B**: components of the COPII

Ser: serine

SLC35C1: solute carrier family 35 member C1

SLC39A8: solute carrier family 39 member 8

SNP: single nucleotide polymorphism

T: thymine

TAG: triacylglycerol(s)

TEM: transmission electron microscopy

Ter: translation termination codon

TF: transferrin

Thr: threonine

TMEM165: transmembrane protein 165

TMEM199: transmembrane protein 199

Trp: tryptophan

Tyr: tyrosine

UDP: uridine diphosphate

UDP-Gal: uridine-5′-diphospho-galactose

UDP-GalNAc: uridine-5′-diphospho-N-acetylgalactosamine

UDP-GlcA: uridine-5′-diphospho-glucuronic acid

UDP-GlcNAc: uridine-5′-diphospho-N-acetylglucosamine

UMP: uridine monophosphate

UPR: unfolded protein response

Val: valine

VLDL: very low density lipoproteins

vs: versus

XBP1: X-box binding protein 1

Xyl: xylose

1 INTRODUCTION

1.1 Glycosylation

Glycosylation is an abudant type of co- and post-translational modification of both proteins and lipids, with more than 50 % of the human proteome estimated to be glycosylated [1]. It comprises a covalent attachment of either a monosaccharide, oligosaccharide or polysaccharide moiety (the latter two are referred to as „a glycan“) in sequential steps, exerted by a complex molecular machinery of enzymes and other proteins that are involved in production of the glycosylation substrates (i.e., nucleotide-monosaccharides), glycan assembly, transfer of the activated monosaccharide or glycan onto polypeptide/lipid backbone, and further glycan modification. This process is localized in cytosol, nucleus, rough endoplasmic reticulum (ER) and Golgi apparatus, and, in most cases, is followed by secretion of the newly synthesized glycoproteins into extracellular space. Based on the type of glycosidic bond either through amide group (-NH-) of asparagine or hydroxy group (-O-) of threonine/serine/hydroxylysine, protein N- and O-glycosylation is distinguished, respectively. These two major classes will be the main focus of the thesis. Other types of mammalian protein glycosylation include C-mannosylation (C-C bond via Trp) and glypiation (attachment of glycosylphosphatidylinositol anchor) [2]. Lipid glycosylation yielding glycosphingolipids begins with stepwise addition of monosaccharides to ceramide, but shares some glycosylation enzymes with protein glycosylation. While the biosynthetic pathway for the addition of N-glycans is highly conserved and all N-glycoproteins share a common glycan core structure, a great variability exists in O-glycan composition and the corresponding enzymatic reactions to produce them; the schematic overview with a brief description is shown in Fig. 2a, b and Table 1. The major classes of the synthesized vertebrate glycan structures are depicted in Fig. 1.

1.1.1 The biological importance of glycosylation

Glycosylation produces an extremely diverse spectrum of glycoproteins and glycolipids that execute various biological functions such as cell-cell communication, immune response, signal transduction, have hormonal and enzymatic activity or help maintain structural integrity [3] (Fig. 3). In general, genetic modifications affecting the initial glycan biosynthesis steps are embryonically lethal in mice [4], demonstrating the critical role of glycosylation for survival. The presence of glycans promotes physicochemical properties (e.g., the correct conformation, stability, solubility) that are often necessary for acurrate

function of the glycoconjugates. Secondly, specific recognition of glycans by other molecules, either by intrinsic or extrinsic glycan-binding proteins, is an important mechanism mediating numerous ligand-receptor interactions (e.g., the clearance of serum glycoproteins by hepatic asialoglycoprotein receptor [5], leukocyte capture and rolling mediated by selectin-selectin ligand interactions [6], etc.) or might be exploited by pathogens for host cell infection [7]. Well established is the role of N-glycans in facilitating protein folding within a calnexin (CNX)-calreticulin (CRT) cycle [8], which is a part of ER quality control system. Briefly, monoglucosylated intermediate is recognized by lectin-chaperones CNX and CRT (bound to oxidoreductase ERp57 - endoplasmic reticulum resident protein 57), enhancing protein folding efficiency by preventing aggregation and mediating formation of disulfide bonds. This is coupled to a de- and reglucosylation cycle, retaining the glycoprotein within ER lumen until it acquires a proper conformation and can be transported to Golgi for further processing. If the glycoprotein fails to fold properly, it is directed to ER-associated degradation pathway (ERAD) and eventually gets degraded by proteasome.

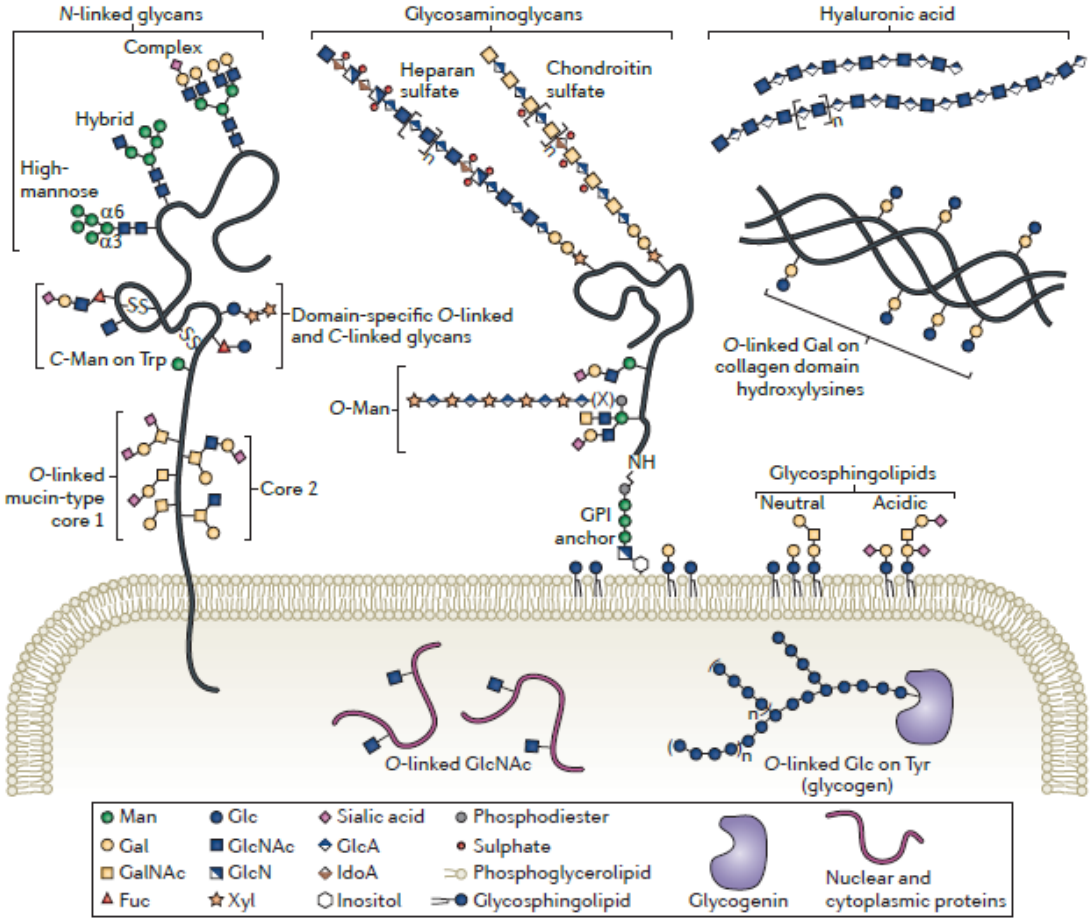

Fig. 1: The major classes of vertebrate glycan structures. Glycoconjugates, i.e. glycoproteins, proteoglycans and glycolipids, contain oligosaccharide chains (glycans) of various structures covalently bound to the protein or lipid. The picture schematically shows the structures of the most common glycans found in humans. Source: [9].

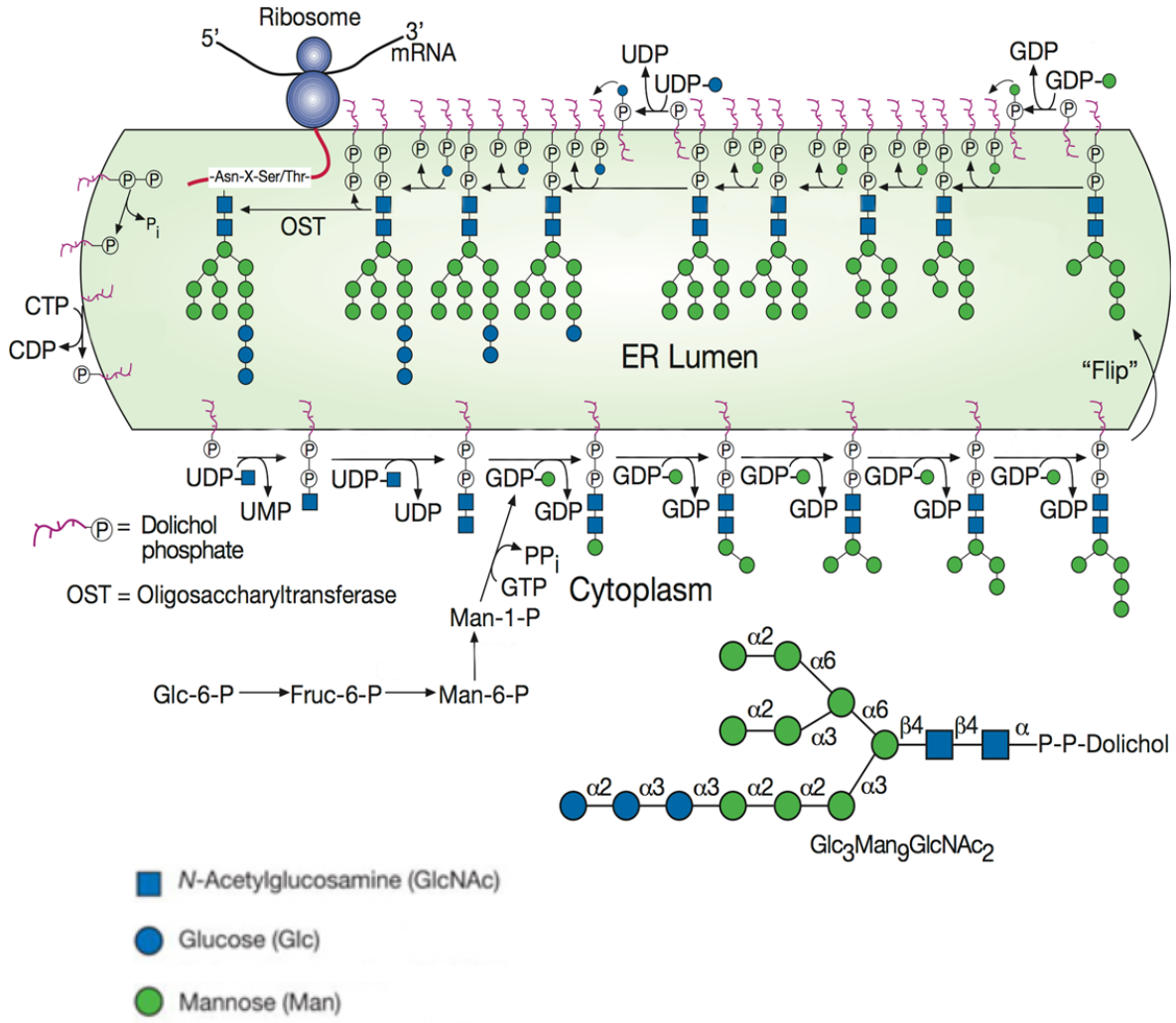

Fig. 2a: N-glycosylation, part 1: N-glycan assembly and its transfer onto nascent polypeptide. N-glycan assembly starts with the transfer of GlcNAc-1-P from cytosolic UDP-GlcNAc onto ER-membrane bound dolichol-P (Dol-P) to generate Dol-P-P-GlcNAc, which is extended by addition of another GlcNAc and, subsequently, five mannose residues (from GDP-Man), forming Dol-P-P-$GlcNAc_2Man_5$. This structure is then „flipped" across the ER membrane into its lumen, where further elongation of the glycan takes place: four more mannose units are added from Dol-P-Man and three glucose residues from Dol-P-Glc (both Dol-P-Man and Dol-P-Glc are first synthesized facing cytosol and then „flipped" into ER lumen to serve as sugar donors). This is followed by the transfer of the generated oligosaccharide $Glc_3Man_9GlcNAc_2$ from dolichol to asparagine of the newly synthesized polypeptide chain, catalyzed by the enzyme oligosaccharyltransferase (OST). Modified from: [4].

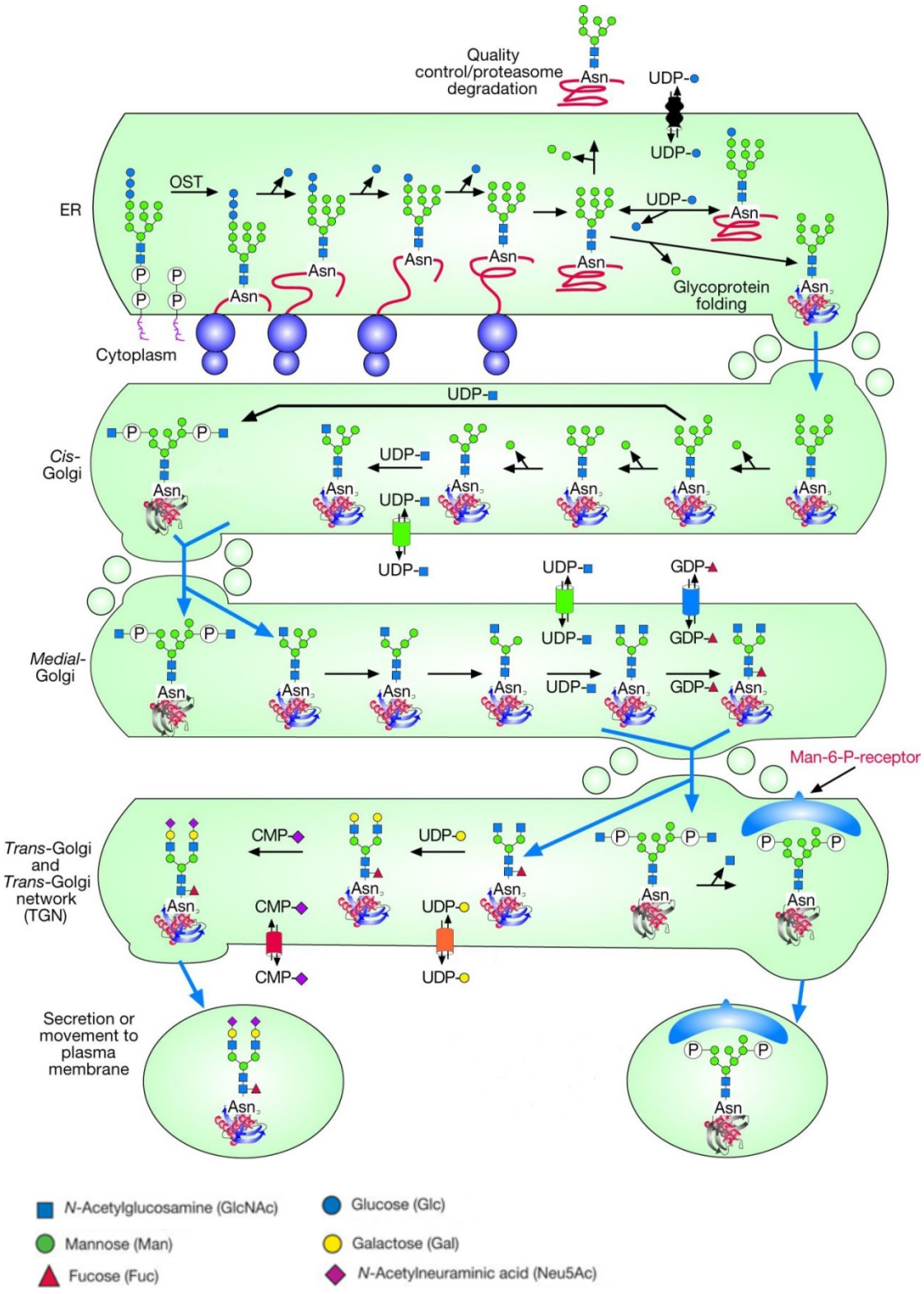

Fig. 2b: N-glycosylation, part 2: N-glycan processing in endoplasmic reticulum and Golgi. Attached to a polypeptide, the N-glycan is modified to $Glc_1Man_9GlcNAc_2$ before entering calnexin-calreticulin cycle. The correctly folded N-glycoproteins are then transported via vesicles to Golgi cisternae, where further processing occurs: mannose trimming, addition of N-acetylglucosamine, generation of the Man-6-P tag (lysosomal proteins), fucosylation, galactosylation and sialylation. Fully glycosylated proteins are eventually secreted to plasma membrane. Modified from: [4].

Table 1: Seven types of O-glycosylation based on the first monosaccharide attached.

The first monosaccharide	**Initiated in***	**Glycan structure** (source: [4, 9, 10])
O-linked GalNac (mucin-type) †	Golgi	(R)-GalNAcα1-Ser/Thr
O-linked Xyl (glycosaminoglycans)	Golgi	(R)-GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-Ser
O-linked GlcNAc	Cytosol, nucleus‡	GlcNacβ1-Ser/Thr
O-linked Gal	ER	(Glcα1-2)Galβ1-Hyl
O-linked Man	ER	(NeuAcα2-3)Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr
O-linked Glc	ER	(Xylα1-3Xylα1-3)Glcβ1-Ser or (R)-Glcα1-Tyr
O-linked Fuc	ER	Glcβ1-3Fucα1-Ser/Thr or (NeuAcα2-6Galβ1-4GlcNAcβ1-3)Fucα1-Thr/Ser

R – variable residue
*subcellular compartment where the addition of the first sugar takes place [11]
†the most frequent O-glycosylation type in humans
‡to a lesser extent also in mitochondria

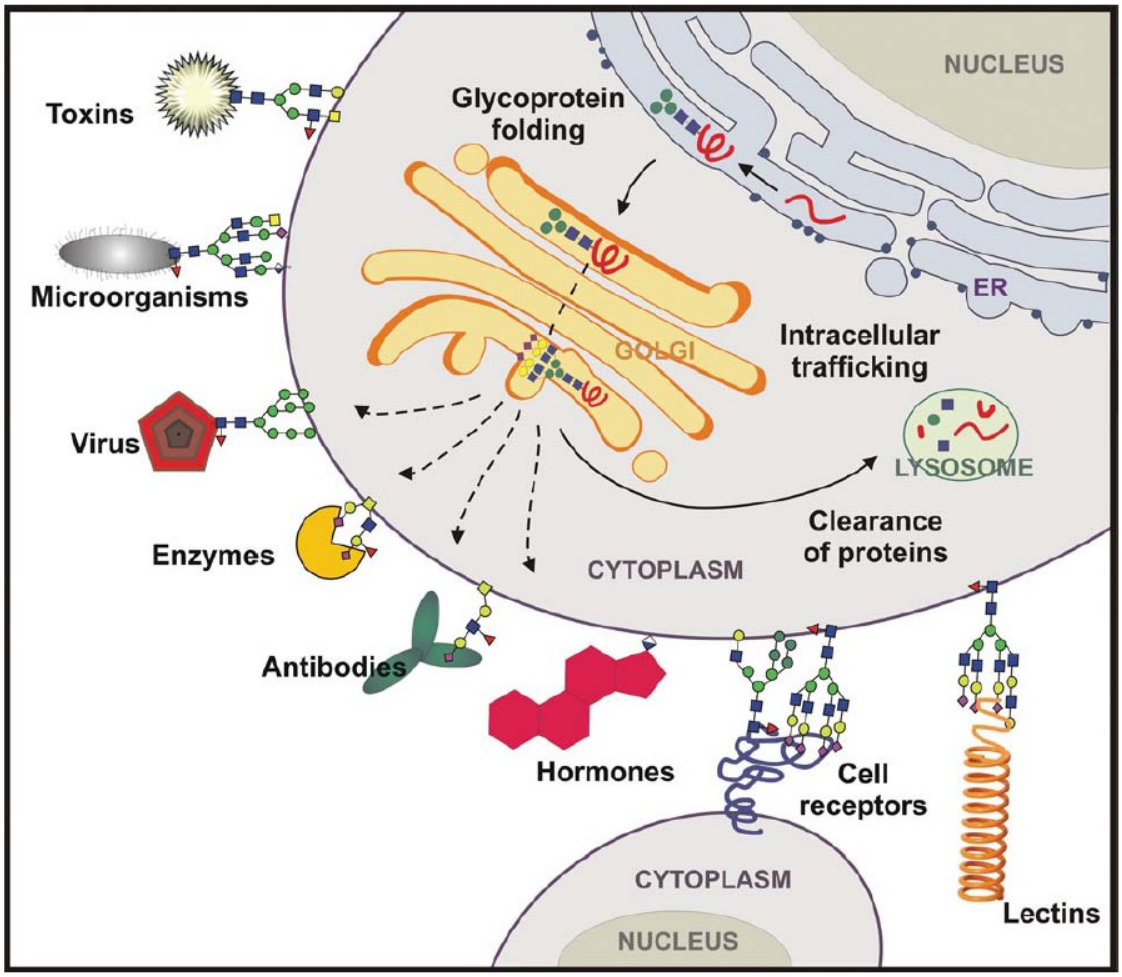

Fig. 3: Schematic representation of various biological functions that glycans participate in. Glycans facilitate protein folding, regulate intracellular trafficking, play role in host-pathogen interactions and determine the function of various proteins including enzymes, hormones, antibodies or receptors. Source: [12].

1.1.2 Regulation of glycosylation

Due to its critical physiological function and complexity, glycosylation is a dynamic and highly regulated process. It is modulated by many factors: the rates of polypeptide translation and protein folding, localization and cellular concentration of nucleotide-monosaccharides, localization of and competition between multiple glycosyltransferases/glycosidases, and membrane trafficking [13]. The precise mechanism of Golgi trafficking, including action of the resident glycosylation enzymes which show a polarized distribution, has been debated for a long time. The present evidence favours the so-called cisternal maturation model [14, 15]. This scheme postulates that after the newly synthesized and folded proteins leave ER in vesicles, they fuse and subsequently mature into Golgi cisternae stacks (progressing from cis, through medial to trans compartments, i.e. in the anterograde direction). While the cargo proteins remain in the cisternal lumen, the Golgi-resident glycosylation enzymes are transported in vesicles by retrograde trafficking and recycled, gradually modifying the passing cargo proteins, thus forming the final composition of the glycans. The expression of enzymes determining glycan structure is cell type-specific and developmentally modulated [16], and these physiological changes in glycosylation are functionally important for processes such as embryogenesis and cell activation [17, 18]. An enormous amount of data exists regarding glycosylation alterations in reponse to pathophysiological stimuli or in various disease conditions. The primary, genetically determined defects of glycosylation in humans comprise a group of rare diseases labeled as congenital disorders of glycosylation (CDG), which are reviewed in Chapter 1.2 and constitute the main topic of this thesis. Secondary changes in protein glycosylation (of non-CDG etiology) are then shortly described in Chapter 1.5.

1.1.3 Unfolded protein response

The characteristic oxidizing conditions and a high calcium concentration in the ER lumen provide a unique environment for efficient protein folding, which relies on the coordinated action of molecular chaperones (classical such as BiP/GRP78 (binding immunoglobulin protein/78 kDa glucose-regulated protein) or carbohydrate-binding, e.g. CNX or CRT; the activity of both classes is dependent on Ca^{2+}), oxidoreductases (protein disulfide isomerase family or PDIs, e.g. ERp57) and peptidyl-prolyl cis/trans isomerases [19]. A variety of physiological or pathological stimuli might disturb ER homeostasis, thus overwhelming ER protein-folding capacity and causing ER stress. These include calcium and redox imbalances, hypoxia, nutrient deprivation or diverse pharmacological compounds such as tunicamycin which blocks protein N-glycosylation by inhibiting the transfer of N-acetylglucosamine-1-

phosphate from UDP-N-acetylglucosamine to dolichol phosphate (the first step of N-glycan assembly; see Fig. 2a) [4]. To cope with the load of accumulated misfolded or unfolded proteins resulting from these perturbations, ER has evolved complex adaptive signalling pathways collectively termed „the unfolded protein response“ (UPR); see Fig. 4. UPR pathway has three branches, initiated by the stress sensors protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6). During physiological conditions, BiP chaperone is bound to these sensors, keeping them inactive. ER stress leads to its dissociation, due to interaction of BiP with exposed hydrophobic regions of the misfolded proteins, and subsequent activation of the three signalling arms. While the main function of the PERK pathway is attenuation of protein translation, IRE1 and ATF6 are mostly involved in upregulation of the UPR proteins, such as molecular chaperones, to increase protein-folding capacity. Upon unresolved, severe and prolonged ER stress, UPR induces apoptosis via two critical mediators c-Jun N-terminal protein kinase (JNK) and C/EBP homologous protein (CHOP) to protect organism by eliminating the damaged cells.

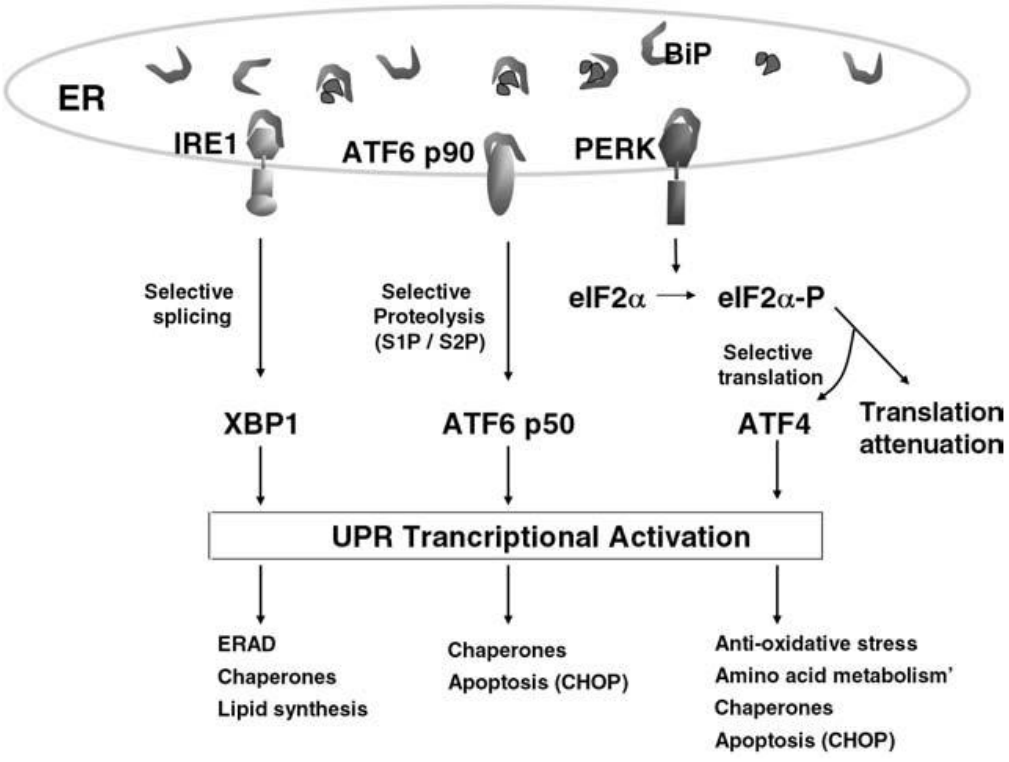

Fig. 4: Unfolded protein response. Under conditions of ER stress, BiP binds to unfolded proteins, what is accompanied by its release from IRE1, ATF6 and PERK. This leads to activation of these three main signal transducers of UPR and, subsequently, upregulation of their downstream targets, whose role is to increase protein-folding capacity and decrease the biosynthetic burden of ER. *BiP: binding immunoglobulin protein; IRE1: inositol-requiring protein 1; ATF6: activating transcription factor 6; PERK: protein kinase RNA-like ER kinase; XBP1: X-box binding protein 1; eIF2α: eukaryotic translation initiation factor 2α; ATF4: activating transcription factor 4.* Source: [20].

1.2 Congenital disorders of glycosylation: a general overview

The first reported case of a patient with a congenital disorder of glycosylation (CDG) dates back to the year 1984, when Jaeken et al. described a novel syndrome in twin sisters, who manifested with deficient sialylation of plasma and cerebrospinal fluid transferrin [21]. Later, the biochemical basis of this disease was identified as the phosphomannomutase 2 (PMM2) deficiency due to mutations in the *PMM2* gene [22]. In this disorder, the conversion of mannose-6-phosphate to mannose-1-phosphate is affected, a step necessary for the subsequent formation of the activated substrate GDP-mannose, which is utilized as a building block in glycosylation pathways. Using the analysis of serum glycoproteins, more subtypes of what was at the time called „carbohydrate-deficient glycoprotein syndrome“ have been discovered, and based on the transferrin profile from isoelectric focusing (see Chapter 1.3.1.1), they were divided into two groups: CDG types I and II. While type I pattern was suggestive of a defect in the first biosynthetic steps of N-glycan assembly, type II indicated impaired processing of N-glycoproteins occurring later in Golgi. The individual genetic defects were denoted by lowercase letters (e.g., CDG-Ia), whereas the unsolved cases were (and still are) labeled as CDG-Ix or CDG-IIx. Modern technologies (e.g., next-generation sequencing) significantly accelerated identification of new glycosylation defects, some of which could not be assorted using the original classification [23]. One such example is the defect of multiple glycosylation pathways and possibly also other biochemical processes caused by the deficiency of the ATP6V0A2 protein, a subunit of a vacuolar-type proton pump (v-ATPase), which is essential for acidification of Golgi and, thus, necessary for its proper function by maintaining pH homeostasis. Novel nomenclature was therefore accepted, using symbol for the causative gene plus the „-CDG“ suffix [24].

To this date, PMM2-CDG (formerly known as CDG-Ia) is the most frequent type of CDG with estimated prevalence of 1:20 000 [25], accounting for the majority of all of the diagnosed CDG patients. More than 100 types of CDG have been described up until now, which should not come as a surprise since it is predicted that a considerable part of the whole genome, approximately 1-2 %, is involved in glycosylation [26]. From the functional point of view, primary glycosylation disorders arise due to a defect in i) genes encoding glycosyltransferases; ii) genes involved in the biosynthesis of the donor substrates; iii) genes required for the translocation of the donor substrates; iv) genes regulating glycosyltransferases localization; and v) genes affecting Golgi milieu. Because of the ubiquitous character of glycoproteins and their diverse functions, CDGs generally manifest

as multi-system diseases. The clinical presentation typically begins in infancy and ranges from very mild to severe, with substantial childhood mortality (of approx. 25 % in PMM2-CDG [27]). Failure to thrive, dysmorphy, microcephaly, psychomotor retardation, hypotonia, ataxia, seizures, coagulopathy and hepatopathy are frequent features. In certain types, strabismus, inverted nipples and abnormal fat distribution might serve as a helpful clinical hint for CDG diagnosis. Patients often suffer from endocrine dysfunction, show growth retardation, urogenital abnormalities and impaired immunity with recurrent infections. Sometimes, a typical dermatological symptoms and skeletal deformities are present.

Usually, CDG syndromes are divided into categories according to the type of the glycosylation reaction affected (i.e., disorders of protein N- or O-glycosylation, lipid glycosylation and glycosylphosphatidylinositol anchor glycosylation, and defects in multiple glycosylation pathways), and they sometimes share a similar phenotype. Each of these groups will be shortly discussed below. The classification is not rigid, since the glycosylation reactions might overlap and affect each other, as is continuously being unraveled by new research studies. Because of the broadness of this family of metabolic diseases, a proper review covering every CDG type is not possible within the scope of this thesis. However, the list of CDG types reported up until now can be found in the Supplement: Tables Sa-d.

1.2.1 Disorders of protein N-glycosylation

Defects limited to N-glycosylation often present with intellectual disability, hypotonia and seizures, like the most frequent ALG6-CDG (the deficiency of α-1,3-glucosyltransferase 1) with at least 30 patients reported in literature [28]. Apart from the enzymes catalyzing stepwise attachment of the monosaccharides (GlcNAc, Man and Glc) to the growing dolichol-bound N-glycan in ER encoded by *DPAGT1* and *ALG 1*, *2*, *3*, *6*, *8*, *9*, *11-14* [29-39], other proteins might be affected such as those participating in flipping of the glycan from cytosol into ER lumen (in RFT1-CDG, the deficiency of $Man_5GlcNac_2$-PP-Dol flippase [40]; with sensorineural deafness as a typical feature in the patients) or different subunits of the oligosaccharyltransferase (OST) complex, which transfers the glycan onto nascent protein, e.g. in TUSC3-CDG or MAGT1-CDG [41], both manifesting as a non-syndromic intellectual disability. Additionaly, defects in enzymes modifying protein-bound oligosaccharide have been described, e.g. GCS1-CDG (the deficiency of α-1,2-glucosidase 1 [42]), MAN1B1-CDG (α-1,2-mannosidase deficiency [43]) or MGAT2-CDG (GlcNAc-transferase II deficiency), which was the first recognized CDG type II [44]. A special type of

CDG was recently discovered, NGLY1-CDG (the deficiency of N-glycanase 1 [45]), that is sometimes called a „disorder of deglycosylation". NGLY1 protein specifically deglycosylates the misfolded N-linked glycoproteins in cytosol and assists in their degradation within ERAD, which explains the finding of cytoplasmic accumulation of storage material in liver biopsy of the NGLY1-CDG patients [46]. Besides the common developmental delay and hypotonia, a distinct symptom, alacrima - absence of the secretion of tears - was noted as in the patients [46]. I-cell disease, albeit historically not classified as CDG but rather as a lysosomal storage disease (mucolipidosis II), is caused by defective GlcNAc-1-P transferase (*GNPTA* gene), whose action is critical for targeting lysosomal enzymes into lysosomes [47]. The patients show gingival hypertrophy, delayed development, hepatosplenomegaly, dysmorphism, and severe skeletal abnormalities [48].

1.2.2 Disorders of protein O-glycosylation

Defects in protein O-mannosylation are characterized by aberrant α-dystroglycan (α-DG) glycosylation, thereby receiving collective name „α-dystroglycanopathies". Glycosylation of α-DG is crucial for its binding to laminin, thus linking cytoskeleton to extracellular matrix in tissues such as muscle, brain, nerve and heart. Disruption of its function is reflected in the clinical manifestation of the patients, who typically suffer from muscle dystrophy and developmental brain and eye abnormalitiess. The severity of symptoms is variable ranging from mild limb girdle muscle dystrophy to severe, often lethal congenital malformations in Walker-Warburg syndrome, and it at least partly depends on the mutated genes. The most common defect is in fukutin-related protein (*FKRP* gene [49]), others involve fukutin (*FKTN* [50]), protein O-mannosyltransferases 1/2 encoded by *POMT1/POMT2* [51, 52], protein O-mannosyl-β-1,2-N-acetylglucosaminyltransferase 1 (*POMGNT1* [53]), N-acetylglucosaminyltransferase-like protein (*LARGE* [54]) or isoprenoid synthase domain containing (*ISPD* [55]). Dystroglycanopathy was also diagnosed in patients with mutations in *POMGNT2*, *TMEM5*, *B3GALNT2*, *POMK*, *B3GAT1* and *GMPPB* [56-61].

Defects in glycosaminoglycans (GAG) assembly are associated with skeletal and connective tissue disorders such as multiple hereditary exostoses (caused by mutations in *EXT1*/*EXT2* encoding exostosin glycosyltransferases 1/2 [62-64]) or different forms of Ehler-Danlos syndrome (e.g., due to mutations in *B3GALT6* and *B4GALT7* encoding galactosyltransferases that participate in GAG synthesis [65-67]). Similarly, phenotypes involving bone, skin and eye abnormalities result from defects in GAG sulfation (e.g., mutations in genes encoding various sulfotransferases, such as *CHST3*, *CHST6, CHST8* or

CHST14 [68-71]).

Reticular hyperpigmentation (Dowling-Degos disease) is a typical feature in autosomal dominant defects of *POFUT1* and *POGLUT1* genes, catalyzing protein O-fucosylation and O-galactosylation, respectively [72, 73]. Developmentally crucial Notch signalling is impaired in the defect of *LFNG* gene (coding Lunatic Fringe, which transfers N-acetylglucosamine to O-linked fucose on Notch receptors), leading to spondylocostal dysostosis with severe vertebral anomalies [74]. Elongation of O-linked fucose by adding a glucose residue on thrombospondin type 1 repeats is compromised in Peters Plus syndrome due to mutations in *B3GALTL* gene [75]. While epidermal growth factor (EGF) domain-specific O-linked N-acetylglucosamine (O-GlcNAc) transferase (EOGT) is defective in Adams-Oliver syndrome [76], mutations in nucleocytoplasmic O-GlcNAc transferase (OGT) have been very recently discovered to cause X-linked intellectual disability [77]. In mammals, there is a family of 20 N-acetylgalactose transferases (GalNAc-Ts) catalyzing the first step of mucin type O-glycosylation [11], however so far only one *GALNT* gene, *GALNT*3, has been shown to underlie disease in human, defined as familial tumoral calcinosis [78]. Mutations in *PLOD* gene, coding for an enzyme that hydroxylates Lys residues in collagens, indirectly lead to an O-galactosylation defect and manifest as Ehler-Danlos syndrome in the patients [79].

1.2.3 Disorders of lipid and glycosylphosphatidylinositol anchor glycosylation

GM3 synthase, an enzyme catalyzing the initial step of lactosylceramide conversion to complex gangliosides (lipid glycosylation), is affected due to *ST3GAL5* defect in Amish infantile epilepsy [80] and in salt and pepper syndrome [81], which is named due to the characteristic skin appearance. Mutations in *B4GALNT1* give rise to complex hereditary spastic paraplegia [82]. Neurological phenotype present in all the disordes above only stresses the critical role of glycans for the function of brain, where most of them are found in the form of glycosphingolipids [83].

11 defects in glycosylphosphatidylinositol (GPI) anchor glycosylation have been described up until now, the majority genetically characterized in the last 5 years. They are caused either by a defect in proteins involved in ER-localized biosynthesis of GPI-anchor (encoded by *PIGA*, *PIGY*, *PIGL*, *PIGW*, *PIGM*, *PIGV*, *PIGN*, *PIGO*, *PIGT* [84-93]) or disrupted lipid remodeling steps of GPI-anchor maturation in Golgi (*PGAP2*, *PGAP3* [94, 95]). The major symptoms of the patients include intellectual disability, epilepsy and multiple congenital

anomalies; laboratory finding of hyperphosphatasia is also common. Interestingly, *PIGA* defect (GlcNAc-PI synthesis protein deficiency) has been found with both somatic and X-linked inheritance, presenting as complement-mediated hemolysis (paroxysmal nocturnal hemoglobinuria [84]) or clinically heterogeneous spectrum of disorders including severe phenotype with multiple congenital anomalies and infantile lethality, respectively [85, 96].

1.2.4 Disorders of (potentially) multiple glycosylation pathways

Donor substrates for the glycan assembly include 11 building blocks, 9 out of which are nucleotide-activated and two are dolichol-phosphate linked monosaccharides; besides their utilization in protein/lipid glycosylation, their metabolism is connected with other biochemical pathways such as glycolysis/gluconeogenesis, glycogen metabolism, nucleotide metabolism or pentose phosphate pathway. Defects in the biosynthesis of donor substrates represent the great majority of patients diagnosed with CDG, predominantly the already mentioned PMM2 deficiency (defect of Man-6-P to Man-1-P conversion). In terms of the onset of the disease and its clinical presentation, three types are distinguished: infantile multisystem, late-infantile and childhood ataxia-intellectual disability, and adult stable disability [97]. PMM2-CDG patients show psychomotor retardation, axial hypotonia, cerebellar hypoplasia, they often have failure to thrive, dysmorphy, strabismus or excessive subcutaneous fat. Seizures and stroke-like episodes might occur, and adults exhibit impaired sexual development. Unfortunately, the current therapy consists of only management of the symptoms, but prenatal genetic counselling is available. It is noteworthy that based on the outcome of at-risk pregnancies, even though the autosomal recessive inheritance manner would suggest 1/4 risk of having an affected child, in reality it is closer to 1/3 [97]. Production of Man-6-P from Fru-6-P is defective in MPI-CDG (phosphomannose isomerase deficiency [98]), a relatively frequent CDG type which lacks the neurological involvement present in most CDG syndromes and mainly manifests as hepatic-intestinal disease (protein-losing enteropathy). Other examples of glycosylation defects due to reduced substrate availability result from pathogenic mutations in *PGM1* [38, 99] and *PGM3* [100] (phosphoglucomutase 1 and 3 deficiencies). While the first one has a characteristic hyposialylated transferrin pattern and a recognizable clinical phenotype (bifid uvula, growth retardation, myopathy, cardiomyopathy [101]), the latter manifests as immunodeficiency with neurocognitive impairment (plus skeletal dysplasia in some cases [102]) and shows normal transferrin glycosylation [100]. Intriguingly, PGM1-CDG also belongs to the group of glycogen storage diseases (GSD), demonstrating that there might be a blurred line in diagnosis of CDG. Some authors even consider GSD type Ib a glycosylation disorder (CDG),

as the detected aberrant neutrophils' glycosylation is predicted to profoundly contribute to neutrophil dysfunction characteristic for this disease [103]. *GNE* gene encodes UDP-GlcNAc-2-epimerase/ManAc kinase, catalyzing the rate-limiting step in the biosynthesis of sialic acid. Its defect leads to inclusion body myopathy (quadriceps-sparing proximal and distal muscle weakness; autosomal recessive disorder) [104] and sialuria with dominant inheritance [105].

Abnormal biosynthesis of dolichol and dolichol-P-mannose have also been identified to underlie glycosylation disorders, and result from mutations in *SRD5A3*, *DHDDS*, *DOLK* and *DPM1-3* [106-111]. The symptoms range from retinis pigmentosa to intellectual disability with hypotonia and various malformations. Dol-P-Man produced by Dol-P-Man synthase complex (encoded by *DMP1-3*) is utilized in N-glycosylation, GPI-anchor biosynthesis and O-mannosylation, integrating disorders of N-glycosylation, GPI-anchor glycosylation disorders and α-dystroglycanopathies. Analogically, MPDU1-CDG (ichthyosis syndrome) is caused by the defect in *MPDU1* encoding enzyme required for utilization of Dol-P-Man in these glycosylation pathways [112].

Another family of disorders potentially affecting multiple glycosylation pathways include deficiencies of Golgi transporters of activated monosaccharides: GDP-Fuc, CMP-NeuAc, UDP-Gal and UDP-GlcNAc (encoded by *SLC35C1*, *SLC35A1*, *SLC35A2* and *SLC35A3* [113-117], respectively). The patients have different clinical features, but intellectual disability/autism and seizures are usually present; other symptoms perhaps reflect the importance of the implied monosaccharide for cellular function, e.g., the role of fucose for immunity as demonstrated by susceptibility for infections in SLC35C1-CDG. Decreased transport of UDP-GlcA and UDP-GalNAc from cytosol to ER due to mutations in *SLC35D1* (Schneckenbecken dysplasia [118]) leads to defective chondroitin sulfate biosynthesis (GAG assembly defect) and possibly other glycosylation pathways.

The last group of defects is related to Golgi structure, function (trafficking) and milieu disturbances. Conserved oligomeric Golgi (COG) complex is a heteromeric complex constituting of 8 subunits, and it is important for proper glycosylation in Golgi by tethering transport vesicles to its membranes, thus determining the correct localization of Golgi glycosylation enzymes [119-121]. Up until now, the deficiencies of all but one (COG3) subunits have been identified to underlie human diseases [122-128]. Interestingly, a study which addressed the differential effects of lobe A (COG1-4) and lobe B (COG5-8) on the

stability of selected glycosylatransferases in HeLa cells showed that while lobe A depletion (COG3 knockdown) resulted in dramatic changes of Golgi structure, lobe B depletion (COG7 knockdown) severely altered localization and steady-state level of the analyzed enzymes [129]. The most severe manifestations (severe neurological impairment, infantile lethality) have been observed in COG(6,7,8)-CDG, while COG(1,2,4,5)-CDG usually showed milder phenotypes, even though it seems rather dependent on how the mutations affect overall COG functionality than on the subunit itself. Coat protein complex II (COPII)-coated vesicles transport secretory proteins from ER to Golgi, and mutations affecting their essential components SEC23A and SEC23B have been described to cause cranio-lenticulo-sutural dysplasia and congenital dyserythropoietic anemia type II, respectively [130, 131]. Golgi trafficking is also affected in achondrogenesis type 1A due to defect of *TRIP11* [132]. On the other hand, Golgi milieu is disrupted in the deficiencies of ATP6V0A2, TMEM165, SLC39A8, TMEM199 and CCDC115 [133-137]. ATP6V0A2 is a subunit of ATP-dependent proton pump essential for acidification of membrane enclosed organelles including Golgi apparatus, and the lack of its activity in *ATP6V0A2* defect leads to N- and O-glycosylation abnormalities observed in the patients [133, 138]. These show a distinct phenotype of cutis laxa (loose skin) or wrinkly skin syndrome, with hyperlaxity of the joints, dysmorphy and varying neurological involvement [138]. The exact mechanism for hypoglycosylation in ATP6V0A2-CDG has not yet been definitely elucidated, however some studies have confirmed a critical role for Golgi pH gradient in determining correct distribution of glycosyltransferases, where even a minor change in pH caused their mislocalization [139, 140]. The major symptoms in the patients with the deficiency of TMEM165 include psychomotor retardation, short stature, dysmorphy and profound skeletal dysplasia [134, 141]. While first it was suggested that TMEM165 participates in calcium/proton transport [142], the latest studies provide evidence that the glycosylation defect caused by its deficiency results from disrupted manganese homeostasis in Golgi [143]. The pathobiochemical mechanism is presumably similar to the one implied in the deficiency of SLC39A8, a transporter responsible for manganese uptake into the cell, where the function of manganese-dependent enzymes, such as β-1,4-galactosyltransferase, is impaired [135].

1.3 Diagnostic approach for CDG

Due to non-specifity and extreme variability of the clinical manifestations, CDG should be suspected by clinicians (mostly pediatricians) in any patient suffering from unexplained symptoms with multi-organ involvement. Coagulopathy, characterized by low serum coagulation factors VIII, IX, XI, XIII, antithrombin III, protein C or protein S, is a common sign and might point to a glycosylation disorder. Other laboratory findings from routine blood panels that have been reported in multiple CDG patients include hypoglycemia, elevated transaminases, hypoalbuminemia, hypocholesterolemia and increased creatine kinase. To selectively search for a glycosylation defect, the first choice of test and a gold standard used in CDG screening is the analysis of serum markers, N- and O- glycoproteins, transferrin (TF) and apolipoprotein C-III (ApoC-III).

1.3.1 Biochemical screening of congenital disorders of glycosylation

1.3.1.1 Isoelectric focusing of transferrin

Human transferrin (TF) is an abundant 79 kDa liver-derived serum N-glycoprotein, whose main biological function is the transport of iron and its delivery to target cells. It carries two N-linked bi- or tri- (with minor fraction of tetra-) antennary glycans of the complex type, and each antennae terminates with negatively charged sialic acid residue [144]. Up to 7 isoforms of sialylated TF are observed in serum (asialo- to hexasialo-), and their relative distribution can be investigated by isoelectric focusing (IEF) with immunofixation, which is used in CDG screening. Whereas tetrasialo- is the predominant form in physiological conditions (> 50 % of total TF), various CDGs affecting protein N-glycosylation show pathological pattern characterized by relative decrease of the fully glycosylated isoforms (tetra- to hexa-), accompanied by relatively increased levels of tri-, di-, mono- and asialotransferrin [145]; see Fig. 5. Alternatively, TF microheterogeneity might be addressed using different methods, e.g. analysis by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), high-performance liquid chromatography (HPLC), capillary electrophoresis or mass spectrometry (MS) [146-150]. Additional serum glycoproteins such as thyroxine-binding globulin, α-1-antitrypsin, haptoglobin or antithrombin III can be analyzed to confirm globally reduced N-glycosylation [146, 151, 152].

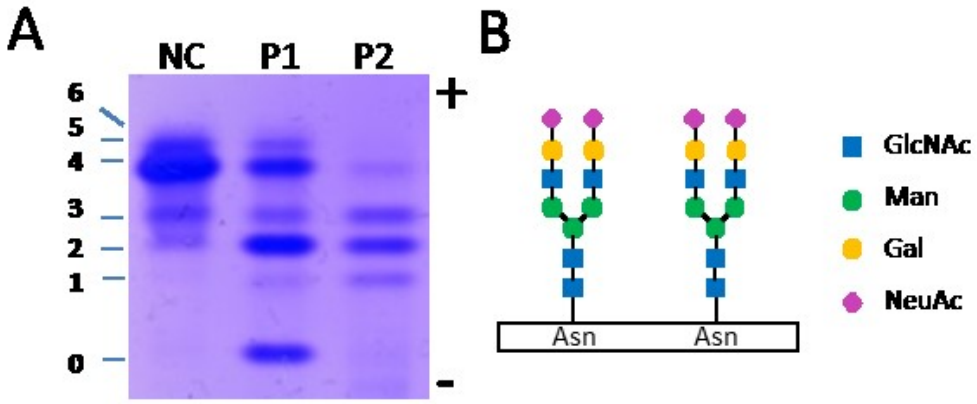

Fig. 5: Analysis of serum transferrin sialylation by isoelectric focusing. A] Sera from a negative control (NC; a healthy individual) and patients with glycosylation disorders affecting N-glycosylation (P1, P2) were separated by isoelectric focusing (pH 5-7), transferrin was labeled by immunofixation and stained by Coomassie. In both patients, there is a marked relative decrease of fully sialylated (most notably tetrasialo-) transferrin. While P1 shows a CDG type I transferrin (TF) pattern, i.e. a relative increase of di- and asialoTF typical for disorders of N-glycan assembly in ER, the relative increase of tri-, di- and monosialoTF in P2 is characteristic for CDG type II with impaired N-glycan processing in Golgi. *Numbers on the left indicate sialic acid content (asialo- to hexiasialo-) of the individual transferrin isoforms*. **B]** Monosaccharide composition of the most dominant transferrin glycan form under physiological conditions (tetrasialoTF).

In the pre-analytical phase of IEF TF, the serum samples are saturated with iron to prevent misinterpretation of the results due to varying Fe^{3+} load. To exclude the contribution of pI-altering TF polypeptide variants to the pathological profile, IEF analysis is repeated after neuraminidase treatment of samples. Regarding sex- and age-dependent differences of the relative amounts of sialylated TF isoforms, no statistically significant changes were found between males and females, neither between age categories within the age interval 2-18 years [145]. However, the experience of CDG experts has shown that hypoglycosylated TF might be observed even in healthy newborns and normalization of their profile can take up to several weeks (personal communication, CDG Orphan Course, Nijmegen, 2013). On the contrary, normal TF glycosylation was detected in affected fetus (at 19 weeks of gestation), proving CDG screening from fetal blood is not possible [153]. Pathological TF profile was also found in various non-CDG conditions such as galactosemia, hereditary fructose intolerance (HFI), severe liver disease or alcohol abuse [154-156]; see Chapter 1.5. All the factors above need to be considered in CDG screening evaluation.

1.3.1.2 Isoelectric focusing of apolipoprotein C-III

To search for disturbances in O-glycosylation, the analysis of serum apolipoprotein C-III (Apo C-III) by IEF and Western blot was introduced as a complementary test to IEF TF [157]. ApoC-III is a core 1 mucin type O-glycoprotein of approx. 9 kDa, synthesized predominantly in liver. It acts as an inhibitor of lipoprotein and hepatic lipases, i.e. of VLDL or LDL clearance. ApoC-III contains one O-linked glycan attached to its polypeptide chain, and three sialylated isoforms can be observed by IEF analysis: asialo-, monosialo- and disialoApoC-III; see Fig. 6.

Aberrant ApoC-III sialylation, defined by relatively decreased disialo- with relatively increased monosialo- and/or asialoApoC-III, has been detected in some of the disorders of multiple glycosylation pathways, such as the deficiencies of COG subunits or *ATP6V0A2* defect. Similarly to TF, ApoC-III sialylation is influenced by various factors, such as age or certain pathophysiological conditions (e.g., the acute phase of hemolytic uremic syndrome) [158]. Interestingly, not only the level of ApoC-III, but also its glycosylation status is responsive to biochemical alterations associated with metabolic syndrome [159, 160]. This is all discussed in more detail in Chapters 1.5, 4.1.4 and 4.3.2.

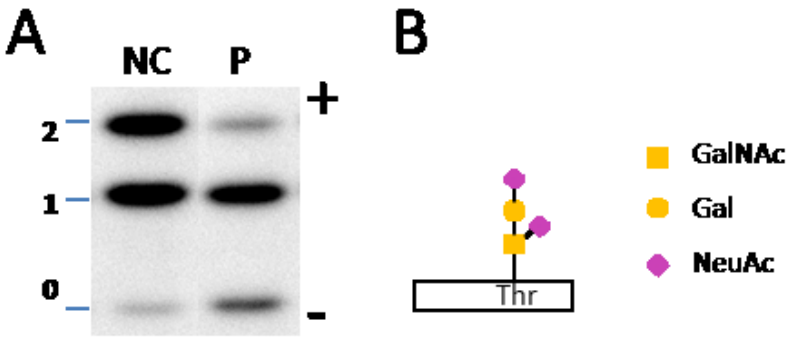

Fig. 6: Analysis of serum apolipoprotein C-III sialylation by isoelectric focusing. A] Sera from a negative control (NC; a healthy individual) and a patient with glycosylation disorder affecting mucin O-glycosylation (P) were separated by isoelectric focusing (pH 3.5-5), proteins were blotted onto nitrocellulose membrane and immunodetected using antibody to apolipoprotein C-III. While di- and monosialoApoC-III are the most abundant forms with approximately 1:1 ratio in healthy controls, the patients show variably decreased di- and increased mono- and/or asialoApoC-III (relative to total ApoC-III). *Numbers on the left indicate sialic acid content (asialo-. monosialo-, disialo-) of the individual apolipoprotein C-III isoforms*. **B]** Monosaccharide composition of the fully glycosylated glycan (disialoApoC-III).

1.3.2 Further biochemical analyses

After a pathological type I pattern is detected using IEF TF, enzyme activities of phosphomannomutase (PMM) and phosphomannose isomerase (MPI) are first measured in leukocytes (preferentially [161]) or fibroblasts of the patients, as these are amongst the most common CDG types. If PMM and MPI activities are normal, the next step is lipid linked (i.e., dolichol-bound) oligosaccharide (LLO) analysis. Briefly, fibroblasts are metabolically labeled, LLO's are extracted and released oligosaccharides are then analyzed by HPLC. Noteworthily, study in ALG11-CDG fibroblasts suggests that prior to metabolic labeling, the patients' cells need to be starved in low glucose (0.5 mM) medium for pathological LLO pattern - accumulation of shortened intermediates - to appear, otherwise the defect might be masked [162]. For CDG type II, further characterization depends on mass spectrometry (with MALDI as the preferred ionization method) analysis of the structure of N- and O-glycans, isolated from specific glycoproteins or whole plasma/serum [163-165]. Recently, a rapid and sensitive method, especially suitable for medical diagnostic setting, was developed by van Scherpenzeel et al. [149], who employed nanoLC-chip [C8]-QTOF MS for protein specific glycoprofiling of intact transferrin, allowing for a diagnosis of a number of CDG subtypes in a single assay. Additionaly, other approaches might be applied in unsolved CDGs, such as staining/fluorescent-labeling techniques using lectins or specific antibodies. Labeling with fluorescently tagged peanut agglutinin (PNA) lectin, which binds to non-sialylated core 1 O-glycans [166], is used to reveal hypoglycosylation in fibroblasts from patients with combined N- and O-glycosylation disorder such as the deficiency of COG subunits [122]. A functional test to detect the retrograde trafficking delay characteristic for COG deficiency can be performed by immunocytochemical analysis using antibody to a Golgi membrane protein (e.g., giantin) after incubating the cells with fungal toxin brefeldin A, which induces extensive retrograde transport of Golgi components to ER [167]. The group of α-dystroglycanopathies show reduced staining for α-dystroglycan using glycan-epitope recognizing antibodies in immunohistochemical analyses of the muscle biopsies. Moreover, the binding of α-DG to laminin-2 is compromised, what can be detected by Western blot using laminin overlay assay [168]. When a suspicion is narrowed down to a specific CDG type by combining clinical and laboratory findings, selected diagnostic tests might be performed according to the character of the individual defect; e.g., the suspected enzyme activity measurement, analysis of plasma polyprenols (increased in SRD5A3-CDG [169]), detection of oligosaccharides in urine (a specific tetrasaccharide is found in GCS1-CDG [42]), assessment of neutrophil adhesion (deficient in SLC35C1-CDG [170]), analysis of GPI and GPI-anchored proteins expression in granulocytes (decreased in GPI anchor

glycosylation defects [171, 172]), etc. However, cases differ and while some patients show certain biochemical abnormalities, others with the same CDG type might have borderline/normal results. Thus, diagnosis confirmation at the genetic level is always necessary. It is also important to note that most of the above analyses are done on a research basis, and because of the broad spectrum of CDG defects and the need for variety of tests, they often require a colaboration with other laboratories that provide the needed technical equipment and expertise.

1.3.3 Genetic analyses

Most of CDG types are inherited in an autosomal recessive manner, however there are a few exceptions with an X-linked inheritance (e.g., MAGT1-CDG, ALG13-CDG), autosomal dominant inheritance (e.g., hereditary multiple exostoses caused by mutated *EXT1* gene) or somatic inheritance (e.g., Tn-Syndrome with mutations in *COSMC*). De novo mutations have also been identified [116, 173]. When clinical picture and laboratory analyses provide substantial evidence for a known defect in a specific gene, Sanger sequencing of the candidate gene is performed in the proband and their relatives, respectively. This might be the case of e.g. ATPV6V0A2-CDG, which presents with specific phenotype (cutis laxa) and biochemical analyses show combined N- and O-glycosylation abnormalities [174], or PMM2-CDG after finding positive IEF of TF (type I pattern) and reduced PMM enzymatic activity. PMM2-CDG is also, as the most prevalent CDG, the best studied in terms of genotype-phenotype correlations. Majority (> 80 %) of the detected mutations in *PMM2* are missense, and the patients are usually compound heterozygotes [175]; homozygosity is likely incompatible with life [176]. In PMM2-CDG individuals of European ancestry, approx. 40 % carry a pathogenic variant p.Arg141His. Generally, all patients, regardless of their genotypes, exhibit the typical clinical features (i.e., developmental delay, cerebellar atrophy, peripheral neuropathy etc.), however some variants have been recognized to be associated with milder or more severe phenotypes [177-179].

In individuals, in whom there is no clear hint suggestive of a defined CDG type, multi gene panel (next generation sequencing, NGS) can be performed as a cost effective alternative to single gene testing. Using NGS, the researchers might choose between either genome or exome sequencing, or a (more specific) disease-targeted panel with selected genes. Exome sequencing approach was successfully applied for identification of e.g. a *PIGV* defect in hyperphosphatasia mental retardation syndrome [90] or *DHDDS* mutations in patients with retinis pigmentosa [107]. Limitations of NGS methods include uncomplete and uneven

gene/exon coverage, a nonquantitative character (it is problematic to detect deletions/duplications), as well as the possibility of "wrong hits" - thus, the following functional studies are always necessary to confirm the gene defect as causative. Other methods for identifying the genetic basis of CDGs, albeit perhaps not as powerful as NGS, have been used historically, such as homozygosity mapping to discover the *FKTN* defect in patients with Fukuyama-type congenital muscular dystrophy [50] or comparative genome hybridization to detect the defect of *B3GALTL* gene underlying Peters Plus syndrome [75].

1.4 Therapy in CDG

For the majority of CDGs, the treatment is, currently, purely symptomatic. In general, it is important to maintain proper nutrition and caloric intake, which in some cases requires the use of nasogastric or gastrostomy tube feeding. Depending on the specific conditions present, some patients will benefit from antiepileptic drugs, hormonal replacement therapy, physio-, speech, language and occupational therapy or various surgical procedures (e.g., to correct ophthalmological or skeletal abnormalities). There are four CDG types, for which more or less effective treatment was developed: MPI-CDG, SLC35C1-CDG, PIGM-CDG and PGM1-CDG. In individuals with MPI-CDG, oral ingestion of mannose has been shown to normalize their protein-losing entheropathy and coagulation abnormalities, as well as the TF hypoglycosylation [98, 180]. Nevertheless, in some cases a progressive liver disease developed despite the mannose therapy [181, 182]. A successful liver transplantation was performed in one therapy-resistant MPI-CDG patient, whose condition dramatically improved afterwards [182]. Mannose administration was also tested in PMM2-CDG, unfortunately no clinical improvement was noted [183]. However, the research studies to find a novel therapy approach continue and some show promising results, such as prenatal mannose treatment in mouse PMM2-CDG models [184] or chemical inhibition of MPI enzymatic activity (to divert Man-6-P towards glycosylation) in PMM2-CDG fibroblasts [185]. Defective function of GDP-fucose transporter in SLC35C1-CDG (also known as LAD II) can be partially corrected by oral fucose supplementation, which led to reduced peripheral neutrophil counts in the subjects studied by Marquardt et al. [186]. However, not all of the patients are responsive to this therapy, as it is dependent on the mutations and the associated residual activity of SLC35C1 [187]. A striking improvement was observed in a girl with PIGM-CDG (PIGM transfers the first mannose from dolichol-phosphate-mannose to phosphatidylinositol) after sodium phenylbutyrate therapy, which restored her GPI biosynthesis by modulating histone acetylation and the patient could walk again, interact and

became seizure-free [188]. The most recent is the therapeutic strategy designed for patients with PGM1-CDG (phosphoglucomutase 1 deficiency), a glycosylation disorder that was discovered just a few years ago, but might become one of the most frequent CDG types with already more than 19 patients described worldwide. Analyses in the patients' fibroblasts revealed a relative decrease of the activated galactose (UDP-Gal), which was in line with the hypogalactosylation of serum glycoproteins detected by mass spectrometry [101]. Encouraging experiments in PGM1-deficient cell lines cultivated in the presence of galactose were followed by a trial in the patients, who showed variable alleviation of the symptoms after a few weeks of daily galactose ingestion [189]. The latest observational study in 10 PGM1-CDG patients, who received oral galactose supplementation for 18 weeks, shows restoration of liver function (transaminanses values), coagulation abnormalities and improvement of endocrine status as well as cessation of rhabdomyolitic episodes [190].

1.5 Secondary glycosylation disturbances (of non-CDG etiology)

Various non-CDG diseases present with more or less specific changes in glycosylation pattern as a secondary phenomenom, even though their contribution to the pathophysiology is often unclear. The most extensively studied example is cancer, where aberrant glycan structures - typically with increased branching, sialylation and fucosylation - are a universal feature in multiple cancer types, and result from down- and up-regulation of a subset of genes involved in glycan synthesis [4]. One such example is hepatocellular carcinoma (HCC), where elevation of fucosylated glycoproteins, including transferrin, and its abnormal branching was detected in sera from the patients [191, 192]. Moreover, significant correlations are observed between glycosylation status and tumor progression, and it is currently well established that altered tumor-cell glycosylation promotes metastasis [193]. For instance, liver cirrhosis progression to HCC was found to be accompanied by increased degree of haptoglobin fucosylation [194].

Historically, the analysis of transferrin glycosylation or the so-called carbohydrate-deficient transferrin (CDT) test was originally applied to detect excessive alcohol (ethanol) consumption and it has been used for almost 40 years now [156]. Elevated CDT (i.e., reduced transferrin glycosylation) in alcoholic patients was explained by the reduced activity of Gal- and GlcNAc- transferases measured in their serum [195], and the inhibitory effect of acetaldehyde on the activity of hepatic glycosyltransferases was confirmed by experiments in rats [196].

Different autoimmune and inflammatory diseases manifest with complex glycosylation abnormalities, what was first observed in patients with rheumatoid arthritis (RA) who showed increased agalactosylated serum immunoglobulins G (IgGs) [197]. Variable changes in glycosylation, predominanty studied on main serum proteins (alpha1-acid glycoproten, IgG, IgA, transferrin, haptoglobin, C-reactive protein), were also found in ankylosing spondylitis, systemic lupus erythematosus, Sjögren syndrome or ulcerative colitis [198]. Altered protein glycosylation in RA involves galactosylation, fucosylation, mannosylation, sialylation and branching, and its extent is associated with the disease activity [199]. In primary IgA nephropathy, aberrant O-linked glycosylation of IgA1 is a consistent finding [200]. Moreover, alterations in serum glycosylation have been reported in acquired, common diseases associated with metabolic „derangement“. Significant differences in N-glycan composition were detected by MS analysis of serum from patients with type 2 diabetes compared to healthy controls, and specific N-glycan structures strongly correlated with parameters of metabolic syndrome [201]. In other study with the aim to analyze apolipoprotein composition in patients with metabolic syndrome, the authors found reduced glycosylation of ApoC-III, as well as hypoglycosylation of ApoE and SAA4 [160].

Changes in serum glycosylation were also documented in monogenic disorders other than CDGs, namely HFI (fructose-1-phosphate aldolase deficiency) and galactosemia (galactose-1-phosphate uridyltransferase deficiency) [155, 202]. The CDG type I-like pathologic IEF profile of transferrin seen in untreated frutosemia [155] can be explained by the inhibitory effect of excess Fru-1-P on MPI [203]. N-glycans of serum transferrin in untreated galactosemic patients are deficient in galactose and sialic acid, as well as exhibit increased branching and fucosylation [154, 204]. The reason for TF hypoglycosylation is unclear, but thought to be either inhibition of galactosyltransferase by the accumulated Gal-1-P or decreased availability of the substrate UDP-Gal [154, 205]. Moreover, global changes in both N- and O-glycome in plasma from the patients were detected by MS [206]. Since in both disorders the transferrin pattern normalizes with therapy (galactose- or fructose-free diet), it can be used as a marker of treatment efficiency [207].

Lastly, hyposialylation of TF and ApoC-III (and presumably other serum glycoproteins) is a feature in patients with a rare but distinct type of hemolytic uremic syndrome, caused by neuraminidase released into circulation by *Streptococcus pneumoniae* [158].

1.6 Glycosylation disorders: future perspectives

With respect to what can be anticipated in the research of glycosylation-related diseases in the near future, extremely interesting and relevant seems the hypothesis put forward by Rhodes, who suggests that an inherited factor combined with various environmental factors might produce a spectrum of different phenotypes [208]. Based on the observation of similar glycosylation abnormalities in inflammatory bowel disease and colonic cancer, he specifically speculated that genetically determined alteration in O-glycosylation simultaneously predisposes to increased risk of colon cancer, Crohn' s disease and ulcerative colitis, and the outcome depends on additional factors and their interactions, e.g. infection with pathogenic bacteria, smoking, etc. In support of this concept, multiple studies have recently linked *FUT2* (fucosyltransferase 2 encoding gene) non-secretor status with the susceptibility to Crohn' s disease [209-211], and it was also implicated as a risk factor for developing chronic pancreatitis [212]. In addition, Hansen et al. in their recent study points out that while most of the known CDGs are caused by defects in glycosylation-involved genes without predicted functional redundancy, deleterious mutations in glycosyltransferases with high degree of potential genetic „backup" (i.e., in large homologous families, such as GalNAc-Ts) might produce rather subtle phenotypes in homozygous and compound heterozygous form [213]. For instance, genome-wide association studies (GWAS) have implicated *GALNT2* as a candidate gene regulating plasma lipid levels and propose that its defect is associated with cardiovascular disease [214]. It can be expected that many more such examples will be discovered in the upcoming years.

2 AIMS OF THE THESIS

Since the beginning of our research on inherited glycosylation disorders, we have learnt that for CDG diagnosis, a close cooperation between clinicians, biochemists and molecular geneticists is crucial. Even though a therapy is currently available only for a few CDG types, giving accurate diagnosis enables prenatal testing and genetic counselling in the affected families. Moreover, broadening the knowledge on pathophysiology of the defects in glycosylation might potentially help develop novel therapeutic approaches, not only for CDG, but also for a wide spectrum of other diseases where glycosylation disturbances have been discovered.

The specific aims of the thesis were:

A.) To introduce, optimize and perform simple and rapid **screening methods for the detection of congenital disorders of glycosylation (CDG)** in a large group of clinically suspected patients from Czech and Slovak Republic.

B.) To apply laboratory approaches for **further biochemical and molecular characterization of the patients** with glycosylation defect detected in screening, to establish the genetic diagnosis and to describe pathophysiology of their disease at cellular level.

C.) To study **alterations in protein glycosylation of non-CDG etiology**, in order to both improve the diagnostic process and to better understand the implications of the secondary glycosylation changes for the health condition of the analyzed patients.

3 MATERIAL AND METHODS

I. Ad published results, see individual articles/manuscript in the Supplement.

II. Material and methods related to the unpublished results:

3.1 Material

Patients' and controls' fibroblasts derived from skin biopsies were cultivated in DMEM medium (E15-843, PAA Laboratories GmbH) supplemented with 10 % fetal bovine serum (FBS; SV30180.03 Hyclone) and antibiotics (P11-002, PAA Laboratories GmbH), at 37 °C in 5 % CO_2 environment. Control primary dermal fibroblasts (3 lines, denoted as C1-3) were purchased commercially from ATTC (Manassas, Virginia); the donors were all male newborns. The analyzed patients are shortly characterized below; the listed age is the patient's age when the biopsy was performed.

3.1.1 Patients

PMM2-CDG: Male, 2 years old. Hypotrophy, facial dysmorphy, arthrogryposis, atypical fat pads and inverted nipples were observed since birth. There was no psychomotor development, but he had central hypotonic syndrome, hepatomegaly, nephropathy, hepatopathy and severe coagulopathy. Type I pathological transferrin pattern (relatively increased di- and asialoTF) was found in CDG screening. The measurement of PMM enzyme activity in the lymphocytes did not reveal decreased values, however, in the following analysis of *PMM2* gene by Sanger sequencing in his blood, two heterozygous missense mutations were detected, c.422G>A (p.Arg141His) and c.691G>A (p.Val231Met), which are considered detrimental to the protein function.

ALG8-CDG: Female, 2 months old. Since the birth at 29+0 weeks of pregnancy, she presented with multiple severe complications including acute renal failure, hypertonia, gastrointestinal bleeding, protein losing enteropathy, ascites, cardiomyopathy and progressive hepatosplenomegaly; laboratory findings showed anemia, trombocytopenia and coagulopathy. Despite continuous intensive therapy, the patient died at 2.5 months of age due to multi-organ failure. CDG screening revealed type I pathological TF pattern. The analysis of PMM enzyme activity was normal, and no pathological mutations were found in *PMM2* gene. After LLO analysis, which revealed accumulation of dolichol-PP-$GlcNAc_2Man_9$ and dolichol-PP-$GlcNAc_2Man_9Glc$, and review of the literature, Sanger

sequencing of *ALG8* gene was suggested. Two heterozygous missense variations were found in *ALG8* gene in the patient's blood: pathogenic mutation c.139A>C (p.Thr47Pro) and a novel mutation c.1090C>T (p.Arg364Ter), resulting in premature stop codon; the detected mutations are thought to be detrimental to the protein function.

RFT1-CDG: Male, 16 years old. His clinical picture included psychomotor delay, hypotonia, ataxia and bilateral hearing loss; hepatosplenomegaly and coagulopathy were noted. The analysis of TF glycosylation in CDG screening showed type I pathological pattern. Based on the LLO analysis (showing accumulation of dolichol-PP-$GlcNAc_2Man_5$) performed in fibroblasts from the patient' s sister with the same disorder, analysis of the gene encoding $Man_5GlcNac_2$-PP-Dol flippase (protein RFT1 homolog) was indicated. Two heterozygous missense mutations c.1222A>G (p.Met408Val) and c.1325G>A (p.Arg442Gln) were found in *RFT1* gene by Sanger sequencing in the patient' s blood, and the following restriction fragment length polymorphism (RFLP) analysis of the detected variations in 150 healthy controls confirmed that the mutations were not polymorphisms (thus, were likely pathogenic).

CDG-IIx(1): Male, 3 months old. Admitted to hospital at 10 weeks for severe jaundice, hepatosplenomegaly, ascites and cutis laxa; laboratory tests showed conjugated hyperbilirubinemia, hepatopathy, coagulopathy and hypercholesterolemia. The patient died at 3 months of age due to liver failure. CDG screening revealed pathological TF profile (type II pattern; with relatively increased tri-, di-, mono- and asialoTF) and marked ApoC-III hyposialylation, indicating combined N- and O-glycosylation defect.

CDG-IIx(2): Male, 23 years old. He manifested with growth retardation, skeletal dysplasia including scoliosis, short trunk, genu valgum and broad phalanges, ptosis and convergent strabismus. Coagulopathy was present. CDG screening showed relatively increased trisialoTF and asialoApoC-III, indicating combined N- and O-glycosylation defect.

3.2 Methods

Only fibroblasts with passage number < 10 were used for the analysis. With the exception of transmission electron microscopy, the cells from controls and patients were cultivated on glass coverslips to reach, if possible, the confluence of 70 - 80 % before the individual experiments were performed. For these methods, epifluorescence microscope Nikon Diaphot

200 with the software Viewfinder (version 3.0.1, Pixera Corporation) were used. In all assays, multiple pictures were taken and the representative images were chosen for evaluation and discussion.

3.2.1 Golgi labeling

Media from the cells grown on coverslips were discarded, they were washed with phosphate-buffered saline (PBS; BE17-517Q BioWhittaker, Lonza; 10 times diluted with distilled water), fixed with 4 % paraformaldehyde (PFA; 19943 Affymetrix) for 10 min at 4 °C, washed in PBS twice and permeabilized with 0.1 % (V/V) Triton X-100 (T9284 Sigma) in PBS for 20 min at room temperature (RT). After washing with PBS (2x), a blocking solution (5 % (V/V) FBS in PBS) was applied, in which the cells were incubated for 1 hour (RT). Blocking solution was then replaced by a solution of primary antibody (1:200 giantin antibody, ab37266 Abcam, in blocking solution) and left to incubate at 4 °C overnight. The cells were thoroughly washed with PBS (3x), followed by incubation with solution of fluorescently labeled secondary antibody (1:1000 anti-mouse IgG1-Alexa Fluor 488, A21121 Invitrogen; diluted in blocking solution) for 2 hours, RT. Following washing with PBS (2x), nucleus was stained with DAPI solution (D1306 Invitrogen, 10 μg/ml in PBS) for 10 min, RT. Finally, the cells were washed with PBS (3x) and the green/blue fluorescent signal was observed and imaged at automatic exposure mode.

3.2.2 PNA staining

For each tested cell line, fibroblasts were cultivated on two coverslips. Prior to labeling, one of them was incubated with neuraminidase (11585886001 Roche, 50 mU in 0.25 ml media) for 1 hour at 37 °C. Then media from both coverslips were discarded, the cells were washed with PBS and fixed with 1 % (V/V) PFA in PBS (10 min, RT). After washing with PBS, the solution containing Alexa Fluor 488 conjugated peanut agglutinin (PNA) lectin (L21408 Invitrogen, 5 μg/ml in PBS with 1 % (V/V) FBS) was added and left for 1 hour at 37 °C. The cells were washed with PBS (3x) and the green fluorescent signal was observed and imaged at manually set exposure. The neuraminisade-treated cells serve as a control of the experiment.

3.2.3 In vivo ROS (reactive oxygen species) detection

The fibroblasts were incubated with dihydroethidium (DHE; D1168, Invitrogen) solution (5 μM in PBS) for 10 min at 37 °C, then washed with PBS (2x) and the red fluorescent

signal was observed and imaged at manually set exposure.

3.2.4 Catalase staining

Media from the cells grown on coverslips were discarded, they were washed with PBS, fixed with 4 % PFA for 15 min at 4 °C, washed in PBS twice and permeabilized with 0.1% (V/V) Triton X-100 in PBS for 10 min at room temperature (RT). After washing with PBS (2x), a blocking solution (10 % (V/V) FBS in PBS) was applied, in which the cells were incubated for 1 hour (RT). Blocking solution was then replaced by a solution of primary antibody (1:100 catalase antibody, A21987 Invitrogen; diluted in blocking solution) and left to incubate at 4 °C overnight. The cells were thoroughly washed with PBS (3x), followed by incubation with solution of fluorescently labeled secondary antibody (1:1000 anti-mouse IgG1-Alexa Fluor 488 in blocking solution) for 2 hours, RT. Finally, the cells were washed with PBS (3x) and the green fluorescent signal was observed and imaged at at manually set exposure.

3.2.5 Transmission electron microscopy (TEM)

The fibroblasts were fixed in 2 % (w/V) potassium permanganate in PBS for 15 min at RT and dehydrated by ethanol series: 50%, 70%, 90% ethanol for 10 min each step and 100% ethanol for 30 minutes. Dehydrated cells were incubated in propylene oxide for 15 min (twice) and embedded into Durcupan Epon (Electron Microscopy Sciences) at 60 °C, overnight. Samples were sectioned by microtome Ultracut III ultramicrotome (Reichert) into thick cuts ranging from 600 to 900 Å. Cuts were stained with lead citrate and uranyl acetate [215]. Finally, pictures were taken using transmission electron miscroscope Jeol JEM 1200Ex.

4 RESULTS AND DISCUSSION

4.1 Results and discussion related to the aim A.)

Screening methods for the detection of congenital disorders of glycosylation (CDG).

The list of publications related to the aim A.):

1] Guillard M, Wada Y, Hansikova H, Yuasa I, Vesela K, Ondruskova N, Kadoya M, Janssen A, Van den Heuvel LP, Morava E, Zeman J, Wevers RA, Lefeber DJ. ***Transferrin mutations at the glycosylation site complicate diagnosis of congenital disorders of glycosylation type I.*** *J Inherit Metab Dis. 2011Aug;34(4):901-6.*

2] Honzík T, Magner M, Krijt J, Sokolová J, Vugrek O, Belužić R, Barić I, Hansíkova H, Elleder M, Veselá K, Bauerová L, Ondrušková N, Ješina P, Zeman J,Kožich V. ***Clinical picture of S-adenosylhomocysteine hydrolase deficiency resembles phosphomannomutase 2 deficiency.*** *Mol Genet Metab. 2012 Nov;107(3):611-3.*

3] Van Scherpenzeel M, Timal S, Rymen D, Hoischen A, Wuhrer M, Hipgrave-Ederveen A, Grunewald S, Peanne R, Saada A, Edvardson S, Grønborg S, Ruijter G, Kattentidt-Mouravieva A, Brum JM, Freckmann ML, Tomkins S, Jalan A, Prochazkova D, Ondruskova N, Hansikova H, Willemsen MA, Hensbergen PJ, Matthijs G, Wevers RA,Veltman JA, Morava E, Lefeber DJ. ***Diagnostic serum glycosylation profile in patients with intellectual disability as a result of MAN1B1 deficiency.*** *Brain.2014 Apr;137(Pt 4):1030-8.*

4] Ondrušková N, Honzík T, Kytnarová J, Matoulek M, Zeman J, Hansíková H. ***Isoelectric Focusing of Serum Apolipoprotein C-III as a Sensitive Screening Method for the Detection of O-glycosylation Disturbances.*** *Prague Med Rep. 2015;116(2):73-86.*

Author' s contribution: biochemical analyses of selected glycoproteins (1-4), molecular genetic analysis (1) and manuscript preparation (4).

4.1.1 A rare transferrin mutation at the glycosylation site hampers the screening of CDG.

1] A 7-year-old boy suffered from cyclic vomiting accompanied by ketosis and metabolic acidosis. He also had atypical fat pads and inverted nipples, what raised a clinical suspicion of CDG. Analysis of serum TF revealed a pattern with decreased relative amount of tetrasialo- (31.3 %, vs. reference range 51.1-59.2 %) and increased disialoform (33.5 %, vs. ref. range 4.7-8.5 %), not resembling any profiles typically found in CDG patients. No polymorphic variants were found by IEF of TF after neuraminidase treatment. SDS-PAGE of TF showed two bands of approximately the same intesity, one representing the fully glycosylated TF and the lower one corresponding to a TF carrying only one glycan. The biochemical analysis is shown in Fig. 7. A similar IEF pattern was detected in his healthy mother, suggesting a non-pathogenic cause. We thus decided to sequence the *TF* gene by Sanger method. Besides detecting 4 polymorphisms which had already been described elsewhere, a novel mutation was found in exon 16 of the patient´s *TF* gene, causing amino acid alteration at one of the two TF N-glycosylation sites (heterozygous c.1889A>C; p.Asn630Thr). This was confirmed by our collaborators' MALDI-MS analysis of the immunopurified TF from the patient and his mother. The present case demonstrates the potential pitfalls, albeit rarely occuring, of CDG screening method using the analysis of TF glycosylation.

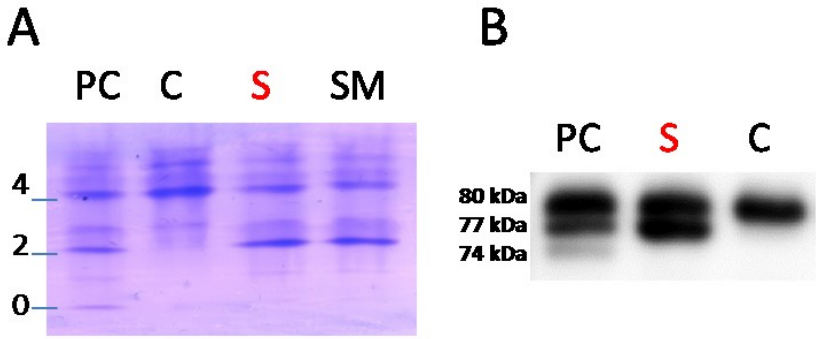

Fig. 7: CDG screening in the patient with rare transferrin polymorphism. Serum analyzed by **A]** isoelectric focusing and **B]** SDS-PAGE of transferrin. PC: PMM2-CDG, C: a control, S: the subject and SM: the subject's mother. *Numbers on the left (A) indicate sialic acid content (asialo-, disialo-, hexiasialo-) of the individual transferrin isoforms*.

4.1.2 A case of S-adenosylhomocysteine hydrolase deficiency clinically resembles PMM2-CDG.

2] A female patient manifested with neonatal onset of hypotonia, psychomotor retardation, hepatopathy and strabism. Severe coagulopathy and the absence of elevated methionine and homocysteine at the age of 2 months prompted us to search for CDG. However, IEF of serum transferrin was normal, as well as the acitivities of oxidative phosphorylation complexes in the patient' s fibroblasts, excluding both an N-glycosylation defect and a mitochondrial disorder. After 8 months, gradual elevation of methione (259-547 μmol/l, controls 12-45) and total homocysteine (16.1-22.4, controls 3.5-10) appeared, but it was only after acute decompensation at the age of 4.5 years when the patient showed markedly increased plasma levels of S-adenosylhomocysteine (6.8 μmol/l, controls 0.004-0.081) and S-adenosylmethione (3.2 μmol/l, controls 0.013-0.141), that a suspicion of S-adenosylhomocysteine hydrolase (SAHH) deficiency was raised. The SAHH activity measured in the patient's erythrocytes and fibroblasts was decreased to 11 % of the control levels. Molecular analysis of the *AHCY* gene revealed a compound heterozygosity for pathogenic mutations c.145C>T (p.Arg49Cys) and c.211G>A (p.Gly71Ser), confirming the diagnosis of SAHH deficiency. The striking resemblance of this patient's clinical picture to those that we have seen in our PMM2-CDG patients can be a useful note for clinicians (see Table 2). Also, our case shows that SAHH deficiency should be considered even if the levels of homocysteine and methionine are normal in neonatal and infantile period.

Table 2: Comparison of the clinical data: the SAHH subject vs. PMM2-CDG patients.

Symptoms	**SAHH-deficient patient**	**PMM2-deficient patients**
	Current report	*Czech patients (n = 17)*
Hypotonia	++	17/17
Psychomotor retardation	+	17/17
Cerebellar hypoplasia	-	13/13
Strabism	+	17/17
Hypo/Areflexia	+	7/13
Seizures	-	6/17
Microcephaly	+	14/17
Hepatomegaly/Hepatopathy	++	14/17
White matter abnormalities	++	1/9
Coagulopathy	++	17/17

4.1.3 A novel rapid diagnostic test for the diagnosis of MAN1B1-CDG from serum.

3] In 2011, whole-exome sequencing identified *MAN1B1* (encoding α-1,2-mannosidase) as a causative gene underlying autosomal-recessive intellectual disability [43]. MAN1B1-CDG shows a typical type II pattern in IEF of transferrin with a relative increase of trisialoform, however, CDG-IIx are generally more complicated to further characterize than CDG-Ix due to lack of specific diagnostic methods. Our collaborators have applied a novel high resolution mass spectrometry (MS) method for direct glycoprofiling of intact plasma transferrin, and observed a unique pattern of hybrid type N-glycans in MAN1B1 deficiency. The aim of the study was to test this approach as a novel diagnostic test which could be used for a rapid and specific screening in a cohort of 100 patients with CDG-IIx. The group included our 14 year-old male patient, in whom we previously detected relatively increased trisialotransferrin. Rapid analysis of immunopurified transferrin by nanochip-C8-QTOF-MS showed an abnormal presence of two hybrid type N-glycans at average mass of 79224 Da and 79062 Da (see Fig. 8) in 12 patients - including ours - from the analyzed group, and this was in agreement with deficient processing of protein-bound glycans by MAN1B1. Sanger sequencing of *MAN1B1* gene was performed in those 12 individuals, and pathogenic mutations were identified in all of them, confirming the efficiency of the novel functional diagnostic assay. Clinically, the group of MAN1B1-CDG patients all presented with intellectual disability with delayed motor and speech development, however variable associated findings were observed such as hypotonia, truncal obesity and macrocephaly (in ~ 65 %). Also, a distinct facial dysmorphy (thin lateral eyebrows, bulbous nose tip, thin upper lip) was noted in about half of the patients. We believe MAN1B1-CDG might be a fairly common intellectual disability syndrome and the presented method is a valuable tool for its diagnosis.

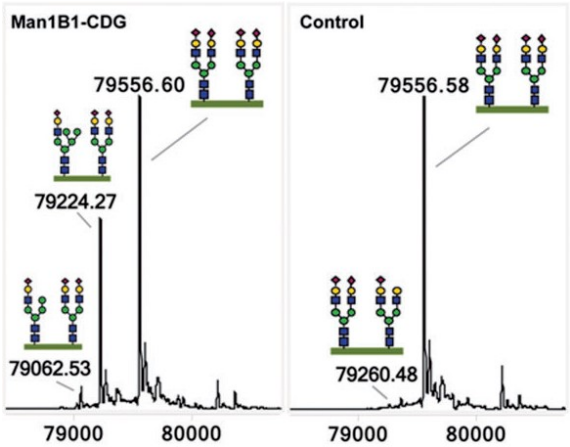

Fig. 8: A representative mass spectrometry profile of intact transferrin analyzed by nanochip-C8-QTOF-MS in serum from MAN1B1-CDG patient versus control. *x-axis: the corresponding mass of the glycans (represented by individual peaks) in Da; y-axis: their relative abundance.*

4.1.4 ApoC-III isoelectric focusing is a sensitive method for detection of O-glycosylation defects.

4] Isoelectric focusing and Western blot detection of serum ApoC-III, a method originally developed by Wopereis et al. [157], was introduced in our laboratory and performed in 170 healthy subjects of both genders in the age interval 1 day-42 years. The statistically evaluated distribution of the three ApoC-III sialylated isoforms in different sexes and age categories is shown in Fig. 9. No statistically significant changes were found between males and females. However, we observed a relative decrease of ApoC-III_2 and an increase of ApoC-III_0 with growing age. Additionally, ApoC-III was analyzed in 25 patients with various selected metabolic diseases (Prader-Willi syndrome (s.), Rett s., Silver-Russell s., DiGeorge s., Gapo s., Schnitzler s., Marfan s., Stickler s., dyschondrosteosis, chronic renal dysfunction, PMM2-CDG, EXT1-CDG, NgBR deficiency, PGM1-CDG and MAN1B1-CDG) to examine possible secondary O-glycosylation disturbances. Mild ApoC-III hypoglycosylation, mostly with relatively elevated ApoC-III_1, was found in 4/10 of our patients with Prader-Willi s.(PWS). While the previous study by Munce et al. [216] reported a correlation between the aberrant ApoC-III profiles in PWS patients and sleep abnormalities, we found no such relationship. However, we noted that the positive individuals had higher serum triacylglycerol levels (≥ 1.4 mmol/l) than those with normal profiles. The first step of ApoC-III glycosylation is specifically catalyzed by the enzyme encoded by the *GALNT2* gene, and, interestingly, it seems that its expression might be regulated by various metabolic factors such as hyperglycemia in diabetes [217]. Moreover, certain SNPs in the *GALNT2* gene were found to be associated with the levels of plasma triacylglycerols and cholesterol [214]. Thus, we suggested that the increased triacylglycerols (TAG) in PWS might be relevant in the etiology of their ApoC-III hypoglycosylation. Indeed, this assumption was very recently validated by the study performed in the group of non-diabetic adolescent participants by Yassine et al., who concluded that ApoC-III_0 (both the aglycosylated form and the one containg the whole glycan without any sialic acid residues) and ApoC-III_1 appear to be under metabolic control, and their relative abundance is associated with fasting plasma TAG [218]. Other metabolic disorders where we have shown, for the first time, borderline/slightly pathological ApoC-III included PGM1-CDG and MAN1B1-CDG. We conclude that the analysis of ApoC-III by isoelectric focusing is a simple and sensitive method to detect mucin O-glycosylation disturbances. However, our results indicate that the glycosylation status of ApoC-III might vary intraindividually depending on the patient's metabolic condition, and therefore analyses at multiple chronological points in the same individual are recommended.

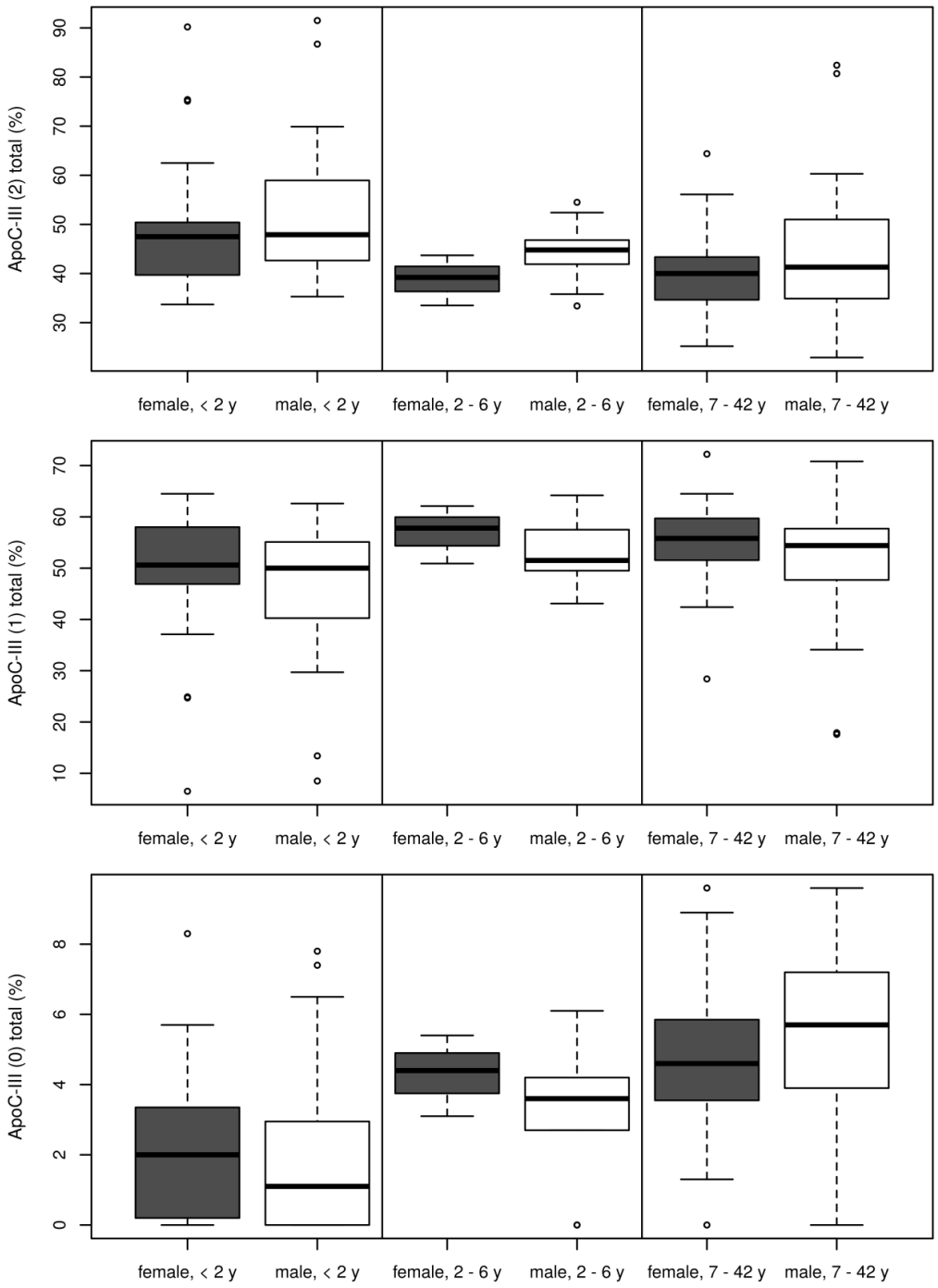

Fig. 9: Evaluation of gender- and age-dependent differences in the distribution of ApoC-III sialylated isoforms separated by isoelectric focusing of serum samples, determined in a group of healthy individuals (n = 170) of both genders (89 males, 81 females) in three different age categories: 1) < 2 y (n = 70), 2) 2-6 y (n = 16) and 3) 7-42 y (n = 84). The bold line inside box-plots represents median. 1) Wilcoxon test results (female vs. male) for a) ApoC-III$_2$: p = 0.238 (< 2 y); 0.157 (2-6 y); 0.345 (7-42 y), b) ApoC-III$_1$: p = 0.226 (< 2 y); 0.281 (2-6 y); 0.181 (7-42 y) and c) ApoC-III$_0$: p = 0.522 (< 2 y); 0.380 (2-6 y); 0.165 (7-42 y). 2) Kruskal-Wallis test results (age dependent differences) for a) ApoC-III$_2$: p = 0.002 (female - F); 0.008 (male - M), b) ApoC-III$_1$: p = 0.041 (F); 0.110 (M) and c) ApoC-III$_0$: p < 0.001 (F); < 0.001 (M).

4.2 Results and discussion related to the aim B.)

Further biochemical and molecular characterization of CDG patients.

I. The list of publications related to the aim B.):

1] Ondruskova N, Vesela K, Hansikova H, Magner M, Zeman J, Honzik T. ***RFT1-CDG in adult siblings with novel mutations.*** *Mol Genet Metab. 2012 Dec;107(4):760-2.*

2] Ondruskova N, Honzik T, Vondrackova A, Tesarova M, Zeman J, Hansikova H. ***Glycogen storage disease-like phenotype with central nervous system involvement in a PGM1-CDG patient.*** *Neuro Endocrinol Lett. 2014;35(2):137-41.*

3] Park EJ, Grabińska KA, Guan Z, Stránecký V, Hartmannová H, Hodaňová K, Barešová V, Sovová J, Jozsef L, Ondrušková N, Hansíková H, Honzík T, Zeman J, Hůlková H, Wen R, Kmoch S, Sessa WC. ***Mutation of Nogo-B receptor, a subunit of cis-prenyltransferase, causes a congenital disorder of glycosylation.*** *Cell Metab. 2014 Sep 2;20(3):448-57.*

II. Additional unpublished experiments related to the aim B.):

4] The changes in subcellular structure, ultrastructure of organelles and pathobiochemistry in the cultivated skin fibroblasts from selected patients with congenital disorders of glycosylation.

Author' s contribution: biochemical analyses of selected glycoproteins (1-3), molecular genetic analysis (2), manuscript preparation (2) and analysis of fibroblasts by immunocytochemical and immunofluorescent methods (4).

4.2.1 Siblings with novel mutations in RFT1 gene show a milder phenotype of RFT1-CDG.

1] Two siblings, a boy (older) and a girl, were born at term and had an uneventful postnatal adaptation. The onset of their first symptoms began at 8 and 6 months, respectively. They gradually presented with psychomotor delay, hypotonia, seizures and ataxia. A bilateral hearing loss was recognized in the boy, while the girl developed no hearing impairment. In both, a mild hepatosplenomegaly was detected using abdominal ultrasound, and laboratory findings revealed coagulopathy with decreased levels of protein C and factor XI (plus factor VIII and XI in the girl). The diagnosis of CDG was made when they reached a young adult age, after IEF of serum transferrin showed type I pattern. Following the detection of normal PMM and MPI activities in lymphocytes and cultivated fibroblasts, LLO analysis was performed in the girl's fibroblasts confirming the accumulation of dolichol-PP-$GlcNAc_2Man_5$. Because the analysis of protein-linked glycan was normal, sequencing of the *RFT1* gene - encoding $Man_5GlcNac_2$-PP-Dol flippase - was suggested. In both patients, two novel heterozygous missense mutations were found in *RFT1*, c.1222A>G (p.Met408Val) and c.1325G>A (p.Arg442Gln); see Fig. 10. At the time of article publication, the patients (19 and 21 years old) showed profound intellectual disability (IQ < 20), dysmorphic features, hypotonia, mild coagulopathy and epilepsy (well controlled), similarly to the previously described RFT1-CDG patients. In contrast, the 6 patients reported elsewhere had, additionally, feeding problems, failure to thrive and poor visual contact. The milder phenotype observed in our patients can perhaps be explained by the position of their mutations in *RFT1* gene, which lead to amino acid change in the transmembrane region, as opposed to defects in the protein sections facing the ER lumen in patients with more severe symptoms.

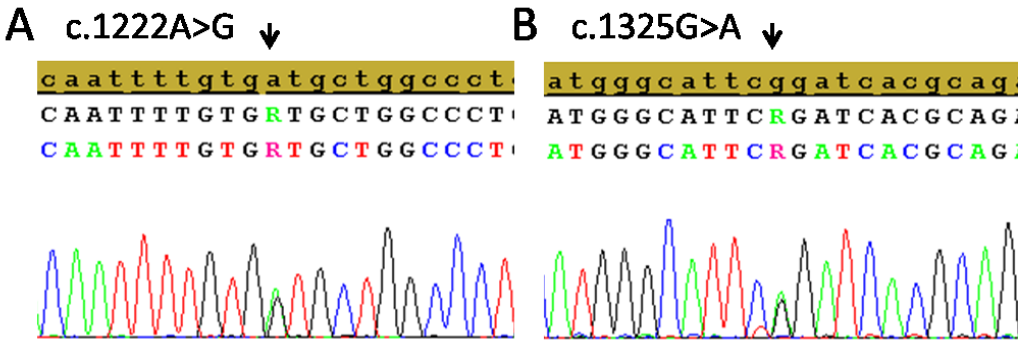

Fig. 10: Sequence profiles with the pathogenic *RFT1* mutations in the affected siblings. Chromatogram of the two causative mutations in exon 12 (heterozygous c.1222A>G, p.Met408Val and c.1325G>A, p.Arg442Gln) from the Sanger sequencing analysis of the *RTF1* gene in the female RFT1-CDG patient; the corresponding reference sequence is shown at the top (brown background).

4.2.2 A boy diagnosed with PGM1-CDG presents with a novel feature of neurological impairment.

2] A 10-year-old boy with a cleft palate and short stature manifested with mutli-systemic symptoms including hepatopathy, coagulapathy, cholecystolithiasis, myopathy and microcephaly. In addition, laboratory findings of hypoglycemia, hyperlipidemia, hypothyroidism and hyperuricemia were noted. While IEF of TF revealed relatively increased levels of its di-, mono- and asialoform, SDS-PAGE and Western blot of TF showed the presence of low-Mw smear corresponding to transferrin polypeptide carrying incomplete glycan chains; see Fig. 11. Because ApoC-III glycosylation was normal to borderline, the results overall indicated a defect affecting (predominantly) the processing of N-glycans. Shortly before our diagnosis was made, an article was published reporting identification of a novel CDG type due to phoshoglucomutase 1 (PGM1) deficiency in two patients using whole-genome sequencing [38]. Interestingly, their IEF TF pattern was quite characteristic and very similar to that seen in our patient. Furthermore, both of the two diagnosed individuals had a distinct feature: a cleft palate. We therefore decided to measure the enzyme activity of PGM, using a spectrophotometric method, in our patient's fibroblasts. Indeed, we detected markedly decreased PGM activity (< 5 %) compared to healthy controls. Based on the following molecular analysis of *PGM1* exons by Sanger sequencing, our patient was found to be a compound heterozygote for c.1010C>T (p.Thr337Met) and c.1508G>A (p.Arg503Gln). *In silico* tools confirmed the pathogenicity of these mutations, and PCR-RFLP analysis did not detect any of them in our group of 100 healthy controls. Moreover, protein alignment showed that the affected codons are evolutionary conserved, supporting the presumed causality of the detected variations. In reponse to studies suggesting a therapeutic effect of galactose supplementation in the PGM1-CDG patients [219], we started our patient on a lactose-rich diet with a dose of 40-50 g of lactose per day (due to impossibility to medically prescribe galactose in Czech republic). Unfortunately, neither clinical nor laboratory improvement resulted from this intervention. Compared to other PGM1-CDG cases, our patient showed a novel feature of mild neurological impairment.

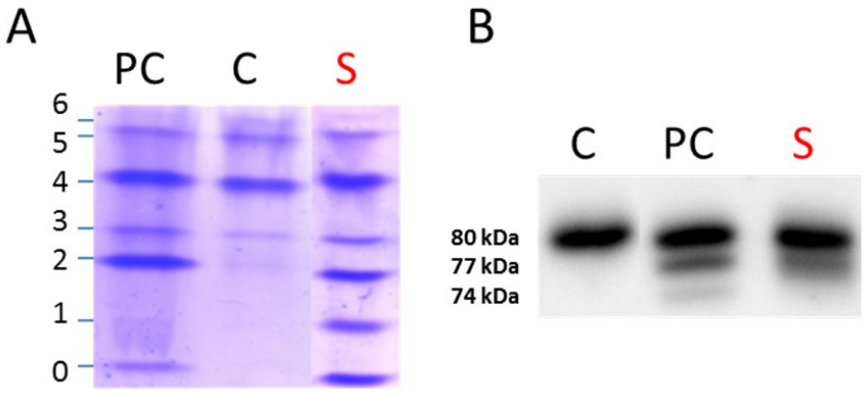

Fig. 11: CDG screening in the PGM1-CDG patient. Serum analyzed by **A]** isoelectric focusing and **B]** SDS-PAGE of transferrin. C: a control, PC: PMM2-CDG and S: the subject. *Numbers on the left (A) indicate sialic acid content (asialo- to hexiasialo-) of the individual transferrin isoforms*.

4.2.3 A novel CDG type due to dolichol biosynthesis defect is caused by mutations in NGBR gene.

3] Two male siblings of Roma origin presented with congenital scoliosis, severe neurological involvement, hypotonia refractory epilepsy, hearing deficit and visual impairment. Patient 1 died at the age of 29 months, patient 2 was still alive (4 years old) at the time of article publication, however died soon afterwards at the age of 6 years. The exoms of the probands and their parents were sequenced and four genetic variants were discovered, out of which the homozygous missense mutation c.869 G>A (p.Arg290His) in the *NUS1* (*NGBR*) gene was evaluated as the one most likely to be responsible for the clinical phenotype; the unrelated parents and healthy siblings were heterozygous for this variation. The affected aminoacid is located in an evolutionarily conserved C-terminal domain of NgBR, and the mutation was, using *in silico* tools, predicted to be damaging for the protein function. In order to characterize the effect of the detected mutation on cellular pathophysiology, fibroblasts were isolated from the patients and used in the following experiments. The levels of NgBR mRNA and protein were found to be not significantly different, demonstrating that the protein translation and processing were not altered by the p.Arg290His mutation. Next, the known aspects of NgBR function were assessed: free cholesterol levels, cis-prenyltransferase (cis-PT) activity and glycosylation. Filipin staining showed increased accumulation of cholesterol in both patients, while microsomal cis-PT activity using isolated membranes from the patients' cells was detected to be less than 20 % of the control's. Addressing glycosylation, mannose incorporation into glycoproteins was measured and found to be markedly lower in the affected siblings. While Western blot in the fibroblasts revealed hypoglycosylation of glycoproteins LAMP1 and ICAM-1, serum

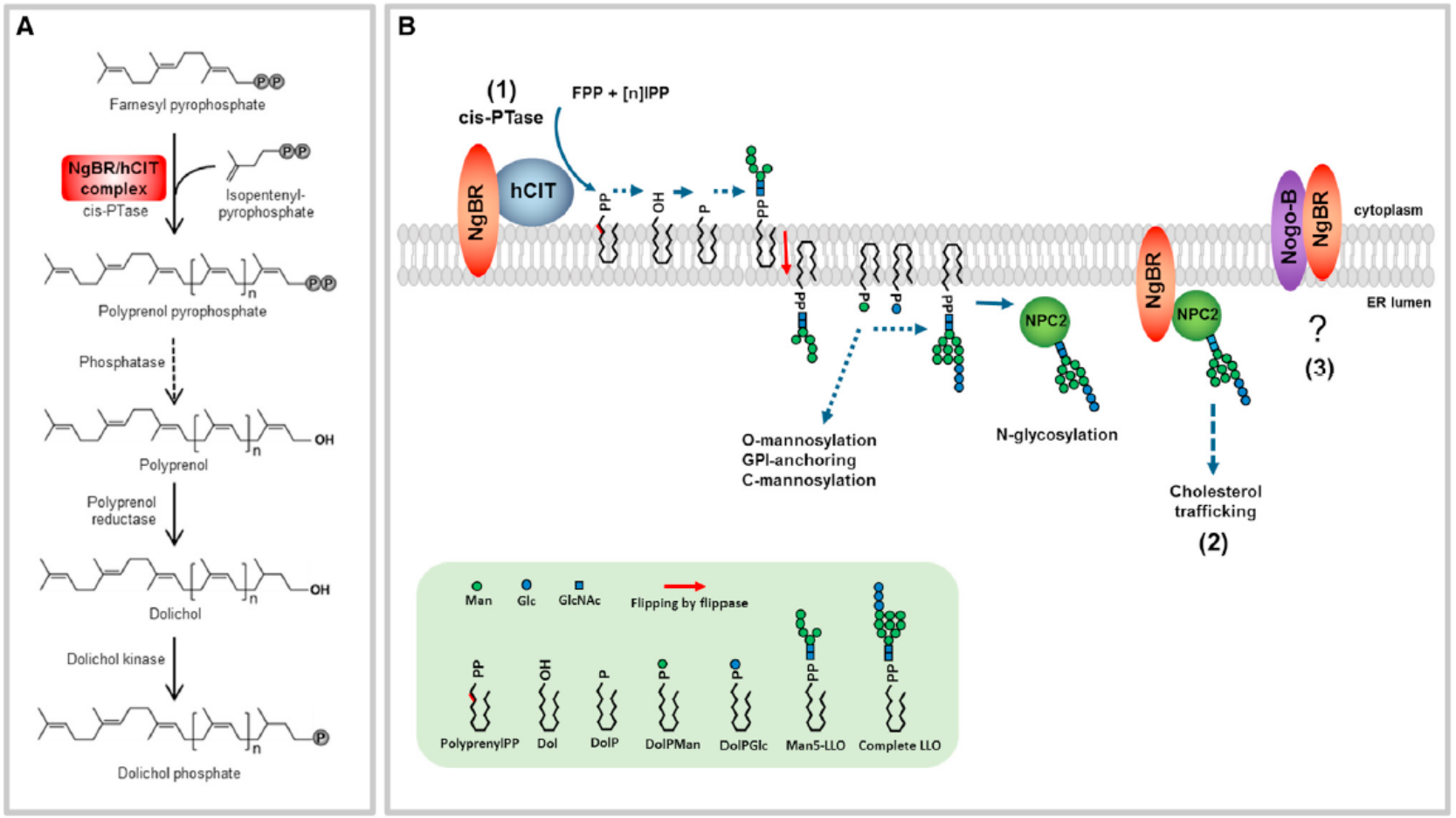

Fig. 12: The function of NgBR/hCIT complex. A] NgBR/hCIT complex promotes cis-prenyltransferase activity, catalyzing the formation of polyprenol pyrophosphate. **B]** NgBR/hCIT complex is essential for the synthesis of dolichol phosphate, which serves as a membrane anchor for the assembly of N-glycans. NgBR influences cholesterol trafficking by directly interacting with NPC2 (intracellular cholesterol transporter) and indirectly via modifying NPC2 glycosylation.

markers TF and ApoC-III analyzed in the patient 2 were normal (not shown in article). Similarly to the described DHDDS-CDG cases, the patients with NgBR defect also had altered ratios of dolichol in urine and blood as assessed by mass spectrometry. In addition, our collaborators demonstrated the necessity of both NgBR and hCIT (DHDDS) for dolichol biosynthesis by experiments in mice, mice embryonic fibroblasts and yeast models. Overall, the published data provide evidence for the essential role of NgBR in dolichol synthesis and protein glycosylation, and report a novel type of CDG due to NgBR defect. Postulated functions of the NgBR/hCIT complex in cellular metabolism are illustrated in Fig. 12.

4.2.4 Altered subcellular structure and pathophysiology found in fibroblasts from CDG patients.

4] Cultivated skin fibroblasts from a group of our patients with either genetically determined CDG-I (n = 3; PMM2-CDG, ALG8-CDG and RFT1-CDG) or undiagnosed CDG-IIx (n = 2) were analyzed to characterize the subcellular structure, ultrastructure of specific organelles and selected aspects of the cellular pathobiochemistry in these disorders. First, the morphology of Golgi apparatus as the main site of glycosylation was investigated by means of immunocytochemistry using antibody to human giantin (Golgi membrane protein); see Fig. 13a. Compared to the control cell lines, abnormal Golgi structure was found in both CDG-IIx cultures, defined by its increased dilatation (in CDG-IIx(1)) and fragmentation (in CDG-IIx(2)). Golgi dilatation was also observed in RFT1-CDG, however it must be noted that this could be related to the overall poor growth of the fibroblasts. Morphological changes of Golgi were previously described in various CDG type II disorders affecting multiple glycosylation pathways, such as the deficiency of COG subunits or TMEM165 [125, 134]. To examine the organelles in detail, we analyzed the fibroblasts by transmission electron microscopy (Fig. 13b) and in CDG-I lines we saw a normal organization of Golgi stacks, what we failed to detect in CDG-IIx cells. In addition, there was an increased occurence of swollen endoplasmic reticulum in PMM2-CDG and ALG8-CDG, perhaps reflecting ER stress in the cells. In the next experiment using fluorescently labeled PNA lectin, the increased signal in both CDG-IIx patients pointed to reduced sialylation of mucin O-glycoproteins, confirming a combined N- and O-glycosylation defect (Fig. 13c). Strikingly, immunofluorescent assay using dihydroethidium (DHE) showed a markedly higher intensity of the red fluorescent signal in all studied CDG lines, in comparison to controls (Fig. 13d). DHE is used to monitor reactive oxygen species (ROS) in tissues *in vivo*, forming a red fluorescent product upon reaction with superoxide anions [220]. We assume that the elevated cytosolic ROS levels we detected in CDG could be a consequence of the

activation of UPR (unfolded protein response), which was studied by Lecca et al. by means of transcriptome analysis in primary fibroblasts from 9 CDG-I patients (4x ALG6-CDG, 3x DPM1-CDG and 2x ALG12-CDG) [221]. While the UPR gene expression profiles were quite uniform in their tested group, the extent of the responses appeared to mirror the severity of the individual defects. The overexpression of *DNAJC3/P58IPK*, encoding a PERK inhibitor and a cochaperone $p58^{IPK}$ [222, 223], was a consistent finding in all CDG-I types. Repression of the PERK pathway (thus, protein translation maintenance) was in agreement with the detection of no significant overexpression of CHOP (which is downstream of PERK), as well as observed induction of amino acid biosynthesis, and the author suggested that this could be a key mechanism to ensure cell survival in CDG fibroblasts. The exact mechanism how ER stress (UPR) triggers oxidative stress is not well understood; ROS are generated as a by-product of oxidative folding in ER, but their production might also be increased as a result of ER-mitochondrial communication involving calcium signaling [224]. We can only speculate about the origin of the markedly increased ROS levels that we found in CDG, but it seems possible that inhibition of PERK signaling could play a role. Apart from phosphorylation of eIF2α, PERK also targets nuclear respiratory factor 2 (NRF2), which upon phosphorylation binds to the antioxidant response element (ARE) to activate transcription of genes encoding enzymes that promote resistance to oxidative stress [225]. Indeed, PERK (-/-) cells (mouse embryonic fibroblasts) accumulated ROS when exposed to ER stress [226]. Another unexpected and novel finding was our immunocytochemical detection of decreased catalase signal within peroxisomes in CDG-IIx(1) (Fig. 13e), what is a feature of certain inherited peroxisomal disorders [227]. Relevant to this observation might be a study by Murakami et al., who showed the existence of an integrated system in mammals to utilize catalase - a key antioxidant enzyme which catalyzes the decomposition of H_2O_2 - by changing its localization from peroxisomes to cytosol in response to increased cytosolic H_2O_2 level [228]. It is, however, unclear to us why the aberrant catalase distribution was seen only in CDG-IIx(1) and not in the other analyzed CDG patients who also displayed a significant (and some of them even greater) ROS accumulation. Possibly the reduced peroxisomal catalase staining in the patient CDG-IIx(1) is directly related to his disorder, however, further elaboration is difficult as his genetic defect has not been identified so far.

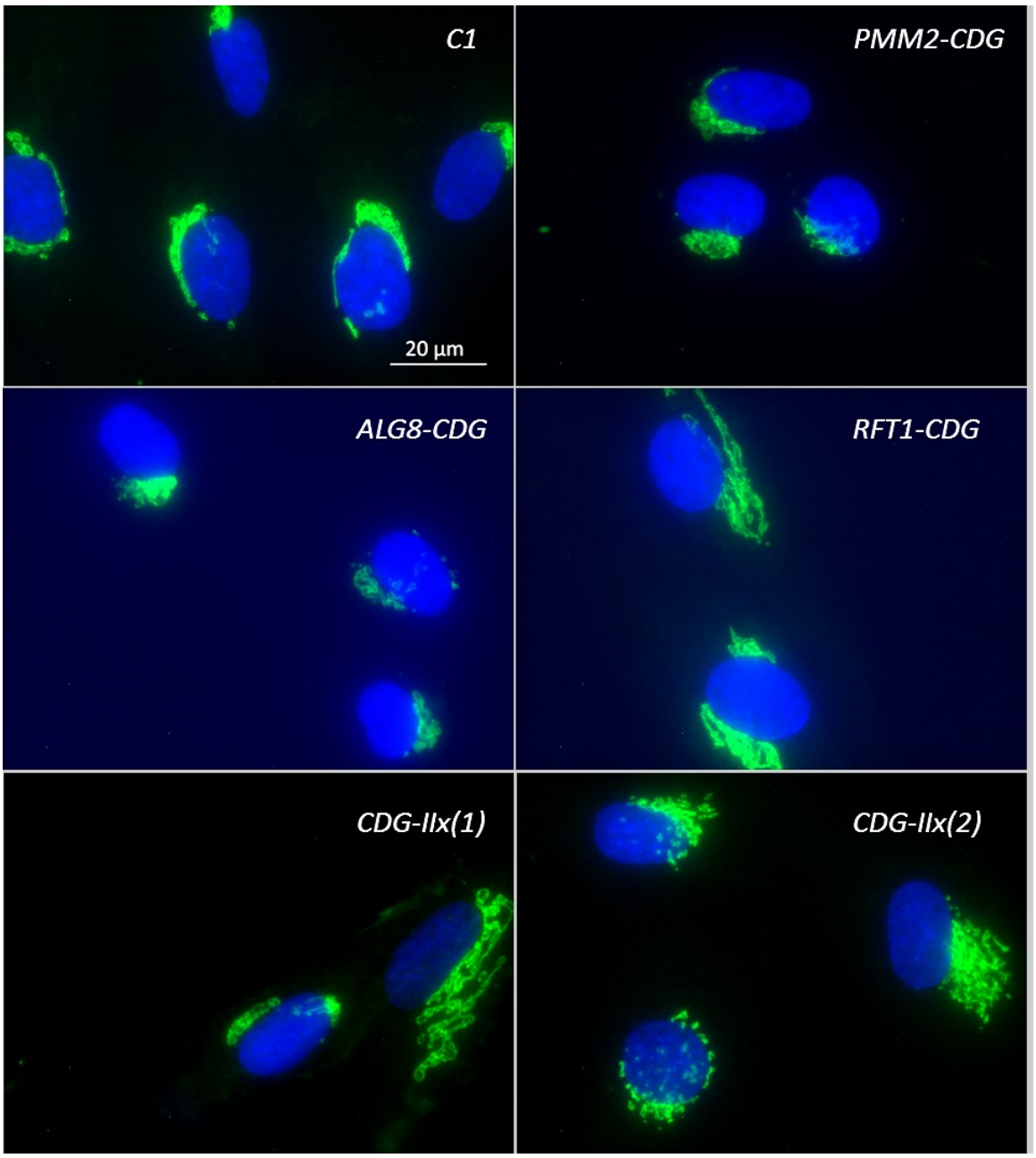

Fig. 13a: Altered Golgi morphology in fibroblasts from selected CDG patients.
Golgi structure was visualized in the cells by immunocytochemical analysis using antibody to giantin, a Golgi membrane protein (green signal), and nucleus was stained with DAPI (blue); the final images were created by merging the two channels. While a normal juxta-nuclear Golgi localization can be seen in the control and CDG type I patients (PMM2-CDG, ALG8-CDG), fibroblasts from CDG type II patients show aberrant Golgi morphology with increased dilatation (CDG-IIx(1)) and fragmentation (CDG-IIx(2)). Abnormal Golgi structure in RFT1-CDG might be related to the observed poor growth of the cell line.

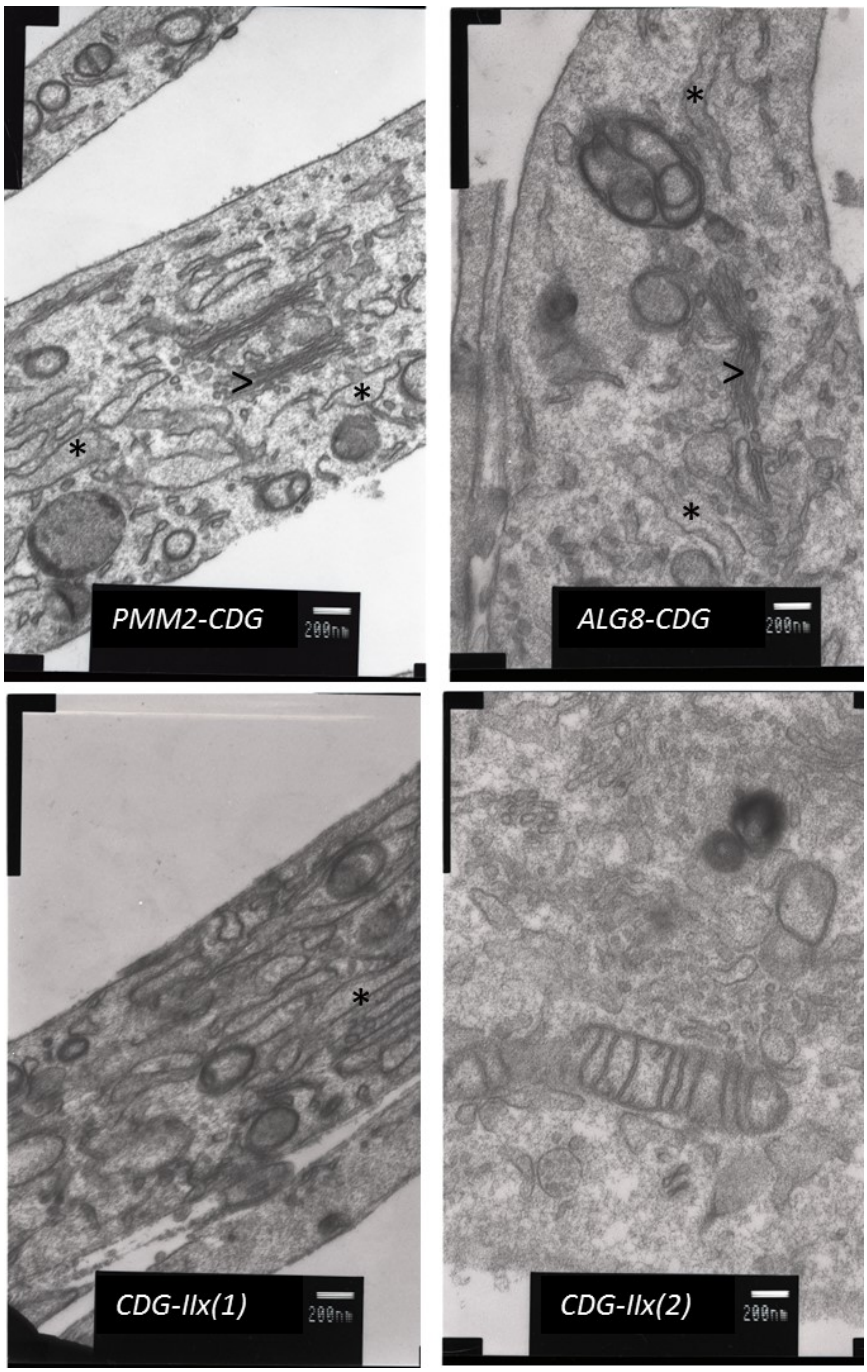

Fig. 13b: Disorganization of organelles in fibroblasts from selected CDG patients revealed by ultrastructure analysis. The cells were analyzed by transmission electron microscopy, using 25 000x magnification. In CDG type I (PMM2-CDG, ALG8-CDG), we saw increased occurrence of swollen endoplasmic reticulum (*). While they showed a normal organization of Golgi stacks (>), this could not be detected in CDG type II fibroblasts.

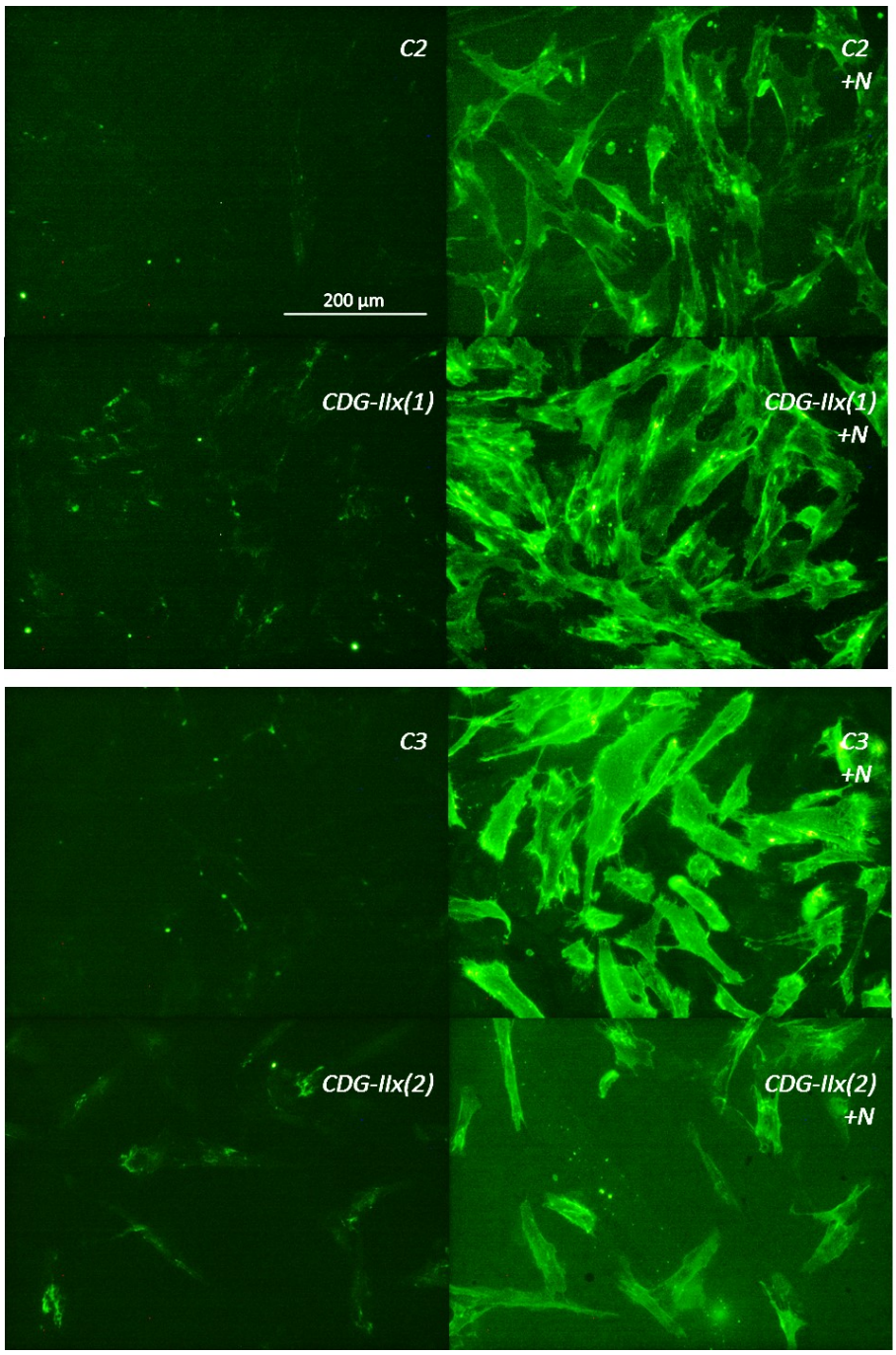

Fig. 13c: Affected mucin type O-glycosylation in fibroblasts from selected CDG patients. Reduced terminal sialylation of mucin type O-glycans was detected in both of our patients with uncharacterized CDG type II by assay using fluorescently labeled peanut agglutinin (PNA) lectin (binds to the exposed Gal residues). The images show cells without (left) and with (right; +N) neuraminidase treatment prior to PNA labeling.

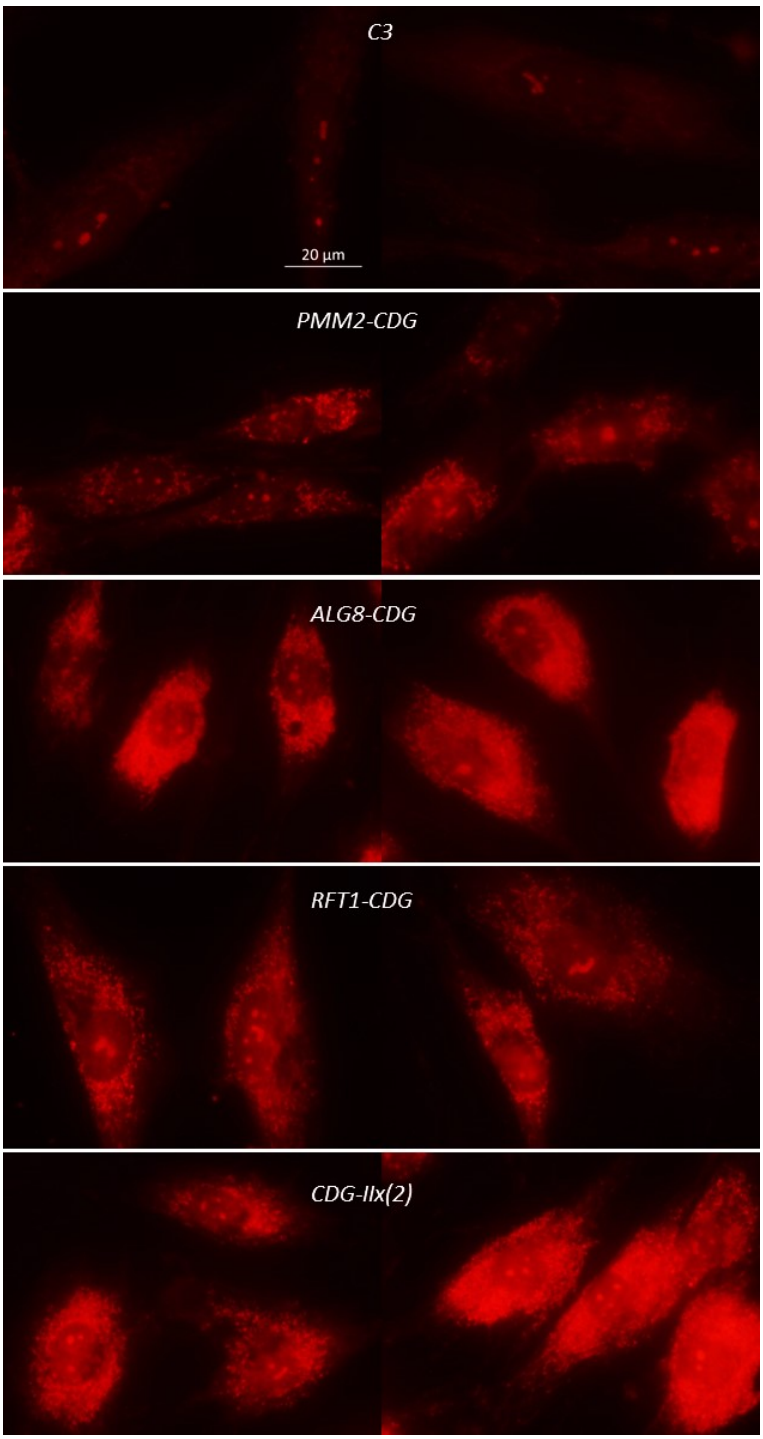

Fig. 13d (part 1/2): Detection of ROS levels in fibroblasts from selected CDG patients. Reactive oxygen species (ROS) were detected in the analyzed cells by immunofluorescent assay using dihydroethidium (DHE). Red fluorescent signal, which reflects the intracellular levels of superoxide anions, was observed to be significantly increased in all studied CDG cell lines. The analysis consisted of two separate experiments (part 1, part 2 of the figure) and for each tested cell line, two representative images are shown.

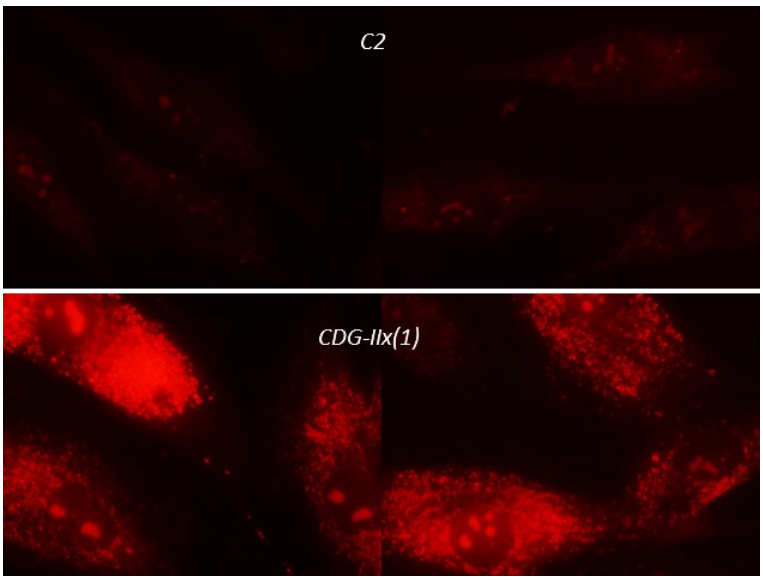

Fig. 13d (part 2/2): Detection of ROS levels in fibroblasts from selected CDG patients. Reactive oxygen species (ROS) were detected in the analyzed cells by immunofluorescent assay using dihydroethidium (DHE). Red fluorescent signal, which reflects the intracellular levels of superoxide anions, was observed to be significantly increased in all studied CDG cell lines. The analysis consisted of two separate experiments (part 1, part 2 of the figure) and for each tested cell line, two representative images are shown.

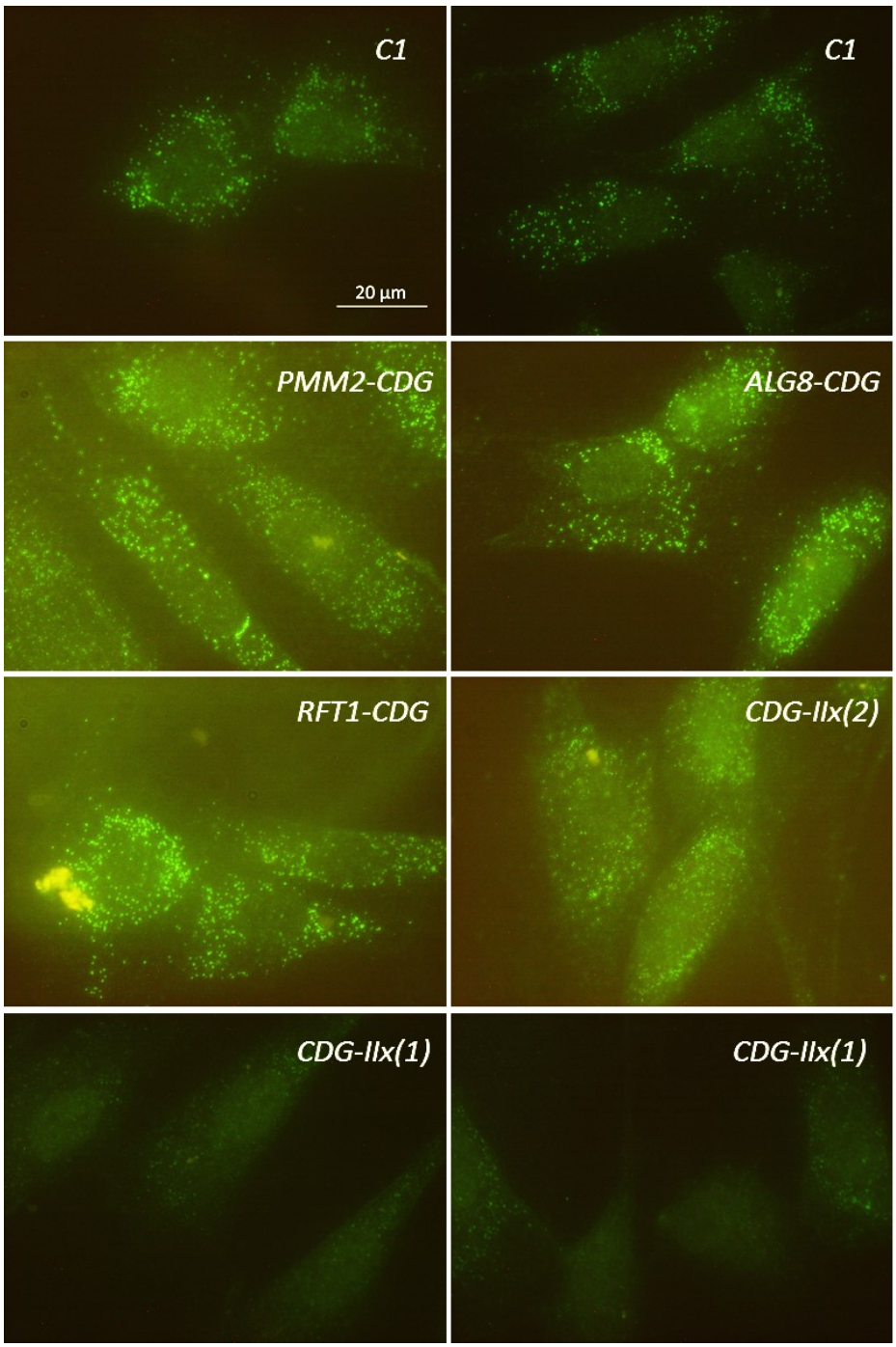

Fig. 13e: Catalase staining in fibroblasts from selected CDG patients. The marker enzyme of peroxisomes, catalase, was detected using immunocytochemical method (green fluorescent signal). Its characteristic localization in peroxisomes - a dotted pattern - was observed to be reduced in the CDG-IIx(1) patient.

4.3 Results and discussion related to the aim C.)

Alterations in protein glycosylation of non-CDG etiology.

I. Publication related to the aim C.):

1] Ondrušková N, Honzík T, Kytnarová J, Matoulek M, Zeman J, Hansíková H. ***Isoelectric Focusing of Serum Apolipoprotein C-III as a Sensitive Screening Method for the Detection of O-glycosylation Disturbances.*** *Prague Med Rep. 2015;116(2):73-86.*

II. Manuscript (under revision) related to the aim C.):

2] Ondruskova N, Honzik T, Kolarova H, Zeman J, Hansikova H. ***ApoC-III hypoglycosylation in glycogen storage diseases: the role of UDP-GalNAc depletion?*** *J Inherit Metab Dis. 2016; xx(x):x.*

Author' s contribution: biochemical analyses of selected glycoproteins and manuscript preparation.

4.3.1 Prader-Willi patients with elevated triacylglycerols show ApoC-III hypoglycosylation.

1] Mild ApoC-III hypoglycosylation was found in 4/10 of the analyzed patients with Prader-Willi syndrome in our study, and we suggest that this might be linked to their increased plasma triacylglycerols (for more detail, see Chapter 4.1.4 and the article in Supplement).

4.3.2 Novel observation of ApoC-III hypoglycosylation in various glycogen storage diseases.

2] The recategorization of phosphoglucomutase 1 (PGM1) deficiency, which originally belonged to the group of glycogen storage diseases (precisely, GSD type XIV) and since 2012 has been classified as CDG, has inspired us to examine possible glycosylation abnormalities in serum from our group of 30 patients with previously, enzymatically or genetically, established GSD diagnosis. Our aim was to analyze the glycosylation status of CDG screening markers, N- and O-glycoproteins TF and ApoC-III, by isoelectric focusing followed by immunofixation or Western blot in sera from the following group of GSD patients: 2x type 0, 7x Ia, 3x non-Ia (Ib), 6x II, 7x III, 1x VI and 4x IX. While most of the patients had normal and few of them had borderline TF glycosylation, mild to profound ApoC-III hypoglycosylation was an unexpected and repeated finding in subjects with GSD Ia (6/7), non-Ia (2/3), III (7/7), VI (1/1) and IX (4/4). GSD type III, VI and IX patients showed the most pathological profile, with relatively decreased amount of disialoApoC-III (mean values: 22 % vs 43.4 % in controls), accompanied by relatively increased monosialoApoC-III (68.2 % vs 53.3 %) and/or asialoApoC-III (9.8 % vs 3.3 %). To find out whether the detected ApoC-III_0 fraction represented ApoC-III with shortened glycan chain or no glycan at all, we run the samples on SDS-PAGE, along with a control sample after neuraminidase treatment. Based on the position of the bands, we presume that (in GSD patients) there is an increased abundance of ApoC-III_0 which carries no glycan, i.e. is aglycosylated; see Fig. 14. Because dyslipidemia is a frequent finding in GSD and in our previous work we had hypothesized a role of elevated triacylglycerols (TAG) in etiology of the slight ApoC-III hypoglycosylation seen in our Prader-Willi patients, four individuals with hypertriglyceridemia (1.7 – 14.1 mmol/l) of different origin were subsequently tested. Their normal to borderline ApoC-III sialylation indicated that TAG could not be the causative factor for the observed markedly hypoglycosylated ApoC-III in GSD. It is relevant to note that GSD III, VI and IX share a common defect in metabolic conversion of glycogen to Glc-1-P, suggesting that the reason for ApoC-III hypoglycosylation could be the resulting reduced availability of glycosylation substrates, namely UDP-GalNAc (the first monosaccharide bound to ApoC-III polypeptide; not present in TF glycan). As for GSD type

Ia and non-Ia, intriguingly, there seemed to be a connection between the detected ApoC-III hypoglycosylation and the extent of their metabolic (de)compensation - characterized by evaluating multiple parameters: their glycemia, lipidemia, hepatic function or various clinical complications. It is known that the first step of ApoC-III O-glycosylation is specifically catalyzed by polypeptide N-acetylgalactosaminyltransferase 2 encoded by *GALNT2*, and expression of *GALNT2* was previously found to be decreased e.g. in the patients with type 2 diabetes, perhaps due to their hyperglycemia [217]. Furthermore, there is a relationship between *GALNT2* expression and TAG concentration; GWAS studies implicated a link between certain SNPs in *GALNT2* and TAG/cholesterol levels [214], and probands with *GALNT2* mutations leading to reduced catalytic activity of the enzyme showed lower TAG and elevated HDL [229]. In our analysis, however, no single metabolic factor was identified as the molecular link underlying reduced ApoC-III glycosylation. Overall, our results not only can be useful in differential diagnosis, but also might help broaden the knowledge of GSD pathophysiology (in particular, of GSD type III, VI and IX). In addition, they suggest that ApoC-III glycosylation status could potentially serve as a marker in monitoring the disease course in GSD type Ia and non-Ia.

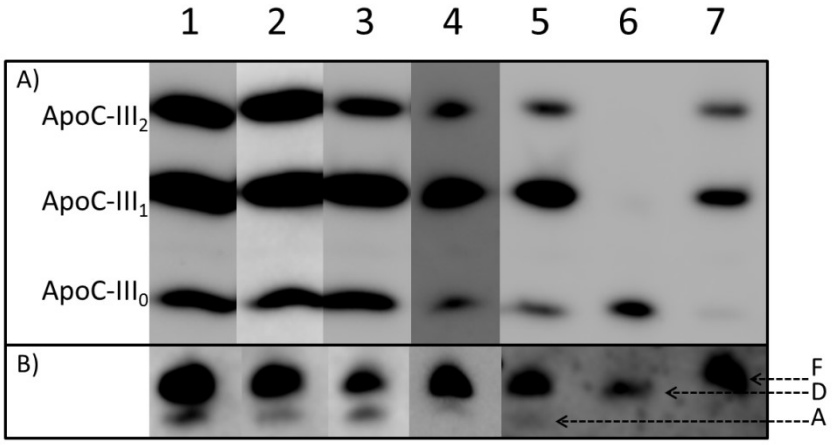

Fig. 14: ApoC-III glycosylation in selected samples from patients with various types of GSD. Serum analyzed by **A]** isoelectric focusing and **B]** SDS-PAGE of ApoC-III. Lane 1: GSD type Ia, lane 2: GSD non-Ia, lane 3: GSD III, lane 4: GSD VI, lane 5: GSD IX, lane 6/7: a healthy control, serum with/without previous neuraminidase treatment. *ApoC-III$_{2,1,0}$: asialo-, monosialo- and disialoApoC-III. Arrows on the right indicate fully glycosylated form (F), desialylated form (D) and aglycosylated form (A) of ApoC-III, as judged by their position.*

5 CONCLUSION

The overall goal of the present dissertation was to study the biochemical and molecular genetic aspects of congenital disorders of glycosylation (CDG) in the context of the Czech and Slovak Republic, what makes a unique research project within our geographic region. The contributions of this work include introducing laboratory methods to screen and further biochemically characterize CDG, establishing genetic diagnosis of specific glycosylation disorders in a group of probands, as well as bringing original findings in terms of differential diagnosis, the cellular pathophysiology and clinical manifestations of various CDG types. The core of the thesis is based on the published articles from the author and her collaborators, extended by some unpublished experiments. After elaboration of the research problem, three specific aims of the thesis were proposed and each of them was then addressed in the corresponding subsection of the Chapter 4 (Results and Discussion).

The first section focused on selective CDG screening, which involves the biochemical analysis of serum markers, transferrin and apolipoprotein C-III, by isoelectric focusing. In a boy with unexplained symptoms, we report a finding of an unusual novel *TF* mutation, which leads to the loss of one glycosylation site and thus hampers the use of TF analysis for detecting an N-glycosylation defect. The next study, on the other hand, shows the benefit of this simple screening test helping to exclude N-glycosylation disorder despite a strong clinical suspicion of PMM2-CDG in a female neonate, who was later diagnosed with S-adenosylhomocysteine hydrolase deficiency. In collaboration with Van Scherpenzeel et al., we successfully diagnosed our CDG-IIx male patient by means of a new methodical approach that allowed for a direct diagnosis of MAN1B1-CDG from serum based on the patient's distinct MS spectrum pattern. The last article describes the introduction of ApoC-III analysis for the screening of O-glycosylation disturbances in our laboratory; we also make a noteworthy observation of the relationship between the relative distribution of ApoC-III glycoforms and the levels of serum triacylglycerols.

The middle part dealt with further laboratory characterization of CDG suspected patients: the first article discusses two siblings with the rare RFT1-CDG syndrome (only six patients had been previously reported worldwide) and the second one describes a boy with the treatable phosphoglucomutase 1 deficiency (PGM1-CDG). All three patients were the first diagnosed cases of these rare disorders in Czech republic, and both studies make novel contributions by extending the knowledge on genotype and phenotype spectrum of CDG. Next, in collaboration with other groups, we identify a new glycosylation disorder due to

defect of NgBR - a protein involved in dolichol biosynthesis. This is followed by the author's unpublished experiments that examine cellular structure and pathobiochemistry in the cultivated fibroblasts from selected patients with glycosylation disorders, involving the detection of significantly increased levels of cellular ROS in CDG that has not yet been described elsewhere.

In the last section, serum glycosylation abnormalities of non-CDG origin were investigated in a group of patients with various types of glycogen storage diseases. The novel finding of pathological ApoC-III glycosylation in certain GSD types is a useful information for CDG screening, as well as potentially uncovers previously unrecognized aspects of GSD pathophysiology.

In conclusion, the proposed aims of the thesis were accomplished. Since the beginning of our CDG research, we have biochemically and genetically diagnosed 32 patients, with 10 different types of CDG (PMM2-CDG: n = 20; EXT1/EXT2-CDG: n = 3; RFT1-CDG: n = 2; NGBR-CDG: n = 2; ALG8-CDG: n = 1; DPAGT1-CDG: n = 1; SRD5A3-CDG: n = 1; MAN1B1-CDG: n = 1; PGM1-CDG: n = 1). The present work has substantially extended the spectrum of applied methods to identify the underlying cause of specific glycosylation disorders in patients with clinical suspicion of CDG, and thus has improved the diagnostic options for these diseases in the Czech Republic. Simultaneously, it has enabled the prenatal testing and genetic counselling in the affected families. And last but not least, the presented results obtained within international collaboration have strengthened our laboratory's position in the worldwide CDG community.

REFERENCES

1. Apweiler, R., Hermjakob, H., and Sharon, N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim Biophys Acta *1473*, 4-8.

2. Spiro, R.G. (2002). Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology *12*, 43R-56R.

3. Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. Glycobiology *3*, 97-130.

4. Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., and Etzler, M.E. (2009). Essentials of Glycobiology. 2nd edition., (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press).

5. Spiess, M. (1990). The asialoglycoprotein receptor: a model for endocytic transport receptors. Biochemistry *29*, 10009-10018.

6. Sperandio, M., Gleissner, C.A., and Ley, K. (2009). Glycosylation in immune cell trafficking. Immunol Rev *230*, 97-113.

7. Karlsson, K.A. (1995). Microbial recognition of target-cell glycoconjugates. Curr Opin Struct Biol *5*, 622-635.

8. Hammond, C., Braakman, I., and Helenius, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc Natl Acad Sci U S A *91*, 913-917.

9. Moremen, K.W., Tiemeyer, M., and Nairn, A.V. (2012). Vertebrate protein glycosylation: diversity, synthesis and function. Nat Rev Mol Cell Biol *13*, 448-462.

10. Wopereis, S., Lefeber, D.J., Morava, E., and Wevers, R.A. (2006). Mechanisms in protein O-glycan biosynthesis and clinical and molecular aspects of protein O-glycan biosynthesis defects: a review. Clin Chem *52*, 574-600.

11. Bennett, E.P., Mandel, U., Clausen, H., Gerken, T.A., Fritz, T.A., and Tabak, L.A. (2012). Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. Glycobiology *22*, 736-756.

12. Defaus, S., Gupta, P., Andreu, D., and Gutiérrez-Gallego, R. (2014). Mammalian protein glycosylation--structure versus function. Analyst *139*, 2944-2967.

13. Hart, G.W., and Copeland, R.J. (2010). Glycomics hits the big time. Cell *143*, 672-676.

14. Glick, B.S., and Luini, A. (2011). Models for Golgi traffic: a critical assessment. Cold Spring Harb Perspect Biol *3*, a005215.

15. Rizzo, R., Parashuraman, S., Mirabelli, P., Puri, C., Lucocq, J., and Luini, A. (2013). The dynamics of engineered resident proteins in the mammalian Golgi complex relies on cisternal maturation. J Cell Biol *201*, 1027-1036.

16. Comelli, E.M., Head, S.R., Gilmartin, T., Whisenant, T., Haslam, S.M., North, S.J., Wong, N.K., Kudo, T., Narimatsu, H., Esko, J.D., et al. (2006). A focused microarray approach to functional glycomics: transcriptional regulation of the glycome. Glycobiology *16*, 117-131.

17. Haltiwanger, R.S., and Lowe, J.B. (2004). Role of glycosylation in development. Annu Rev Biochem *73*, 491-537.

18. Daniels, M.A., Hogquist, K.A., and Jameson, S.C. (2002). Sweet 'n' sour: the impact of differential glycosylation on T cell responses. Nat Immunol *3*, 903-910.

19. Braakman, I., and Hebert, D.N. (2013). Protein folding in the endoplasmic reticulum. Cold Spring Harb Perspect Biol *5*, a013201.

20. Malhotra, J.D., and Kaufman, R.J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal *9*, 2277-2293.

21. Jaeken, J., van Eijk, H.G., van der Heul, C., Corbeel, L., Eeckels, R., and Eggermont, E. (1984). Sialic acid-deficient serum and cerebrospinal fluid transferrin in a newly recognized genetic syndrome. Clin Chim Acta *144*, 245-247.

22. Matthijs, G., Schollen, E., Pardon, E., Veiga-Da-Cunha, M., Jaeken, J., Cassiman, J.J., and Van Schaftingen, E. (1997). Mutations in PMM2, a phosphomannomutase gene on chromosome 16p13, in carbohydrate-deficient glycoprotein type I syndrome (Jaeken syndrome). Nat Genet *16*, 88-92.

23. Matthijs, G., Rymen, D., Millón, M.B., Souche, E., and Race, V. (2013). Approaches to homozygosity mapping and exome sequencing for the identification of novel types of CDG. Glycoconj J *30*, 67-76.

24. Jaeken, J., Hennet, T., Matthijs, G., and Freeze, H.H. (2009). CDG nomenclature: time for a change! Biochim Biophys Acta *1792*, 825-826.

25. Jaeken, J., and Matthijs, G. (2001). Congenital disorders of glycosylation. Annu Rev Genomics Hum Genet *2*, 129-151.

26. Schachter, H., and Freeze, H.H. (2009). Glycosylation diseases: quo vadis? Biochim Biophys Acta *1792*, 925-930.

27. Grunewald, S., Matthijs, G., and Jaeken, J. (2002). Congenital disorders of glycosylation: a review. Pediatr Res *52*, 618-624.

28. Cylwik, B., Naklicki, M., Chrostek, L., and Gruszewska, E. (2013). Congenital disorders of glycosylation. Part I. Defects of protein N-glycosylation. Acta Biochim Pol *60*, 151-161.

29. Wu, X., Rush, J.S., Karaoglu, D., Krasnewich, D., Lubinsky, M.S., Waechter, C.J., Gilmore, R., and Freeze, H.H. (2003). Deficiency of UDP-GlcNAc:Dolichol Phosphate N-Acetylglucosamine-1 Phosphate Transferase (DPAGT1) causes a novel congenital disorder of Glycosylation Type Ij. Hum Mutat *22*, 144-150.

30. Schwarz, M., Thiel, C., Lübbehusen, J., Dorland, B., de Koning, T., von Figura, K., Lehle, L., and Körner, C. (2004). Deficiency of GDP-Man:GlcNAc2-PP-dolichol mannosyltransferase causes congenital disorder of glycosylation type Ik. Am J Hum Genet *74*, 472-481.

31. Thiel, C., Schwarz, M., Peng, J., Grzmil, M., Hasilik, M., Braulke, T., Kohlschütter, A., von

Figura, K., Lehle, L., and Körner, C. (2003). A new type of congenital disorders of glycosylation (CDG-Ii) provides new insights into the early steps of dolichol-linked oligosaccharide biosynthesis. J Biol Chem *278*, 22498-22505.

32. Körner, C., Knauer, R., Stephani, U., Marquardt, T., Lehle, L., and von Figura, K. (1999). Carbohydrate deficient glycoprotein syndrome type IV: deficiency of dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase. EMBO J *18*, 6816-6822.

33. Imbach, T., Burda, P., Kuhnert, P., Wevers, R.A., Aebi, M., Berger, E.G., and Hennet, T. (1999). A mutation in the human ortholog of the Saccharomyces cerevisiae ALG6 gene causes carbohydrate-deficient glycoprotein syndrome type-Ic. Proc Natl Acad Sci U S A *96*, 6982-6987.

34. Chantret, I., Dancourt, J., Dupré, T., Delenda, C., Bucher, S., Vuillaumier-Barrot, S., Ogier de Baulny, H., Peletan, C., Danos, O., Seta, N., et al. (2003). A deficiency in dolichyl-P-glucose:Glc1Man9GlcNAc2-PP-dolichyl alpha3-glucosyltransferase defines a new subtype of congenital disorders of glycosylation. J Biol Chem *278*, 9962-9971.

35. Frank, C.G., Grubenmann, C.E., Eyaid, W., Berger, E.G., Aebi, M., and Hennet, T. (2004). Identification and functional analysis of a defect in the human ALG9 gene: definition of congenital disorder of glycosylation type IL. Am J Hum Genet *75*, 146-150.

36. Rind, N., Schmeiser, V., Thiel, C., Absmanner, B., Lübbehusen, J., Hocks, J., Apeshiotis, N., Wilichowski, E., Lehle, L., and Körner, C. (2010). A severe human metabolic disease caused by deficiency of the endoplasmatic mannosyltransferase hALG11 leads to congenital disorder of glycosylation-Ip. Hum Mol Genet *19*, 1413-1424.

37. Chantret, I., Dupré, T., Delenda, C., Bucher, S., Dancourt, J., Barnier, A., Charollais, A., Heron, D., Bader-Meunier, B., Danos, O., et al. (2002). Congenital disorders of glycosylation type Ig is defined by a deficiency in dolichyl-P-mannose:Man7GlcNAc2-PP-dolichyl mannosyltransferase. J Biol Chem *277*, 25815-25822.

38. Timal, S., Hoischen, A., Lehle, L., Adamowicz, M., Huijben, K., Sykut-Cegielska, J., Paprocka, J., Jamroz, E., van Spronsen, F.J., Körner, C., et al. (2012). Gene identification in the congenital disorders of glycosylation type I by whole-exome sequencing. Hum Mol Genet *21*, 4151-4161.

39. Cossins, J., Belaya, K., Hicks, D., Salih, M.A., Finlayson, S., Carboni, N., Liu, W.W., Maxwell, S., Zoltowska, K., Farsani, G.T., et al. (2013). Congenital myasthenic syndromes due to mutations in ALG2 and ALG14. Brain *136*, 944-956.

40. Haeuptle, M.A., Pujol, F.M., Neupert, C., Winchester, B., Kastaniotis, A.J., Aebi, M., and Hennet, T. (2008). Human RFT1 deficiency leads to a disorder of N-linked glycosylation. Am J Hum Genet *82*, 600-606.

41. Molinari, F., Foulquier, F., Tarpey, P.S., Morelle, W., Boissel, S., Teague, J., Edkins, S., Futreal, P.A., Stratton, M.R., Turner, G., et al. (2008). Oligosaccharyltransferase-subunit mutations in nonsyndromic mental retardation. Am J Hum Genet *82*, 1150-1157.

42. De Praeter CM, Gerwig, G.J., Bause, E., Nuytinck, L.K., Vliegenthart, J.F., Breuer, W., Kamerling, J.P., Espeel, M.F., Martin, J.J., De Paepe AM, et al. (2000). A novel disorder caused by defective biosynthesis of N-linked oligosaccharides due to glucosidase I deficiency. Am J Hum Genet *66*, 1744-1756.

43. Rafiq, M.A., Kuss, A.W., Puettmann, L., Noor, A., Ramiah, A., Ali, G., Hu, H., Kerio, N.A., Xiang, Y., Garshasbi, M., et al. (2011). Mutations in the alpha 1,2-mannosidase gene, MAN1B1, cause autosomal-recessive intellectual disability. Am J Hum Genet *89*, 176-182.

44. Jaeken, J., Schachter, H., Carchon, H., De Cock, P., Coddeville, B., and Spik, G. (1994). Carbohydrate deficient glycoprotein syndrome type II: a deficiency in Golgi localised N-acetyl-glucosaminyltransferase II. Arch Dis Child *71*, 123-127.

45. Need, A.C., Shashi, V., Hitomi, Y., Schoch, K., Shianna, K.V., McDonald, M.T., Meisler, M.H., and Goldstein, D.B. (2012). Clinical application of exome sequencing in undiagnosed genetic conditions. J Med Genet *49*, 353-361.

46. Enns, G.M., Shashi, V., Bainbridge, M., Gambello, M.J., Zahir, F.R., Bast, T., Crimian, R., Schoch, K., Platt, J., Cox, R., et al. (2014). Mutations in NGLY1 cause an inherited disorder of the endoplasmic reticulum-associated degradation pathway. Genet Med *16*, 751-758.

47. Tiede, S., Storch, S., Lübke, T., Henrissat, B., Bargal, R., Raas-Rothschild, A., and Braulke, T. (2005). Mucolipidosis II is caused by mutations in GNPTA encoding the alpha/beta GlcNAc-1-phosphotransferase. Nat Med *11*, 1109-1112.

48. Bargal, R., Zeigler, M., Abu-Libdeh, B., Zuri, V., Mandel, H., Ben Neriah, Z., Stewart, F., Elcioglu, N., Hindi, T., Le Merrer, M., et al. (2006). When Mucolipidosis III meets Mucolipidosis II: GNPTA gene mutations in 24 patients. Mol Genet Metab *88*, 359-363.

49. Brockington, M., Blake, D.J., Prandini, P., Brown, S.C., Torelli, S., Benson, M.A., Ponting, C.P., Estournet, B., Romero, N.B., Mercuri, E., et al. (2001). Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin alpha2 deficiency and abnormal glycosylation of alpha-dystroglycan. Am J Hum Genet *69*, 1198-1209.

50. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., et al. (1998). An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. Nature *394*, 388-392.

51. Beltrán-Valero de Bernabé, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W.B., et al. (2002). Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. Am J Hum Genet *71*, 1033-1043.

52. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabé, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., et al. (2005). POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. J Med Genet *42*, 907-912.

53. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., et al. (2001). Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell *1*, 717-724.

54. Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R.K., Voit, T., Merlini, L., et al. (2003). Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. Hum Mol Genet *12*, 2853-2861.

55. Willer, T., Lee, H., Lommel, M., Yoshida-Moriguchi, T., de Bernabe, D.B., Venzke, D., Cirak, S., Schachter, H., Vajsar, J., Voit, T., et al. (2012). ISPD loss-of-function mutations disrupt dystroglycan O-mannosylation and cause Walker-Warburg syndrome. Nat Genet *44*, 575-580.

56. Manzini, M.C., Tambunan, D.E., Hill, R.S., Yu, T.W., Maynard, T.M., Heinzen, E.L., Shianna, K.V., Stevens, C.R., Partlow, J.N., Barry, B.J., et al. (2012). Exome sequencing and functional validation in zebrafish identify GTDC2 mutations as a cause of Walker-Warburg syndrome. Am J Hum Genet *91*, 541-547.

57. Vuillaumier-Barrot, S., Bouchet-Séraphin, C., Chelbi, M., Devisme, L., Quentin, S., Gazal, S., Laquerrière, A., Fallet-Bianco, C., Loget, P., Odent, S., et al. (2012). Identification of mutations in TMEM5 and ISPD as a cause of severe cobblestone lissencephaly. Am J Hum Genet *91*, 1135-1143.

58. Stevens, E., Carss, K.J., Cirak, S., Foley, A.R., Torelli, S., Willer, T., Tambunan, D.E., Yau, S., Brodd, L., Sewry, C.A., et al. (2013). Mutations in B3GALNT2 cause congenital muscular dystrophy and hypoglycosylation of α-dystroglycan. Am J Hum Genet *92*, 354-365.

59. Jae, L.T., Raaben, M., Riemersma, M., van Beusekom, E., Blomen, V.A., Velds, A., Kerkhoven, R.M., Carette, J.E., Topaloglu, H., Meinecke, P., et al. (2013). Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. Science *340*, 479-483.

60. Buysse, K., Riemersma, M., Powell, G., van Reeuwijk, J., Chitayat, D., Roscioli, T., Kamsteeg, E.J., van den Elzen, C., van Beusekom, E., Blaser, S., et al. (2013). Missense mutations in β-1,3-N-acetylglucosaminyltransferase 1 (B3GNT1) cause Walker-Warburg syndrome. Hum Mol Genet *22*, 1746-1754.

61. Carss, K.J., Stevens, E., Foley, A.R., Cirak, S., Riemersma, M., Torelli, S., Hoischen, A., Willer, T., van Scherpenzeel, M., Moore, S.A., et al. (2013). Mutations in GDP-mannose pyrophosphorylase B cause congenital and limb-girdle muscular dystrophies associated with hypoglycosylation of α-dystroglycan. Am J Hum Genet *93*, 29-41.

62. Ahn, J., Lüdecke, H.J., Lindow, S., Horton, W.A., Lee, B., Wagner, M.J., Horsthemke, B., and Wells, D.E. (1995). Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). Nat Genet *11*, 137-143.

63. Stickens, D., Clines, G., Burbee, D., Ramos, P., Thomas, S., Hogue, D., Hecht, J.T., Lovett, M., and Evans, G.A. (1996). The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. Nat Genet *14*, 25-32.

64. Wuyts, W., Van Hul, W., Wauters, J., Nemtsova, M., Reyniers, E., Van Hul, E.V., De Boulle, K., de Vries, B.B., Hendrickx, J., Herrygers, I., et al. (1996). Positional cloning of a gene involved in hereditary multiple exostoses. Hum Mol Genet *5*, 1547-1557.

65. Nakajima, M., Mizumoto, S., Miyake, N., Kogawa, R., Iida, A., Ito, H., Kitoh, H., Hirayama, A., Mitsubuchi, H., Miyazaki, O., et al. (2013). Mutations in B3GALT6, which encodes a glycosaminoglycan linker region enzyme, cause a spectrum of skeletal and connective tissue disorders. Am J Hum Genet *92*, 927-934.

66. Okajima, T., Fukumoto, S., Furukawa, K., and Urano, T. (1999). Molecular basis for the progeroid variant of Ehlers-Danlos syndrome. Identification and characterization of two mutations in galactosyltransferase I gene. J Biol Chem *274*, 28841-28844.

67. Almeida, R., Levery, S.B., Mandel, U., Kresse, H., Schwientek, T., Bennett, E.P., and Clausen, H. (1999). Cloning and expression of a proteoglycan UDP-galactose:beta-xylose beta1,4-galactosyltransferase I. A seventh member of the human beta4-galactosyltransferase gene family. J Biol Chem *274*, 26165-26171.

68. Thiele, H., Sakano, M., Kitagawa, H., Sugahara, K., Rajab, A., Höhne, W., Ritter, H., Leschik, G., Nürnberg, P., and Mundlos, S. (2004). Loss of chondroitin 6-O-sulfotransferase-1 function results in severe human chondrodysplasia with progressive spinal involvement. Proc Natl Acad Sci U S A *101*, 10155-10160.

69. Akama, T.O., Nishida, K., Nakayama, J., Watanabe, H., Ozaki, K., Nakamura, T., Dota, A., Kawasaki, S., Inoue, Y., Maeda, N., et al. (2000). Macular corneal dystrophy type I and type II are caused by distinct mutations in a new sulphotransferase gene. Nat Genet *26*, 237-241.

70. Cabral, R.M., Kurban, M., Wajid, M., Shimomura, Y., Petukhova, L., and Christiano, A.M. (2012). Whole-exome sequencing in a single proband reveals a mutation in the CHST8 gene in autosomal recessive peeling skin syndrome. Genomics *99*, 202-208.

71. Dündar, M., Müller, T., Zhang, Q., Pan, J., Steinmann, B., Vodopiutz, J., Gruber, R., Sonoda, T., Krabichler, B., Utermann, G., et al. (2009). Loss of dermatan-4-sulfotransferase 1 function results in adducted thumb-clubfoot syndrome. Am J Hum Genet *85*, 873-882.

72. Li, M., Cheng, R., Liang, J., Yan, H., Zhang, H., Yang, L., Li, C., Jiao, Q., Lu, Z., He, J., et al. (2013). Mutations in POFUT1, encoding protein O-fucosyltransferase 1, cause generalized Dowling-Degos disease. Am J Hum Genet *92*, 895-903.

73. Basmanav, F.B., Oprisoreanu, A.M., Pasternack, S.M., Thiele, H., Fritz, G., Wenzel, J., Größer, L., Wehner, M., Wolf, S., Fagerberg, C., et al. (2014). Mutations in POGLUT1, encoding protein O-glucosyltransferase 1, cause autosomal-dominant Dowling-Degos disease. Am J Hum Genet *94*, 135-143.

74. Sparrow, D.B., Chapman, G., Wouters, M.A., Whittock, N.V., Ellard, S., Fatkin, D., Turnpenny, P.D., Kusumi, K., Sillence, D., and Dunwoodie, S.L. (2006). Mutation of the LUNATIC FRINGE gene in humans causes spondylocostal dysostosis with a severe vertebral phenotype. Am J Hum Genet *78*, 28-37.

75. Lesnik Oberstein, S.A., Kriek, M., White, S.J., Kalf, M.E., Szuhai, K., den Dunnen, J.T., Breuning, M.H., and Hennekam, R.C. (2006). Peters Plus syndrome is caused by mutations in B3GALTL, a putative glycosyltransferase. Am J Hum Genet *79*, 562-566.

76. Ogawa, M., Sawaguchi, S., Kawai, T., Nadano, D., Matsuda, T., Yagi, H., Kato, K., Furukawa, K., and Okajima, T. (2015). Impaired O-linked N-acetylglucosaminylation in the endoplasmic reticulum by mutated epidermal growth factor (EGF) domain-specific O-linked N-acetylglucosamine transferase found in Adams-Oliver syndrome. J Biol Chem *290*, 2137-2149.

77. Wells, L. (2015). Identification and characterization of missense mutations in O-GlcNAc transferase associated with X-linked intellectual disability. In 2015 SFG Annual Meeting. (San Francisco, CA).

78. Topaz, O., Shurman, D.L., Bergman, R., Indelman, M., Ratajczak, P., Mizrachi, M., Khamaysi, Z., Behar, D., Petronius, D., Friedman, V., et al. (2004). Mutations in GALNT3, encoding a protein involved in O-linked glycosylation, cause familial tumoral calcinosis. Nat Genet *36*, 579-581.

79. Yeowell, H.N., and Walker, L.C. (2000). Mutations in the lysyl hydroxylase 1 gene that result in enzyme deficiency and the clinical phenotype of Ehlers-Danlos syndrome type VI. Mol Genet Metab *71*, 212-224.

80. Simpson, M.A., Cross, H., Proukakis, C., Priestman, D.A., Neville, D.C., Reinkensmeier, G., Wang, H., Wiznitzer, M., Gurtz, K., Verganelaki, A., et al. (2004). Infantile-onset symptomatic epilepsy syndrome caused by a homozygous loss-of-function mutation of GM3 synthase. Nat Genet *36*, 1225-1229.

81. Boccuto, L., Aoki, K., Flanagan-Steet, H., Chen, C.F., Fan, X., Bartel, F., Petukh, M., Pittman, A., Saul, R., Chaubey, A., et al. (2014). A mutation in a ganglioside biosynthetic enzyme, ST3GAL5, results in salt & pepper syndrome, a neurocutaneous disorder with altered glycolipid and glycoprotein glycosylation. Hum Mol Genet *23*, 418-433.

82. Boukhris, A., Schule, R., Loureiro, J.L., Lourenço, C.M., Mundwiller, E., Gonzalez, M.A., Charles, P., Gauthier, J., Rekik, I., Acosta Lebrigio, R.F., et al. (2013). Alteration of ganglioside biosynthesis responsible for complex hereditary spastic paraplegia. Am J Hum Genet *93*, 118-123.

83. Freeze, H.H., Eklund, E.A., Ng, B.G., and Patterson, M.C. (2015). Neurological aspects of human glycosylation disorders. Annu Rev Neurosci *38*, 105-125.

84. Takeda, J., Miyata, T., Kawagoe, K., Iida, Y., Endo, Y., Fujita, T., Takahashi, M., Kitani, T., and Kinoshita, T. (1993). Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell *73*, 703-711.

85. Johnston, J.J., Gropman, A.L., Sapp, J.C., Teer, J.K., Martin, J.M., Liu, C.F., Yuan, X., Ye, Z., Cheng, L., Brodsky, R.A., et al. (2012). The phenotype of a germline mutation in PIGA: the gene somatically mutated in paroxysmal nocturnal hemoglobinuria. Am J Hum Genet *90*, 295-300.

86. Ilkovski, B., Pagnamenta, A.T., O'Grady, G.L., Kinoshita, T., Howard, M.F., Lek, M., Thomas, B., Turner, A., Christodoulou, J., Sillence, D., et al. (2015). Mutations in PIGY: expanding the phenotype of inherited glycosylphosphatidylinositol deficiencies. Hum Mol Genet *24*, 6146-6159.

87. Ng, B.G., Hackmann, K., Jones, M.A., Eroshkin, A.M., He, P., Wiliams, R., Bhide, S., Cantagrel, V., Gleeson, J.G., Paller, A.S., et al. (2012). Mutations in the glycosylphosphatidylinositol gene PIGL cause CHIME syndrome. Am J Hum Genet *90*, 685-688.

88. Chiyonobu, T., Inoue, N., Morimoto, M., Kinoshita, T., and Murakami, Y. (2014). Glycosylphosphatidylinositol (GPI) anchor deficiency caused by mutations in PIGW is associated with West syndrome and hyperphosphatasia with mental retardation syndrome. J Med Genet *51*, 203-207.

89. Almeida, A.M., Murakami, Y., Layton, D.M., Hillmen, P., Sellick, G.S., Maeda, Y., Richards, S., Patterson, S., Kotsianidis, I., Mollica, L., et al. (2006). Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency. Nat Med *12*, 846-851.

90. Krawitz, P.M., Schweiger, M.R., Rödelsperger, C., Marcelis, C., Kölsch, U., Meisel, C., Stephani, F., Kinoshita, T., Murakami, Y., Bauer, S., et al. (2010). Identity-by-descent filtering of exome sequence data identifies PIGV mutations in hyperphosphatasia mental retardation syndrome. Nat Genet *42*, 827-829.

91. Maydan, G., Noyman, I., Har-Zahav, A., Neriah, Z.B., Pasmanik-Chor, M., Yeheskel, A., Albin-Kaplanski, A., Maya, I., Magal, N., Birk, E., et al. (2011). Multiple congenital anomalies-hypotonia-seizures syndrome is caused by a mutation in PIGN. J Med Genet *48*, 383-389.

92. Krawitz, P.M., Murakami, Y., Hecht, J., Krüger, U., Holder, S.E., Mortier, G.R., Delle Chiaie, B., De Baere, E., Thompson, M.D., Roscioli, T., et al. (2012). Mutations in PIGO, a member of the GPI-anchor-synthesis pathway, cause hyperphosphatasia with mental retardation. Am J Hum Genet *91*, 146-151.

93. Kvarnung, M., Nilsson, D., Lindstrand, A., Korenke, G.C., Chiang, S.C., Blennow, E., Bergmann, M., Stödberg, T., Mäkitie, O., Anderlid, B.M., et al. (2013). A novel intellectual disability syndrome caused by GPI anchor deficiency due to homozygous mutations in PIGT. J Med Genet *50*, 521-528.

94. Hansen, L., Tawamie, H., Murakami, Y., Mang, Y., ur Rehman, S., Buchert, R., Schaffer, S., Muhammad, S., Bak, M., Nöthen, M.M., et al. (2013). Hypomorphic mutations in PGAP2, encoding a GPI-anchor-remodeling protein, cause autosomal-recessive intellectual disability. Am J Hum Genet *92*, 575-583.

95. Howard, M.F., Murakami, Y., Pagnamenta, A.T., Daumer-Haas, C., Fischer, B., Hecht, J., Keays, D.A., Knight, S.J., Kölsch, U., Krüger, U., et al. (2014). Mutations in PGAP3 impair GPI-anchor maturation, causing a subtype of hyperphosphatasia with mental retardation. Am J Hum Genet *94*, 278-287.

96. Fauth, C., Steindl, K., Toutain, A., Farrell, S., Witsch-Baumgartner, M., Karall, D., Joset, P., Böhm, S., Baumer, A., Maier, O., et al. (2015). A recurrent germline mutation in the PIGA gene causes Simpson-Golabi-Behmel syndrome type 2. Am J Med Genet A.

97. Sparks, S.E., and Krasnewich, D.M. (2005 [updated 2015]). PMM2-CDG (CDG-Ia). In GeneReviews® [Internet], Pagon RA, Adam MP, Ardinger, HH and e. al., eds. (Seattle (WA): University of Washington).

98. Niehues, R., Hasilik, M., Alton, G., Körner, C., Schiebe-Sukumar, M., Koch, H.G., Zimmer, K.P., Wu, R., Harms, E., Reiter, K., et al. (1998). Carbohydrate-deficient glycoprotein syndrome type Ib. Phosphomannose isomerase deficiency and mannose therapy. J Clin Invest *101*, 1414-1420.

99. Stojkovic, T., Vissing, J., Petit, F., Piraud, M., Orngreen, M.C., Andersen, G., Claeys, K.G., Wary, C., Hogrel, J.Y., and Laforêt, P. (2009). Muscle glycogenosis due to phosphoglucomutase 1 deficiency. N Engl J Med *361*, 425-427.

100. Zhang, Y., Yu, X., Ichikawa, M., Lyons, J.J., Datta, S., Lamborn, I.T., Jing, H., Kim, E.S., Biancalana, M., Wolfe, L.A., et al. (2014). Autosomal recessive phosphoglucomutase 3 (PGM3) mutations link glycosylation defects to atopy, immune deficiency, autoimmunity, and neurocognitive impairment. J Allergy Clin Immunol *133*, 1400-1409, 1409.e1401-1405.

101. Tegtmeyer, L.C., Rust, S., van Scherpenzeel, M., Ng, B.G., Losfeld, M.E., Timal, S., Raymond, K., He, P., Ichikawa, M., Veltman, J., et al. (2014). Multiple phenotypes in phosphoglucomutase 1 deficiency. N Engl J Med *370*, 533-542.

102. Stray-Pedersen, A., Backe, P.H., Sorte, H.S., Mørkrid, L., Chokshi, N.Y., Erichsen, H.C., Gambin, T., Elgstøen, K.B., Bjørås, M., Wlodarski, M.W., et al. (2014). PGM3 mutations cause a congenital disorder of glycosylation with severe immunodeficiency and skeletal

dysplasia. Am J Hum Genet *95*, 96-107.

103. Hayee, B., Antonopoulos, A., Murphy, E.J., Rahman, F.Z., Sewell, G., Smith, B.N., McCartney, S., Furman, M., Hall, G., Bloom, S.L., et al. (2011). G6PC3 mutations are associated with a major defect of glycosylation: a novel mechanism for neutrophil dysfunction. Glycobiology *21*, 914-924.

104. Eisenberg, I., Avidan, N., Potikha, T., Hochner, H., Chen, M., Olender, T., Barash, M., Shemesh, M., Sadeh, M., Grabov-Nardini, G., et al. (2001). The UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene is mutated in recessive hereditary inclusion body myopathy. Nat Genet *29*, 83-87.

105. Seppala, R., Lehto, V.P., and Gahl, W.A. (1999). Mutations in the human UDP-N-acetylglucosamine 2-epimerase gene define the disease sialuria and the allosteric site of the enzyme. Am J Hum Genet *64*, 1563-1569.

106. Cantagrel, V., Lefeber, D.J., Ng, B.G., Guan, Z., Silhavy, J.L., Bielas, S.L., Lehle, L., Hombauer, H., Adamowicz, M., Swiezewska, E., et al. (2010). SRD5A3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder. Cell *142*, 203-217.

107. Züchner, S., Dallman, J., Wen, R., Beecham, G., Naj, A., Farooq, A., Kohli, M.A., Whitehead, P.L., Hulme, W., Konidari, I., et al. (2011). Whole-exome sequencing links a variant in DHDDS to retinitis pigmentosa. Am J Hum Genet *88*, 201-206.

108. Kranz, C., Jungeblut, C., Denecke, J., Erlekotte, A., Sohlbach, C., Debus, V., Kehl, H.G., Harms, E., Reith, A., Reichel, S., et al. (2007). A defect in dolichol phosphate biosynthesis causes a new inherited disorder with death in early infancy. Am J Hum Genet *80*, 433-440.

109. Kim, S., Westphal, V., Srikrishna, G., Mehta, D.P., Peterson, S., Filiano, J., Karnes, P.S., Patterson, M.C., and Freeze, H.H. (2000). Dolichol phosphate mannose synthase (DPM1) mutations define congenital disorder of glycosylation Ie (CDG-Ie). J Clin Invest *105*, 191-198.

110. Barone, R., Aiello, C., Race, V., Morava, E., Foulquier, F., Riemersma, M., Passarelli, C., Concolino, D., Carella, M., Santorelli, F., et al. (2012). DPM2-CDG: a muscular dystrophy-dystroglycanopathy syndrome with severe epilepsy. Ann Neurol *72*, 550-558.

111. Lefeber, D.J., Schönberger, J., Morava, E., Guillard, M., Huyben, K.M., Verrijp, K., Grafakou, O., Evangeliou, A., Preijers, F.W., Manta, P., et al. (2009). Deficiency of Dol-P-Man synthase subunit DPM3 bridges the congenital disorders of glycosylation with the dystroglycanopathies. Am J Hum Genet *85*, 76-86.

112. Schenk, B., Imbach, T., Frank, C.G., Grubenmann, C.E., Raymond, G.V., Hurvitz, H., Korn-Lubetzki, I., Revel-Vik, S., Raas-Rotschild, A., Luder, A.S., et al. (2001). MPDU1 mutations underlie a novel human congenital disorder of glycosylation, designated type If. J Clin Invest *108*, 1687-1695.

113. Lübke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K., and Körner, C. (2001). Complementation cloning identifies CDG-IIc, a new type of congenital disorders of glycosylation, as a GDP-fucose transporter deficiency. Nat Genet *28*, 73-76.

114. Lühn, K., Wild, M.K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001). The gene defective in leukocyte adhesion deficiency II encodes a putative GDP-fucose transporter. Nat Genet *28*, 69-72.

115. Martinez-Duncker, I., Dupré, T., Piller, V., Piller, F., Candelier, J.J., Trichet, C., Tchernia, G., Oriol, R., and Mollicone, R. (2005). Genetic complementation reveals a novel human congenital disorder of glycosylation of type II, due to inactivation of the Golgi CMP-sialic acid transporter. Blood *105*, 2671-2676.

116. Ng, B.G., Buckingham, K.J., Raymond, K., Kircher, M., Turner, E.H., He, M., Smith, J.D., Eroshkin, A., Szybowska, M., Losfeld, M.E., et al. (2013). Mosaicism of the UDP-galactose transporter SLC35A2 causes a congenital disorder of glycosylation. Am J Hum Genet *92*, 632-636.

117. Edvardson, S., Ashikov, A., Jalas, C., Sturiale, L., Shaag, A., Fedick, A., Treff, N.R., Garozzo, D., Gerardy-Schahn, R., and Elpeleg, O. (2013). Mutations in SLC35A3 cause autism spectrum disorder, epilepsy and arthrogryposis. J Med Genet *50*, 733-739.

118. Hiraoka, S., Furuichi, T., Nishimura, G., Shibata, S., Yanagishita, M., Rimoin, D.L., Superti-Furga, A., Nikkels, P.G., Ogawa, M., Katsuyama, K., et al. (2007). Nucleotide-sugar transporter SLC35D1 is critical to chondroitin sulfate synthesis in cartilage and skeletal development in mouse and human. Nat Med *13*, 1363-1367.

119. Ungar, D., Oka, T., Brittle, E.E., Vasile, E., Lupashin, V.V., Chatterton, J.E., Heuser, J.E., Krieger, M., and Waters, M.G. (2002). Characterization of a mammalian Golgi-localized protein complex, COG, that is required for normal Golgi morphology and function. J Cell Biol *157*, 405-415.

120. Zolov, S.N., and Lupashin, V.V. (2005). Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells. J Cell Biol *168*, 747-759.

121. Shestakova, A., Zolov, S., and Lupashin, V. (2006). COG complex-mediated recycling of Golgi glycosyltransferases is essential for normal protein glycosylation. Traffic *7*, 191-204.

122. Wu, X., Steet, R.A., Bohorov, O., Bakker, J., Newell, J., Krieger, M., Spaapen, L., Kornfeld, S., and Freeze, H.H. (2004). Mutation of the COG complex subunit gene COG7 causes a lethal congenital disorder. Nat Med *10*, 518-523.

123. Foulquier, F., Vasile, E., Schollen, E., Callewaert, N., Raemaekers, T., Quelhas, D., Jaeken, J., Mills, P., Winchester, B., Krieger, M., et al. (2006). Conserved oligomeric Golgi complex subunit 1 deficiency reveals a previously uncharacterized congenital disorder of glycosylation type II. Proc Natl Acad Sci U S A *103*, 3764-3769.

124. Foulquier, F., Ungar, D., Reynders, E., Zeevaert, R., Mills, P., García-Silva, M.T., Briones, P., Winchester, B., Morelle, W., Krieger, M., et al. (2007). A new inborn error of glycosylation due to a Cog8 deficiency reveals a critical role for the Cog1-Cog8 interaction in COG complex formation. Hum Mol Genet *16*, 717-730.

125. Reynders, E., Foulquier, F., Leão Teles, E., Quelhas, D., Morelle, W., Rabouille, C., Annaert, W., and Matthijs, G. (2009). Golgi function and dysfunction in the first COG4-deficient CDG type II patient. Hum Mol Genet *18*, 3244-3256.

126. Paesold-Burda, P., Maag, C., Troxler, H., Foulquier, F., Kleinert, P., Schnabel, S., Baumgartner, M., and Hennet, T. (2009). Deficiency in COG5 causes a moderate form of congenital disorders of glycosylation. Hum Mol Genet *18*, 4350-4356.

127. Lübbehusen, J., Thiel, C., Rind, N., Ungar, D., Prinsen, B.H., de Koning, T.J., van Hasselt, P.M., and Körner, C. (2010). Fatal outcome due to deficiency of subunit 6 of the conserved

oligomeric Golgi complex leading to a new type of congenital disorders of glycosylation. Hum Mol Genet *19*, 3623-3633.

128. Kodera, H., Ando, N., Yuasa, I., Wada, Y., Tsurusaki, Y., Nakashima, M., Miyake, N., Saitoh, S., Matsumoto, N., and Saitsu, H. (2015). Mutations in COG2 encoding a subunit of the conserved oligomeric golgi complex cause a congenital disorder of glycosylation. Clin Genet *87*, 455-460.

129. Peanne, R., Legrand, D., Duvet, S., Mir, A.M., Matthijs, G., Rohrer, J., and Foulquier, F. (2011). Differential effects of lobe A and lobe B of the Conserved Oligomeric Golgi complex on the stability of {beta}1,4-galactosyltransferase 1 and {alpha}2,6-sialyltransferase 1. Glycobiology *21*, 864-876.

130. Boyadjiev, S.A., Kim, S.D., Hata, A., Haldeman-Englert, C., Zackai, E.H., Naydenov, C., Hamamoto, S., Schekman, R.W., and Kim, J. (2011). Cranio-lenticulo-sutural dysplasia associated with defects in collagen secretion. Clin Genet *80*, 169-176.

131. Schwarz, K., Iolascon, A., Verissimo, F., Trede, N.S., Horsley, W., Chen, W., Paw, B.H., Hopfner, K.P., Holzmann, K., Russo, R., et al. (2009). Mutations affecting the secretory COPII coat component SEC23B cause congenital dyserythropoietic anemia type II. Nat Genet *41*, 936-940.

132. Smits, P., Bolton, A.D., Funari, V., Hong, M., Boyden, E.D., Lu, L., Manning, D.K., Dwyer, N.D., Moran, J.L., Prysak, M., et al. (2010). Lethal skeletal dysplasia in mice and humans lacking the golgin GMAP-210. N Engl J Med *362*, 206-216.

133. Kornak, U., Reynders, E., Dimopoulou, A., van Reeuwijk, J., Fischer, B., Rajab, A., Budde, B., Nürnberg, P., Foulquier, F., Lefeber, D., et al. (2008). Impaired glycosylation and cutis laxa caused by mutations in the vesicular H+-ATPase subunit ATP6V0A2. Nat Genet *40*, 32-34.

134. Foulquier, F., Amyere, M., Jaeken, J., Zeevaert, R., Schollen, E., Race, V., Bammens, R., Morelle, W., Rosnoblet, C., Legrand, D., et al. (2012). TMEM165 deficiency causes a congenital disorder of glycosylation. Am J Hum Genet *91*, 15-26.

135. Park, J.H., Hogrebe, M., Grüneberg, M., DuChesne, I., von der Heiden, A.L., Reunert, J., Schlingmann, K.P., Boycott, K.M., Beaulieu, C.L., Mhanni, A.A., et al. (2015). SLC39A8 Deficiency: A Disorder of Manganese Transport and Glycosylation. Am J Hum Genet *97*, 894-903.

136. Jansen, J.C., Timal, S., van Scherpenzeel, M., Michelakakis, H., Vicogne, D., Ashikov, A., Moraitou, M., Hoischen, A., Huijben, K., Steenbergen, G., et al. (2016). TMEM199 Deficiency Is a Disorder of Golgi Homeostasis Characterized by Elevated Aminotransferases, Alkaline Phosphatase, and Cholesterol and Abnormal Glycosylation. Am J Hum Genet *98*, 322-330.

137. Jansen, J.C., Cirak, S., van Scherpenzeel, M., Timal, S., Reunert, J., Rust, S., Pérez, B., Vicogne, D., Krawitz, P., Wada, Y., et al. (2016). CCDC115 Deficiency Causes a Disorder of Golgi Homeostasis with Abnormal Protein Glycosylation. Am J Hum Genet *98*, 310-321.

138. Morava, E., Wopereis, S., Coucke, P., Gillessen-Kaesbach, G., Voit, T., Smeitink, J., Wevers, R., and Grünewald, S. (2005). Defective protein glycosylation in patients with cutis laxa syndrome. Eur J Hum Genet *13*, 414-421.

139. Axelsson, M.A., Karlsson, N.G., Steel, D.M., Ouwendijk, J., Nilsson, T., and Hansson, G.C. (2001). Neutralization of pH in the Golgi apparatus causes redistribution of glycosyltransferases and changes in the O-glycosylation of mucins. Glycobiology *11*, 633-644.

140. Rivinoja, A., Hassinen, A., Kokkonen, N., Kauppila, A., and Kellokumpu, S. (2009). Elevated Golgi pH impairs terminal N-glycosylation by inducing mislocalization of Golgi glycosyltransferases. J Cell Physiol *220*, 144-154.

141. Zeevaert, R., de Zegher, F., Sturiale, L., Garozzo, D., Smet, M., Moens, M., Matthijs, G., and Jaeken, J. (2013). Bone Dysplasia as a Key Feature in Three Patients with a Novel Congenital Disorder of Glycosylation (CDG) Type II Due to a Deep Intronic Splice Mutation in TMEM165. JIMD Rep *8*, 145-152.

142. Demaegd, D., Foulquier, F., Colinet, A.S., Gremillon, L., Legrand, D., Mariot, P., Peiter, E., Van Schaftingen, E., Matthijs, G., and Morsomme, P. (2013). Newly characterized Golgi-localized family of proteins is involved in calcium and pH homeostasis in yeast and human cells. Proc Natl Acad Sci U S A *110*, 6859-6864.

143. Potelle, S., Morelle, W., Dulary, E., Duvet, S., Vicogne, D., Spriet, C., Krzewinski-Recchi, M., Morsomme, P., Jaeken, J., Matthijs, G., et al. (2016). Glycosylation abnormalities in Gdt1p/TMEM165 deficient cells result from a defect in Golgi manganese homeostasis. Human Molecular Genetics *Published online: February 1*, 1-12.

144. de Jong, G., van Dijk, J.P., and van Eijk, H.G. (1990). The biology of transferrin. Clin Chim Acta *190*, 1-46.

145. Ondruskova, N. (2010). Study of transferrin as a marker of congenital disorders of glycosylation. In Department of Biochemistry. (Prague: Faculty of Science, Charles University in Prague).

146. Seta, N., Barnier, A., Hochedez, F., Besnard, M.A., and Durand, G. (1996). Diagnostic value of Western blotting in carbohydrate-deficient glycoprotein syndrome. Clin Chim Acta *254*, 131-140.

147. Carchon, H.A., Chevigné, R., Falmagne, J.B., and Jaeken, J. (2004). Diagnosis of congenital disorders of glycosylation by capillary zone electrophoresis of serum transferrin. Clin Chem *50*, 101-111.

148. Yamashita, K., Ohkura, T., Ideo, H., Ohno, K., and Kanai, M. (1993). Electrospray ionization-mass spectrometric analysis of serum transferrin isoforms in patients with carbohydrate-deficient glycoprotein syndrome. J Biochem *114*, 766-769.

149. van Scherpenzeel, M., Steenbergen, G., Morava, E., Wevers, R.A., and Lefeber, D.J. (2015). High-resolution mass spectrometry glycoprofiling of intact transferrin for diagnosis and subtype identification in the congenital disorders of glycosylation. Transl Res *166*, 639-649.e631.

150. Bergström, J.P., and Helander, A. (2008). Clinical characteristics of carbohydrate-deficient transferrin (%disialotransferrin) measured by HPLC: sensitivity, specificity, gender effects, and relationship with other alcohol biomarkers. Alcohol Alcohol *43*, 436-441.

151. Yuasa, I., Ohno, K., Hashimoto, K., Iijima, K., Yamashita, K., and Takeshita, K. (1995). Carbohydrate-deficient glycoprotein syndrome: electrophoretic study of multiple serum

glycoproteins. Brain Dev *17*, 13-19.

152. Fang, J., Peters, V., Assmann, B., Körner, C., and Hoffmann, G.F. (2004). Improvement of CDG diagnosis by combined examination of several glycoproteins. J Inherit Metab Dis *27*, 581-590.

153. Clayton, P., Winchester, B., Di Tomaso, E., Young, E., Keir, G., and Rodeck, C. (1993). Carbohydrate-deficient glycoprotein syndrome: normal glycosylation in the fetus. Lancet *341*, 956.

154. Charlwood, J., Clayton, P., Keir, G., Mian, N., and Winchester, B. (1998). Defective galactosylation of serum transferrin in galactosemia. Glycobiology *8*, 351-357.

155. Adamowicz, M., and Pronicka, E. (1996). Carbohydrate deficient glycoprotein syndrome--like transferrin isoelectric focusing pattern in untreated fructosaemia. Eur J Pediatr *155*, 347-348.

156. Stibler, H. (1991). Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. Clin Chem *37*, 2029-2037.

157. Wopereis, S., Grünewald, S., Morava, E., Penzien, J.M., Briones, P., García-Silva, M.T., Demacker, P.N., Huijben, K.M., and Wevers, R.A. (2003). Apolipoprotein C-III isofocusing in the diagnosis of genetic defects in O-glycan biosynthesis. Clin Chem *49*, 1839-1845.

158. Wopereis, S., Grünewald, S., Huijben, K.M., Morava, E., Mollicone, R., van Engelen, B.G., Lefeber, D.J., and Wevers, R.A. (2007). Transferrin and apolipoprotein C-III isofocusing are complementary in the diagnosis of N- and O-glycan biosynthesis defects. Clin Chem *53*, 180-187.

159. Kashyap, M.L., Srivastava, L.S., Hynd, B.A., Gartside, P.S., and Perisutti, G. (1981). Quantitation of human apolipoprotein C-III and its subspecie by radioimmunoassay and analytical isoelectric focusing: abnormal plasma triglyceride-rich lipoprotein apolipoprotein C-III subspecie concentrations in hypertriglyceridemia. J Lipid Res *22*, 800-810.

160. Savinova, O.V., Fillaus, K., Jing, L., Harris, W.S., and Shearer, G.C. (2014). Reduced apolipoprotein glycosylation in patients with the metabolic syndrome. PLoS One *9*, e104833.

161. Grünewald, S., Schollen, E., Van Schaftingen, E., Jaeken, J., and Matthijs, G. (2001). High residual activity of PMM2 in patients' fibroblasts: possible pitfall in the diagnosis of CDG-Ia (phosphomannomutase deficiency). Am J Hum Genet *68*, 347-354.

162. Thiel, C., Rind, N., Popovici, D., Hoffmann, G.F., Hanson, K., Conway, R.L., Adamski, C.R., Butler, E., Scanlon, R., Lambert, M., et al. (2012). Improved diagnostics lead to identification of three new patients with congenital disorder of glycosylation-Ip. Hum Mutat *33*, 485-487.

163. Wada, Y. (2006). Mass spectrometry for congenital disorders of glycosylation, CDG. J Chromatogr B Analyt Technol Biomed Life Sci *838*, 3-8.

164. Guillard, M., Morava, E., van Delft, F.L., Hague, R., Körner, C., Adamowicz, M., Wevers, R.A., and Lefeber, D.J. (2011). Plasma N-glycan profiling by mass spectrometry for congenital disorders of glycosylation type II. Clin Chem *57*, 593-602.

165. Xia, B., Zhang, W., Li, X., Jiang, R., Harper, T., Liu, R., Cummings, R.D., and He, M. (2013). Serum N-glycan and O-glycan analysis by mass spectrometry for diagnosis of congenital

disorders of glycosylation. Anal Biochem *442*, 178-185.

166. Chacko, B.K., and Appukuttan, P.S. (2001). Peanut (Arachis hypogaea) lectin recognizes alpha-linked galactose, but not N-acetyl lactosamine in N-linked oligosaccharide terminals. Int J Biol Macromol *28*, 365-371.

167. Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell *56*, 801-813.

168. Michele, D.E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R.D., Satz, J.S., Dollar, J., Nishino, I., Kelley, R.I., Somer, H., et al. (2002). Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. Nature *418*, 417-422.

169. Morava, E., Wevers, R.A., Cantagrel, V., Hoefsloot, L.H., Al-Gazali, L., Schoots, J., van Rooij, A., Huijben, K., van Ravenswaaij-Arts, C.M., Jongmans, M.C., et al. (2010). A novel cerebello-ocular syndrome with abnormal glycosylation due to abnormalities in dolichol metabolism. Brain *133*, 3210-3220.

170. Phillips, M.L., Schwartz, B.R., Etzioni, A., Bayer, R., Ochs, H.D., Paulson, J.C., and Harlan, J.M. (1995). Neutrophil adhesion in leukocyte adhesion deficiency syndrome type 2. J Clin Invest *96*, 2898-2906.

171. Brodsky, R.A., Mukhina, G.L., Li, S., Nelson, K.L., Chiurazzi, P.L., Buckley, J.T., and Borowitz, M.J. (2000). Improved detection and characterization of paroxysmal nocturnal hemoglobinuria using fluorescent aerolysin. Am J Clin Pathol *114*, 459-466.

172. Almeida, A., Layton, M., and Karadimitris, A. (2009). Inherited glycosylphosphatidyl inositol deficiency: a treatable CDG. Biochim Biophys Acta *1792*, 874-880.

173. Losfeld, M.E., Ng, B.G., Kircher, M., Buckingham, K.J., Turner, E.H., Eroshkin, A., Smith, J.D., Shendure, J., Nickerson, D.A., Bamshad, M.J., et al. (2014). A new congenital disorder of glycosylation caused by a mutation in SSR4, the signal sequence receptor 4 protein of the TRAP complex. Hum Mol Genet *23*, 1602-1605.

174. Jones, M.A., Rhodenizer, D., da Silva, C., Huff, I.J., Keong, L., Bean, L.J., Coffee, B., Collins, C., Tanner, A.K., He, M., et al. (2013). Molecular diagnostic testing for congenital disorders of glycosylation (CDG): detection rate for single gene testing and next generation sequencing panel testing. Mol Genet Metab *110*, 78-85.

175. Yuste-Checa, P., Gámez, A., Brasil, S., Desviat, L.R., Ugarte, M., Pérez-Cerdá, C., and Pérez, B. (2015). The Effects of PMM2-CDG-Causing Mutations on the Folding, Activity, and Stability of the PMM2 Protein. Hum Mutat *36*, 851-860.

176. Matthijs, G., Schollen, E., Bjursell, C., Erlandson, A., Freeze, H., Imtiaz, F., Kjaergaard, S., Martinsson, T., Schwartz, M., Seta, N., et al. (2000). Mutations in PMM2 that cause congenital disorders of glycosylation, type Ia (CDG-Ia). Hum Mutat *16*, 386-394.

177. Kjaergaard, S., Schwartz, M., and Skovby, F. (2001). Congenital disorder of glycosylation type Ia (CDG-Ia): phenotypic spectrum of the R141H/F119L genotype. Arch Dis Child *85*, 236-239.

178. de Lonlay, P., Seta, N., Barrot, S., Chabrol, B., Drouin, V., Gabriel, B.M., Journel, H., Kretz, M., Laurent, J., Le Merrer, M., et al. (2001). A broad spectrum of clinical presentations in

congenital disorders of glycosylation I: a series of 26 cases. J Med Genet *38*, 14-19.

179. Barone, R., Carrozzi, M., Parini, R., Battini, R., Martinelli, D., Elia, M., Spada, M., Lilliu, F., Ciana, G., Burlina, A., et al. (2015). A nationwide survey of PMM2-CDG in Italy: high frequency of a mild neurological variant associated with the L32R mutation. J Neurol *262*, 154-164.

180. de Lonlay, P., and Seta, N. (2009). The clinical spectrum of phosphomannose isomerase deficiency, with an evaluation of mannose treatment for CDG-Ib. Biochim Biophys Acta *1792*, 841-843.

181. Mention, K., Lacaille, F., Valayannopoulos, V., Romano, S., Kuster, A., Cretz, M., Zaidan, H., Galmiche, L., Jaubert, F., de Keyzer, Y., et al. (2008). Development of liver disease despite mannose treatment in two patients with CDG-Ib. Mol Genet Metab *93*, 40-43.

182. Janssen, M.C., de Kleine, R.H., van den Berg, A.P., Heijdra, Y., van Scherpenzeel, M., Lefeber, D.J., and Morava, E. (2014). Successful liver transplantation and long-term follow-up in a patient with MPI-CDG. Pediatrics *134*, e279-283.

183. Mayatepek, E., and Kohlmüller, D. (1998). Mannose supplementation in carbohydrate-deficient glycoprotein syndrome type I and phosphomannomutase deficiency. Eur J Pediatr *157*, 605-606.

184. Schneider, A., Thiel, C., Rindermann, J., DeRossi, C., Popovici, D., Hoffmann, G.F., Gröne, H.J., and Körner, C. (2012). Successful prenatal mannose treatment for congenital disorder of glycosylation-Ia in mice. Nat Med *18*, 71-73.

185. Sharma, V., Ichikawa, M., He, P., Scott, D.A., Bravo, Y., Dahl, R., Ng, B.G., Cosford, N.D., and Freeze, H.H. (2011). Phosphomannose isomerase inhibitors improve N-glycosylation in selected phosphomannomutase-deficient fibroblasts. J Biol Chem *286*, 39431-39438.

186. Marquardt, T., Lühn, K., Srikrishna, G., Freeze, H.H., Harms, E., and Vestweber, D. (1999). Correction of leukocyte adhesion deficiency type II with oral fucose. Blood *94*, 3976-3985.

187. Sturla, L., Puglielli, L., Tonetti, M., Berninsone, P., Hirschberg, C.B., De Flora, A., and Etzioni, A. (2001). Impairment of the Golgi GDP-L-fucose transport and unresponsiveness to fucose replacement therapy in LAD II patients. Pediatr Res *49*, 537-542.

188. Almeida, A.M., Murakami, Y., Baker, A., Maeda, Y., Roberts, I.A., Kinoshita, T., Layton, D.M., and Karadimitris, A. (2007). Targeted therapy for inherited GPI deficiency. N Engl J Med *356*, 1641-1647.

189. Morava, E. (2014). Galactose supplementation in phosphoglucomutase-1 deficiency; review and outlook for a novel treatable CDG. Mol Genet Metab *112*, 275-279.

190. Gadomski, T., Wong, S., Honzik, T., Misfeld, A., Koch, D., Brocke, K., Sykut-Cegielska, J., Bowling, F., Kozicz, T., Lefeber, D., et al. (2015). Oral galactose supplementation in PGM1-CDG: as sweet as it sounds? Journal of Inherited Metabolic Disease *38*, S38-S39.

191. Matsumoto, K., Maeda, Y., Kato, S., and Yuki, H. (1994). Alteration of asparagine-linked glycosylation in serum transferrin of patients with hepatocellular carcinoma. Clin Chim Acta *224*, 1-8.

192. Yamashita, K., Koide, N., Endo, T., Iwaki, Y., and Kobata, A. (1989). Altered glycosylation

of serum transferrin of patients with hepatocellular carcinoma. J Biol Chem *264*, 2415-2423.

193. Häuselmann, I., and Borsig, L. (2014). Altered tumor-cell glycosylation promotes metastasis. Front Oncol *4*, 28.

194. Zhu, J., Lin, Z., Wu, J., Yin, H., Dai, J., Feng, Z., Marrero, J., and Lubman, D.M. (2014). Analysis of serum haptoglobin fucosylation in hepatocellular carcinoma and liver cirrhosis of different etiologies. J Proteome Res *13*, 2986-2997.

195. Stibler, H., and Borg, S. (1991). Glycoprotein glycosyltransferase activities in serum in alcohol-abusing patients and healthy controls. Scand J Clin Lab Invest *51*, 43-51.

196. Xin, Y., Lasker, J.M., and Lieber, C.S. (1995). Serum carbohydrate-deficient transferrin: mechanism of increase after chronic alcohol intake. Hepatology *22*, 1462-1468.

197. Parekh, R.B., Dwek, R.A., Sutton, B.J., Fernandes, D.L., Leung, A., Stanworth, D., Rademacher, T.W., Mizuochi, T., Taniguchi, T., and Matsuta, K. (1985). Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. Nature *316*, 452-457.

198. Gornik, O., and Lauc, G. (2008). Glycosylation of serum proteins in inflammatory diseases. Dis Markers *25*, 267-278.

199. Chrostek, L., Cylwik, B., Gindzienska-Sieskiewicz, E., Gruszewska, E., Szmitkowski, M., and Sierakowski, S. (2014). Sialic acid level reflects the disturbances of glycosylation and acute-phase reaction in rheumatic diseases. Rheumatol Int *34*, 393-399.

200. Narita, I., and Gejyo, F. (2008). Pathogenetic significance of aberrant glycosylation of IgA1 in IgA nephropathy. Clin Exp Nephrol *12*, 332-338.

201. Testa, R., Vanhooren, V., Bonfigli, A.R., Boemi, M., Olivieri, F., Ceriello, A., Genovese, S., Spazzafumo, L., Borelli, V., Bacalini, M.G., et al. (2015). N-glycomic changes in serum proteins in type 2 diabetes mellitus correlate with complications and with metabolic syndrome parameters. PLoS One *10*, e0119983.

202. Stibler, H., von Döbeln, U., Kristiansson, B., and Guthenberg, C. (1997). Carbohydrate-deficient transferrin in galactosaemia. Acta Paediatr *86*, 1377-1378.

203. Jaeken, J., Pirard, M., Adamowicz, M., Pronicka, E., and van Schaftingen, E. (1996). Inhibition of phosphomannose isomerase by fructose 1-phosphate: an explanation for defective N-glycosylation in hereditary fructose intolerance. Pediatr Res *40*, 764-766.

204. Sturiale, L., Barone, R., Fiumara, A., Perez, M., Zaffanello, M., Sorge, G., Pavone, L., Tortorelli, S., O'Brien, J.F., Jaeken, J., et al. (2005). Hypoglycosylation with increased fucosylation and branching of serum transferrin N-glycans in untreated galactosemia. Glycobiology *15*, 1268-1276.

205. Ng, W.G., Xu, Y.K., Kaufman, F.R., and Donnell, G.N. (1989). Deficit of uridine diphosphate galactose in galactosaemia. J Inherit Metab Dis *12*, 257-266.

206. Liu, Y., Xia, B., Gleason, T.J., Castañeda, U., He, M., Berry, G.T., and Fridovich-Keil, J.L. (2012). N- and O-linked glycosylation of total plasma glycoproteins in galactosemia. Mol Genet Metab *106*, 442-454.

207. Pronicka, E., Adamowicz, M., Kowalik, A., Płoski, R., Radomyska, B., Rogaszewska, M., Rokicki, D., and Sykut-Cegielska, J. (2007). Elevated carbohydrate-deficient transferrin (CDT) and its normalization on dietary treatment as a useful biochemical test for hereditary fructose intolerance and galactosemia. Pediatr Res *62*, 101-105.

208. Rhodes, J.M. (1996). Unifying hypothesis for inflammatory bowel disease and associated colon cancer: sticking the pieces together with sugar. Lancet *347*, 40-44.

209. McGovern, D.P., Jones, M.R., Taylor, K.D., Marciante, K., Yan, X., Dubinsky, M., Ippoliti, A., Vasiliauskas, E., Berel, D., Derkowski, C., et al. (2010). Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. Hum Mol Genet *19*, 3468-3476.

210. Parmar, A.S., Alakulppi, N., Paavola-Sakki, P., Kurppa, K., Halme, L., Färkkilä, M., Turunen, U., Lappalainen, M., Kontula, K., Kaukinen, K., et al. (2012). Association study of FUT2 (rs601338) with celiac disease and inflammatory bowel disease in the Finnish population. Tissue Antigens *80*, 488-493.

211. Hu, D.Y., Shao, X.X., Xu, C.L., Xia, S.L., Yu, L.Q., Jiang, L.J., Jin, J., Lin, X.Q., and Jiang, Y. (2014). Associations of FUT2 and FUT3 gene polymorphisms with Crohn's disease in Chinese patients. J Gastroenterol Hepatol *29*, 1778-1785.

212. Weiss, F.U., Schurmann, C., Guenther, A., Ernst, F., Teumer, A., Mayerle, J., Simon, P., Völzke, H., Radke, D., Greinacher, A., et al. (2015). Fucosyltransferase 2 (FUT2) non-secretor status and blood group B are associated with elevated serum lipase activity in asymptomatic subjects, and an increased risk for chronic pancreatitis: a genetic association study. Gut *64*, 646-656.

213. Hansen, L., Lind-Thomsen, A., Joshi, H.J., Pedersen, N.B., Have, C.T., Kong, Y., Wang, S., Sparso, T., Grarup, N., Vester-Christensen, M.B., et al. (2015). A glycogene mutation map for discovery of diseases of glycosylation. Glycobiology *25*, 211-224.

214. Kathiresan, S., Melander, O., Guiducci, C., Surti, A., Burtt, N.P., Rieder, M.J., Cooper, G.M., Roos, C., Voight, B.F., Havulinna, A.S., et al. (2008). Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. Nat Genet *40*, 189-197.

215. Brantová, O., Tesarová, M., Hansíková, H., Elleder, M., Zeman, J., and Sládková, J. (2006). Ultrastructural changes of mitochondria in the cultivated skin fibroblasts of patients with point mutations in mitochondrial DNA. Ultrastruct Pathol *30*, 239-245.

216. Munce, T., Heussler, H.S., and Bowling, F.G. (2010). Analysis of N- and O-linked protein glycosylation in children with Prader-Willi syndrome. J Intellect Disabil Res *54*, 929-937.

217. Marucci, A., di Mauro, L., Menzaghi, C., Prudente, S., Mangiacotti, D., Fini, G., Lotti, G., Trischitta, V., and Di Paola, R. (2013). GALNT2 expression is reduced in patients with Type 2 diabetes: possible role of hyperglycemia. PLoS One *8*, e70159.

218. Yassine, H.N., Trenchevska, O., Ramrakhiani, A., Parekh, A., Koska, J., Walker, R.W., Billheimer, D., Reaven, P.D., Yen, F.T., Nelson, R.W., et al. (2015). The Association of Human Apolipoprotein C-III Sialylation Proteoforms with Plasma Triglycerides. PLoS One *10*, e0144138.

219. van Scherpenzeel, M., Timal, S., Raymond, K., Adamowicz, M., Socha, P., van Spronsen, F., Veltman, J., Wevers, R., Morava, E., and Lefeber, D. (2012). PGM1-deficiency with

abnormal protein glycosylation: easy diagnosis and dietary intervention. J Inherit Metab Dis *35 (Suppl 1)*, S20.

220. Owusu-Ansah, E., Yavari, A., and Banerjee, U. (2008). A protocol for *in vivo* detection of reactive oxygen species. (Nature Publishing Group).

221. Lecca, M.R., Wagner, U., Patrignani, A., Berger, E.G., and Hennet, T. (2005). Genome-wide analysis of the unfolded protein response in fibroblasts from congenital disorders of glycosylation type-I patients. FASEB J *19*, 240-242.

222. Yan, W., Frank, C.L., Korth, M.J., Sopher, B.L., Novoa, I., Ron, D., and Katze, M.G. (2002). Control of PERK eIF2alpha kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. Proc Natl Acad Sci U S A *99*, 15920-15925.

223. Rutkowski, D.T., Kang, S.W., Goodman, A.G., Garrison, J.L., Taunton, J., Katze, M.G., Kaufman, R.J., and Hegde, R.S. (2007). The role of p58IPK in protecting the stressed endoplasmic reticulum. Mol Biol Cell *18*, 3681-3691.

224. Cao, S.S., and Kaufman, R.J. (2014). Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. Antioxid Redox Signal *21*, 396-413.

225. Malhotra, J.D., and Kaufman, R.J. (2007). The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol *18*, 716-731.

226. Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., et al. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell *11*, 619-633.

227. Suzuki, Y., Shimozawa, N., Yajima, S., Orii, T., Yokota, S., Tashiro, Y., Osumi, T., and Hashimoto, T. (1992). Different intracellular localization of peroxisomal proteins in fibroblasts from patients with aberrant peroxisome assembly. Cell Struct Funct *17*, 1-8.

228. Murakami, K., Ichinohe, Y., Koike, M., Sasaoka, N., Iemura, S., Natsume, T., and Kakizuka, A. (2013). VCP Is an integral component of a novel feedback mechanism that controls intracellular localization of catalase and H2O2 Levels. PLoS One *8*, e56012.

229. Holleboom, A.G., Karlsson, H., Lin, R.S., Beres, T.M., Sierts, J.A., Herman, D.S., Stroes, E.S., Aerts, J.M., Kastelein, J.J., Motazacker, M.M., et al. (2011). Heterozygosity for a loss-of-function mutation in GALNT2 improves plasma triglyceride clearance in man. Cell Metab *14*, 811-818.

SUPPLEMENT

Tables Sa, Sb, Sc, Sd

List of original articles

The original articles

Table Sa. Disorders of N-glycosylation.

Disorders of N-glycosylation			
Affected gene	**Inheritance**	**Function of the encoded protein**	**NCBI Gene ID**
DPAGT1	AR	GlcNAc-1-P transferase	1798
ALG1	AR	β-1,4-mannosyltransferase	56052
ALG2	AR	α-1,3/1,6-mannosyltransferase	85365
ALG3	AR	α-1,3-mannosyltransferase	10195
ALG6	AR	α-1,3-glucosyltransferase	29929
ALG8	AR	α-1,3-glucosyltransferase	79053
ALG9	AR	α-1,2-mannosyltransferase	79796
ALG11	AR	α-1,2-mannosyltransferase	440138
ALG12	AR	α-1,6-mannosyltransferase	79087
ALG13	X-linked	UDP-GlcNAc transferase subunit	79868
ALG14	AR	UDP-GlcNAc transferase subunit	199857
RFT1	AR	$Man_5GlcNAc_2$-PP-Dol flippase	91869
TUSC3	AR	Oligosaccharyltransferase subunit	7991
MAGT1	X-linked	Magnesium transporter associated with oligosaccharyltransferase	84061
DDOST	AR	Oligosaccharyltransferase subunit	1650
STT3A	AR	Oligosaccharyltransferase subunit	3703
STT3B	AR	Oligosaccharyltransferase subunit	201595
MGAT2	AR	GlcNAc transferase	4247
MOGS	AR	α-1,2-glucosidase	7841
MAN1B1	AR	α-1,2-mannosidase	11253
NGLY1	AR	N-glycanase	55768
SSR4	X-linked, de novo	Translocon-associated protein complex subunit	6748
GNPTA	AR	GlcNAc-1-P transferase	79158
PRKCSH	AD	Glucosidase II subunit	5589

Table Sb. Disorders of O-glycosylation.

Disorders of O-mannosylation (α-dystroglycanopathies)			
Affected gene	**Inheritance**	**Function of the encoded protein**	**NCBI Gene ID**
POMT1	AR	Protein O-mannosyltransferase	10585
POMT2	AR	Protein O-mannosyltransferase	29954
POMGNT1	AR	Protein O-mannose β-1,2-N-acetylglucosaminyltransferase	55624
FKTN	AR	Putative glycosyltransferase	2218
FKRP	AR	Putative glycosyltransferase	79147
LARGE	AR	Xylosyltransferase, glucuronyltransferase	9215
ISPD	AR	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	729920
POMGNT2	AR	Protein O-mannose β-1,4-N-acetylglucosaminyltransferase	84892
TMEM5	AR	Putative glycosyltransferase	10329
B3GALNT2	AR	β-1,3-N-acetylgalactosaminyltransferase	148789
POMK	AR	Protein O-mannose kinase	84197
B3GAT1	AR	β-1,4-glucuronyltransferase	11041
GMPPB	AR	GDP-mannose pyrophosphorylase	29925
Disorders of the glycosaminoglycan assembly (O-Xyl linked)			
XYLT1	AR	Xylosyltransferase	64131
B4GALT7	AR	β-1,4-galactosyltransferase	11285
B3GALT6	AR	β-1,3-galactosyltransferase	126792
B3GAT3	AR	β-1,3-glucuronyltransferase	26229
EXT1	AD	GlcA/GlcNAc transferase	2131
EXT2	AD	GlcA/GlcNAc transferase	2132
PAPSS2	AR	3'-phosphoadenosine 5'-phosphosulfate synthase	9060
SLC26A2	AR	Sulphate transporter	1836
CHST3	AR	Chondroitin 6-O-sulfotransferase	9469
CHST6	AR	GlcNAc-6-O-sulfotransferase	4166
CHST8	AR	GalNAc-4-O-sulfotransferase	64377
CHST14	AR	GalNAc-4-O-sulfotransferase	113189
DSE	AR	Dermatan sulfate epimerase	29940

Table Sb. Disorders of O-glycosylation (continuation).

Disorders of mucin O-glycosylation (O-GalNAc linked)			
Affected gene	**Inheritance**	**Function of the encoded protein**	**NCBI Gene ID**
GALNT3	AR	GalNAc transferase	2591
COSMC	Somatic	Chaperone of β-1,3-galactosyltransferase	29071
Other O-glycosylation disorders (O-Fuc, O-Glc, O-GlcNAc, O-Gal linked)			
POFUT1	AD	Protein O-fucosyltransferase	23509
B3GALTL	AR	β-1,3-glucosyltransferase	145173
LFNG	AR	O-fucosylpeptide β-1,3-GlcNAc ltransferase	3955
POGLUT1	AD	Protein O-glucosyltransferase	56983
EOGT	AR	EGF domain-specific O-linked GlcNAc transferase	285203
OGT	X-linked	O-linked GlcNAc transferase	8473
PLOD1	AR	Lysine hydroxylase	5351

Table Sc. Disorders of lipid and glycosylphosphatidylinositol anchor glycosylation.

Disorders of lipid glycosylation			
Affected gene	**Inheritance**	**Function of the encoded protein**	**NCBI Gene ID**
ST3GAL5	AR	Lactosylceramide α-2,3-sialyltransferase	8869
B4GALNT1	AR	β-1,4-GalNAc transferase	2583
PIGA	Somatic, X-linked	GlcNAc-PI synthesis	5277
PIGY	AR	Part of the GPI-GlcNAc transferase complex	84992
PIGL	AR	De-N-acetylation GlcNAc-PI	9487
PIGW	AR	Acylation of the inositol ring of PI	284098
PIGM	AR	Transfer of the first mannose to GPI	93183
PIGV	AR	Transfer of the second mannose to GPI	55650
PIGN	AR	Transfers phosphoethanolamine to the first mannose of GPI	23556
PIGO	AR	Transfers phosphoethanolamine to the third mannose of GPI	84720
PIGT	AR	Component of the GPI transamidase	51604
PGAP2	AR	Role in GPI anchor maturation	27315
PGAP3	AR	Role in GPI anchor maturation	93210

Table Sd. Disorders of (potentially) multiple glycosylation pathways.

Disorders of multiple glycosylation pathways			
Affected gene	**Inheritance**	**Function of the encoded protein**	**NCBI Gene ID**
PMM2	AR	Phosphomannomutase 2	5373
MPI	AR	Mannose phosphate isomerase	4351
PGM1	AR	Phosphoglucomutase 1	5236
PGM3	AR	Phosphoglucomutase 3	5238
GNE	AR, AD	Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase	10020
GFPT1	AR	Glutamine fructose-6-phosphate transaminase	2673
DHDDS	AR	Dehydrodolichyl diphosphate synthase subunit	79947
DOLK	AR	Dolichol kinase	22845
SRD5A3	AR	Steroid 5 alpha-reductase	79644
NGBR	AR	Promotes cis-prenyltransferase activity	116150
DPM1	AR	Dol-P-Man synthase subunit (catalytic)	8813
DPM2	AR	Dol-P-Man synthase subunit (regulatory)	8818
DPM3	AR	Dol-P-Man synthase subunit (stabilizer)	54344
GMPPA	AR	GDP-mannose pyrophosphorylase	29926
MPDU1	AR	Mannose-P-dolichol utilization	9526
SLC35A1	AR	CMP-sialic acid transporter	10559
SLC35A2	X-linked, de novo	UDP-galactose transporter	7355
SLC35A3	AR	UDP-N-acetylglucosamine transporter	23443
SLC35C1	AR	GDP-fucose transporter	55343
SLC35D1	AR	UDP-GlcA/UDP-GalNAc transporter	23169
B4GALT1	AR	β-1,4-galactosyltransferase	2683
ST3GAL3	AR	α-2,3-sialyltransferase	6487
TRIP11	AR	Role in Golgi structure	9321
SEC23A	AR	Role in Golgi trafficking	10484
SEC23B	AR	Role in Golgi trafficking	10483
SEC63	AD	Role in Golgi trafficking	11231
COG1	AR	Role in Golgi-to-ER retrograde transport	9382

Table Sd. Disorders of (potentially) multiple glycosylation pathways (continuation).

Disorders of multiple glycosylation pathways			
Affected gene	**Inheritance**	**Function of the encoded protein**	**NCBI Gene ID**
COG2	AR	Role in Golgi-to-ER retrograde transport	22796
COG4	AR	Role in Golgi-to-ER retrograde transport	25839
COG5	AR	Role in Golgi-to-ER retrograde transport	10466
COG6	AR	Role in Golgi-to-ER retrograde transport	57511
COG7	AR	Role in Golgi-to-ER retrograde transport	91949
COG8	AR	Role in Golgi-to-ER retrograde transport	84342
ATP6V0A2	AR	Role in Golgi pH regulation	23545
ATP6V1A	AR	Role in Golgi pH regulation	523
TMEM165	AR	Role in Mn^{2+} homeostasis in Golgi	55858
SLC39A8	AR	Role in Mn^{2+} homeostasis in Golgi	64116
TMEM199	AR	Role in Golgi homeostasis (unspecified)	147007
CCDC115	AR	Role in Golgi homeostasis (unspecified)	84317

List of original articles (in chronological order)

Guillard M, Wada Y, Hansikova H, Yuasa I, Vesela K, Ondruskova N, Kadoya M, Janssen A, Van den Heuvel LP, Morava E, Zeman J, Wevers RA, Lefeber DJ. ***Transferrin mutations at the glycosylation site complicate diagnosis of congenital disorders of glycosylation type I.*** *J Inherit Metab Dis. 2011Aug;34(4):901-6.* [IF: 3.808]

Honzík T, Magner M, Krijt J, Sokolová J, Vugrek O, Belužić R, Barić I, Hansíkova H, Elleder M, Veselá K, Bauerová L, Ondrušková N, Ješina P, Zeman J,Kožich V. ***Clinical picture of S-adenosylhomocysteine hydrolase deficiency resembles phosphomannomutase 2 deficiency.*** *Mol Genet Metab. 2012 Nov;107(3):611-3.* [IF: 2.834]

Ondruskova N, Vesela K, Hansikova H, Magner M, Zeman J, Honzik T. ***RFT1-CDG in adult siblings with novel mutations.*** *Mol Genet Metab. 2012 Dec;107(4):760-2.* [IF: 2.834]

Ondruskova N, Honzik T, Vondrackova A, Tesarova M, Zeman J, Hansikova H. ***Glycogen storage disease-like phenotype with central nervous system involvement in a PGM1-CDG patient.*** *Neuro Endocrinol Lett. 2014;35(2):137-41.* [IF: 0.935]

Van Scherpenzeel M, Timal S, Rymen D, Hoischen A, Wuhrer M, Hipgrave-Ederveen A, Grunewald S, Peanne R, Saada A, Edvardson S, Grønborg S, Ruijter G, Kattentidt-Mouravieva A, Brum JM, Freckmann ML, Tomkins S, Jalan A, Prochazkova D, Ondruskova N, Hansikova H, Willemsen MA, Hensbergen PJ, Matthijs G, Wevers RA,Veltman JA, Morava E, Lefeber DJ. ***Diagnostic serum glycosylation profile in patients with intellectual disability as a result of MAN1B1 deficiency.*** *Brain.2014 Apr;137(Pt 4):1030-8.* [IF: 10.226]

Park EJ, Grabińska KA, Guan Z, Stránecký V, Hartmannová H, Hodaňová K, Barešová V, Sovová J, Jozsef L, Ondrušková N, Hansíková H, Honzík T, Zeman J, Hůlková H, Wen R, Kmoch S, Sessa WC. ***Mutation of Nogo-B receptor, a subunit of cis-prenyltransferase, causes a congenital disorder of glycosylation.*** *Cell Metab. 2014 Sep 2;20(3):448-57.* [IF: 16.747]

Ondrušková N, Honzík T, Kytnarová J, Matoulek M, Zeman J, Hansíková H. ***Isoelectric Focusing of Serum Apolipoprotein C-III as a Sensitive Screening Method for the Detection of O-glycosylation Disturbances.*** *Prague Med Rep. 2015;116(2):73-86.* [IF: -]

Ondruskova N, Honzik T, Kolarova H, Zeman J, Hansikova H. ***ApoC-III hypoglycosylation in glycogen storage diseases: the role of UDP-GalNAc depletion?*** *J Inherit Metab Dis. 2016;xx(x):x.*
[IF: 3.365]

Univerzita Karlova v Praze, 1. lékařská fakulta
Kateřinská 32, Praha 2

Prohlášení zájemce o nahlédnutí
do závěrečné práce absolventa studijního programu
uskutečňovaného na 1. lékařské fakultě Univerzity Karlovy v Praze

Jsem si vědom/a, že závěrečná práce je autorským dílem a že informace získané nahlédnutím do zpřístupněné závěrečné práce nemohou být použity k výdělečným účelům, ani nemohou být vydávány za studijní, vědeckou nebo jinou tvůrčí činnost jiné osoby než autora.

Byl/a jsem seznámen/a se skutečností, že si mohu pořizovat výpisy, opisy nebo kopie závěrečné práce, jsem však povinen/a s nimi nakládat jako s autorským dílem a zachovávat pravidla uvedená v předchozím odstavci.

Příjmení, jméno (hůlkovým písmem)	Číslo dokladu totožnosti vypůjčitele (např. OP, cestovní pas)	Signatura závěrečné práce	Datum	Podpis