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PhD thesis summary

Biochemical and molecular studies of the congenital disorders of glycosylation

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ABBREVATIONS

A: adenine

ALG6-CDG: glucosyltransferase I deficiency (a type of CDG)

ALG8-CDG: glucosyltransferase II deficiency (a type of CDG)

ALG12-CDG: mannosyltransferase VIII deficiency (a type of CDG)

ApoC-III: apolipoprotein C-III

ApoC-III$_0$: asialoapolipoprotein C-III

ApoC-III$_1$: monosialoapolipoprotein C-III

ApoC-III$_2$: disialoapolipoprotein C-III

Arg: arginine

Asn: asparagine

C: cytidine

cis-PT: cis-prenyltransferase

CDG: congenital disorders of glycosylation

COG: conserved oligomeric Golgi (subunits 1-8)

Cys: cysteine

DHDDS: dehydrodolichyl diphosphate synthase subunit

DHDDS-CDG: dehydrodolichyl diphosphate synthase deficiency (a type of CDG)

DHE: dihydroethidium

DPAGT1-CDG: N-acetylglucosaminyltransferase I deficiency (a type of CDG)

DPM1-CDG: deficiency of dolichol-phosphate-mannose synthase-1 (a type of CDG)

ER: endoplasmic reticulum

EXT1/EXT2-CDG: exostosin 1/exostosin 2 deficiency (a type of CDG)

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

Gln: glutamine

Gly: glycine

GSD: glycogen storage diseases

GWAS: genome-wide association study

H_2O_2: hydrogen peroxide

HDL: high-density lipoproteins

His: histidine

HPLC: high-performance liquid chromatography

ICAM-1: intercellular adhesion molecule 1

IEF: isoelectric focusing

IQ: intelligence quotient

LAMP1: lysosomal-associated membrane protein 1

LDL: low-density lipoproteins

LLO: lipid linked oligosaccharide(s)

MAN1B1: α-1,2-mannosidase

MAN1B1-CDG: α-1,2-mannosidase deficiency (a type of CDG)

Met: methionine

MPI: phosphomannose isomerase

MPI-CDG: phosphomannose isomerase deficiency (a type of CDG)

mRNA: messenger ribonucleid acid

MS: mass spectrometry

M_w: molecular weight

nanochip-C8-QTOF-MS: high-resolution nano liquid chromatography-chip (C8)-quadrupole time of flight mass spectrometry

NgBR: Nogo-B receptor

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism

PGM(1): phoshoglucomutase (1)

PGM1-CDG: phoshoglucomutase 1 deficiency (a type of CDG)

PMM(2): phosphomannomutase (2)

PMM2-CDG: phosphomannomutase 2 deficiency (a type of CDG)

PNA: peanut agglutinin

PWS: Prader-Willi syndrome

ROS: reactive oxygen species

RFT1-CDG: deficiency of $Man_5GlcNac_2$-PP-Dol flippase (a type of CDG)

SAHH: S-adenosylhomocysteine hydrolase

SDS-PAGE: sodium dodecylsulphate polyacrylamide gel electrophoresis

Ser: serine

SNP: single nucleotide polymorphism

SRD5A3-CDG: polyprenol reductase deficiency (a type of CDG)

TAG: triacylglycerol(s)

TF: transferrin

Thr: threonine

TMEM165: transmembrane protein 165

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

UPR: unfolded protein response

Val: valine

VLDL: very low density lipoproteins

vs.: versus

ABSTRAKT

Dědičné poruchy glykosylace (Congenital disorders of glycosylation, CDG) představují rychle rostoucí skupinu vzácných metabolických onemocnění, které jsou zapříčiněné genetickými defekty vedoucími k poruše procesu glykosylace, tj. enzymatického navázání specifického sacharidového zbytku na proteinovou nebo lipidovou kostru. Kvůli nespecifitě a variabilitě klinických projevů u pacientů je stanovení diagnózy CDG velmi náročné, a kritickou roli sehrává správná biochemická a genetická analýza.

Cílem předkládané disertační práce bylo studium CDG na biochemické a molekulárně-genetické urovni v rámci České a Slovenské republiky. To zahrnovalo zavedení, optimalizaci a provedení metod pro screening CDG u pacientů s klinickým podezřením, stanovení genetické diagnózy u pozitivních pacientů a studium patobiochemických jevů specifických typů CDG na buněčné úrovni, jako i analýzu poruch glykosylace u jiných nemocí. Vybrané glykoproteiny byly analyzovány elektroforetickými technikami s imunodetekcí, molekulárně-genetická podstata onemocnění byla studována pomocí sekvenování a restrikční analýzy a mikroskopie s imunofluorescencí/imunocytochemií byla aplikována k charakterizaci změn na buněčné úrovni; další specifické metody (např. hmotnostní spektrometrie) byly prováděny ve spolupráci se zahraničními pracovišti.

Mezi přínosy předložené práce patří optimalizace isoelektrické fokusace sérového apolipoproteinu C-III (ApoC-III) jako metody pro detekci poruch O-glykosylace, a rovněž popis praktických implikací metod pro screening CDG. Ve skupině více než 20 pacientů jsme stanovili diagnózu na genetické úrovni, a rozšířili jsme tím spektrum známých fenotypů a genotypů daných onemocnění. V neposlední řadě jsme také pozorovali dosud nepopsané patobiochemické aspekty ve fibroblastech pacientů s vybranými typy CDG (např. zvýšenou hladinu reaktivních forem kyslíku).

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 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 greatly relies on accurate biochemical and genetic analyses.

The 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. This involved the introduction, optimization and application of CDG screening methods in clinically suspected individuals, genetic diagnosis of the positive patients and the study of pathobiochemical aspects of specific CDG types at the cellular level, as well as the analysis of glycosylation disturbances of non-CDG etiology. Selected glycoproteins were analyzed by electrophoretic techniques with immunodetection, sequencing and restriction analysis were applied to study the genetic defects and microscopy with immunofluorescence or immunocytochemistry was used to characterize alterations at the cellular level; additional specific methods (e.g., mass spectrometry) were performed by the author' s collaborators.

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. The genetic diagnosis of CDG was determined in more than 20 patients, bringing novel phenotype and genotype findings. Moreover, we made original observations related to various pathobiochemical aspects in fibroblasts from patients with selected CDG types (e.g., the increased level of reactive oxygen species).

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

1 INTRODUCTION

1.1 Glycosylation

Glycosylation is a common 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. 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. Other types of mammalian protein glycosylation include C-mannosylation 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.

1.2 Congenital disorders of glycosylation (CDG): a brief overview

The primary, genetically determined defects of glycosylation in humans comprise a group of rare diseases labeled as congenital disorders of glycosylation (CDG). The first reported case of a patient with 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 [3]. Later, the biochemical basis of this disease was identified as the phosphomannomutase 2 (PMM2) deficiency due to mutations in the *PMM2* gene [4]. 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. To this date, PMM2-CDG (formerly known as CDG-Ia) is the most frequent type of CDG with estimated prevalence of 1:20 000

[5], 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 [6]. 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 [7]). 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.

1.3 Laboratory diagnosis of CDG

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).

Human **transferrin (TF)** is an abundant 79 kDa liver-derived serum N-glycoprotein, which 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 [8]. 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. 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 [9]. Two typical pathological profiles are distinguished: A) **CDG type I** pattern, i.e. a relative increase of di- and asialoTF typical for disorders of N-glycan assembly in ER (the patients with this profile, but yet not genetically diagnosed, are labeled as CDG-Ix), and B) **CDG type II** pattern, i.e. a variable relative increase of tri-, di- and monosialoTF, characteristic for CDG patients with impaired N-glycan processing in Golgi (the unsolved cases are labeled as CDG-IIx).

Apolipoprotein C-III (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- (ApoC-III_0), monosialo- (ApoC-III_1) and disialoApoC-III (ApoC-III_2). Aberrant ApoC-III glycosylation, 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.

After a positive finding in CDG screening, the patient is analyzed by a spectrum of additional biochemical methods, which are chosen based on the character of the detected aberrant profile and the patient' s clinical manifestation. When type I pattern is detected using IEF of TF, enzyme activities of phosphomannomutase (PMM) and phosphomannose isomerase (MPI) are measured in leukocytes or fibroblasts of the patients, as these (PMM2-CDG, MPI-CDG) 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 using HPLC. Accumulation of shortened intermediates might point to deficiencies of specific enzymes involved in N-glycan assembly. For CDG type II, further characterization depends on mass spectrometry analysis of the structure of N- and O-glycans, isolated from specific glycoproteins or whole plasma. When a suspicion is narrowed down to a specific CDG type, other selected tests might be performed (e.g., the detection of oligosaccharides in urine, analysis of plasma polyprenols, etc.). However, due to considerable variability in various biochemical abnormalities, diagnosis confirmation at the genetic level is necessary. Most of CDG types are inherited in an autosomal recessive manner, and a lot of the patients are compound heterozygotes. Sanger sequencing is applied when the findings strongly suggest a specific gene defect; otherwise, next-generation sequencing (genome, exome or disease targeted panel) might be performed.

2 AIMS OF THE THESIS

Congenital disorders of glycosylation (CDG) are a heterogeneous group of metabolic diseases with a broad spectrum of diverse, often multi-systemic clinical manifestations of varying severity. Albeit rare in occurrence, the number of recognized CDG types (i.e., the individual gene defects) has grown rapidly over the last years, especially thanks to the recent advances in diagnostic technologies. The accurate diagnosis is important for prenatal testing and genetic counseling in the affected families, as well as for selecting the right treatment (if available).

The specific aims of the thesis were:

A.) To introduce, optimize and perform **screening methods for the detection of congenital disorders of glycosylation (CDG)**.

B.) In the positive patients selected via CDG screening, to apply laboratory approaches for **further biochemical and molecular characterization of the glycosylation defect**, to establish their genetic diagnosis and to describe pathophysiology of their disease at the cellular level.

C.) To study **alterations in protein glycosylation of non-CDG etiology**.

3 RESULTS AND DISCUSSION

3.1 Results and discussion related to the aim A.)

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

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

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.*

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 an unusual 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 %). 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. A similar IEF pattern was detected in his healthy mother, suggesting a non-pathogenic cause. Sanger sequencing of the patient´s *TF* gene uncovered a novel mutation in exon 16, causing amino acid alteration at one of the two TF N-glycosylation sites (heterozygous c.1889A>C; p.Asn630Thr). The present case demonstrates the potential pitfalls, albeit rarely occuring, of CDG screening method using the analysis of TF glycosylation.

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

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.*

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, excluding N-glycosylation defect. 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 PMM2-CDG patients can be a useful note for clinicians.

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

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.*

In 2011, whole-exome sequencing identified *MAN1B1* (encoding α-1,2-mannosidase) as a causative gene underlying autosomal-recessive intellectual disability [10]. 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 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.

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

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.*

Isoelectric focusing and Western blot detection of serum ApoC-III, a method originally developed by Wopereis et al. [11], was introduced in our laboratory and performed in 170

healthy subjects of both genders in the age interval 1 day-42 years. 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 syndrome. 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.

3.2 Results and discussion related to the aim B.)

Biochemical and molecular characterization of CDG patients.

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

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.*

Two siblings, a boy (older) and a girl, were born at term and had an uneventful postnatal adaptation. Since the age of 8 and 6 months, 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). When they reached a young adult age, IEF of TF was performed and showed type I pattern. Following the detection of normal PMM and MPI activities in lymphocytes and cultivated fibroblasts, LLO analysis in the girl's fibroblasts confirmed the accumulation of dolichol-PP-$GlcNAc_2Man_5$. 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). 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, 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 might 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.

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

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.*

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. Because ApoC-III glycosylation was normal to borderline, the results indicated a defect affecting 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 [12]. Interestingly, their IEF of 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 [13], 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.

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

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.*

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 markers TF and ApoC-III analyzed in the patient 2 were normal. 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, the necessity of both NgBR and DHDDS for dolichol biosynthesis was demonstrated 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.

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

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). 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)). 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 [14, 15]. To examine the organelles in detail, we analyzed the fibroblasts by transmission electron microscopy 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. 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. DHE is used to monitor reactive oxygen species (ROS) in tissues *in vivo*, forming a red fluorescent product upon reaction with superoxide anions [16]. We hypothesize 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) [17]. Another unexpected and novel finding was our immunocytochemical detection of decreased catalase signal within peroxisomes in CDG-IIx(1), what is a feature of certain inherited peroxisomal disorders [18]. 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 [19]. It is, however, unclear to us why the aberrant catalase distribution was seen only in CDG-IIx(1); further elaboration is difficult as his genetic defect has not been identified so far.

3.3 Results and discussion related to the aim C.)

Alterations in protein glycosylation of non-CDG etiology.

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

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.*

Mild ApoC-III hypoglycosylation, mostly with relatively elevated ApoC-III_1, was found in 4/10 of our patients with Prader-Willi s.(PWS). 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 [20]. Moreover, certain SNPs in *GALNT2* were found to be associated with the levels of plasma triacylglycerols (TAG) and cholesterol [21]. Thus, we suggested that the increased 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 and ApoC-III_1 appear to be under metabolic control, and their relative abundance is associated with fasting plasma TAG [22].

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

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.*

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 %). SDS-PAGE analysis of ApoC-III suggested that in GSD patients with pathological ApoC-III profile, there is an increased abundance of ApoC-III_0 which carries no glycan. 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 glucose-l-phosphate, 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 due to their hyperglycemia [20]. Furthermore, GWAS studies implicated a link between certain SNPs in *GALNT2* and TAG/cholesterol levels [21], and probands with *GALNT2* mutations leading to reduced catalytic activity of the enzyme showed lower TAG and elevated HDL [23]. In our analysis, however, no single metabolic factor was identified as the molecular link underlying reduced ApoC-III glycosylation. Overall, our results can be useful in differential diagnosis and 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.

4 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:

- introduction, optimization and realization of CDG screening methods, e.g., introduction of ApoC-III isoelectric focusing as a method for screening O-glycosylation disturbances, or detection of rare transferrin polymorphism which hampers N-glycosylation screening

- biochemical and molecular characterization of patients with various CDG types, including analysis and diagnosis of patients with RFT1-CDG, MAN1B1-CDG and PGM1-CDG, expanding the genotypes and phenotypes associated with the disorders; identification of a novel CDG type due to NgBR defect in two siblings; original observation of increased reactive oxygen species in fibroblasts from patients with selected CDG types

- description of glycosylation defects of non-CDG etiology, such as previously unreported finding of hypoglycosylated ApoC-III in glycogen storage diseases

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 (20x PMM2-CDG, 3x EXT1/EXT2-CDG, 2x RFT1-CDG, 2x NGBR-CDG, 1x ALG8-CDG, 1x DPAGT1-CDG, 1x SRD5A3-CDG, 1x MAN1B1-CDG and 1x PGM1-CDG). 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, as well as enabled the prenatal testing and genetic counselling in the affected families in the Czech Republic. Importantly, the presented results obtained within international collaboration have strengthened our laboratory's position in the worldwide CDG community.

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THE LIST OF ORIGINAL PUBLICATIONS

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b) without IF

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.*

Other publications *in extenso*:

b) without IF

Hansíková H., Ondrušková N., Honzík, T., Veselá K., Horová E., Švecová Š., Tesařová M., Zeman J. ***Aktivita fosfomanomutázy 2 u pacientů s podezřením na dědičnou poruchu glykosylace.*** *Klin Biochem Metab. 2016; 24(25), No.2:68-75.*