SUMMARY OF DOCTORAL THESIS
SCREENING OF CONGENITAL DISORDERS OF GLYCOSYLATION

DOCTORAL PROGRAM STUDY IN PAEDIATRICS

HRADEC KRÁLOVÉ
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OF GLYCOSYLATION

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Disertační práce byla vypracována v rámci prezenčního studia v doktorském studijním programu všeobecné lékařství, obor pediatrie, na Dětské klinice Lékařské fakulty University Karlovy v Hradci Králové.

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S disertační prací je možné se seznámit na děkanátu Lékařské fakulty v Hradci Králové, Šimkova 870, 500 01 Hradec Králové.

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2
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>List of abbreviations</td>
<td>5</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>6</td>
</tr>
<tr>
<td>1.1 Congenital disorders of glycosylation (CDG)</td>
<td>6</td>
</tr>
<tr>
<td>1.2 CDG diagnostics</td>
<td>7</td>
</tr>
<tr>
<td>1.3 Treatment of CDG</td>
<td>8</td>
</tr>
<tr>
<td>2 Aims of the study</td>
<td>10</td>
</tr>
<tr>
<td>3 Material and methods</td>
<td>10</td>
</tr>
<tr>
<td>3.1 Samples</td>
<td>10</td>
</tr>
<tr>
<td>3.2 Isoelectric focusing (IEF)</td>
<td>10</td>
</tr>
<tr>
<td>3.3 High performance liquid chromatography (HPLC)</td>
<td>11</td>
</tr>
<tr>
<td>3.4 Enzyme essay</td>
<td>11</td>
</tr>
<tr>
<td>4 Results</td>
<td>11</td>
</tr>
<tr>
<td>4.1 IEF</td>
<td>12</td>
</tr>
<tr>
<td>4.1.1 Glycosylation abnormalities</td>
<td>14</td>
</tr>
<tr>
<td>4.1.2 Transferrin and α₁-antitrypsin genetic variants</td>
<td>14</td>
</tr>
<tr>
<td>4.2 HPLC</td>
<td>15</td>
</tr>
<tr>
<td>4.3 Enzyme essays</td>
<td>16</td>
</tr>
<tr>
<td>4.4 Our CDG patient</td>
<td>17</td>
</tr>
<tr>
<td>5 Discussion</td>
<td>18</td>
</tr>
<tr>
<td>5.1 Preanalysis</td>
<td>18</td>
</tr>
<tr>
<td>5.2 IEF</td>
<td>19</td>
</tr>
<tr>
<td>5.3 HPLC</td>
<td>20</td>
</tr>
<tr>
<td>5.4 Enzyme essay</td>
<td>20</td>
</tr>
<tr>
<td>5.5 Interpretation of results</td>
<td>21</td>
</tr>
<tr>
<td>5.6 Transferrin and α₁-antitrypsin genetic variants</td>
<td>22</td>
</tr>
<tr>
<td>5.7 Comparison of methods used in CDG screening</td>
<td>23</td>
</tr>
<tr>
<td>5.8 Diagnostic algorithm</td>
<td>25</td>
</tr>
<tr>
<td>6 Conclusions</td>
<td>26</td>
</tr>
<tr>
<td>7 References</td>
<td>27</td>
</tr>
<tr>
<td>8 Publications of the author</td>
<td>29</td>
</tr>
<tr>
<td>9 Summary</td>
<td>30</td>
</tr>
<tr>
<td>10 Souhrn</td>
<td>33</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁-AT</td>
<td>α₁-Antitrypsin</td>
</tr>
<tr>
<td>Apo C-III</td>
<td>Apolipoprotein C-III</td>
</tr>
<tr>
<td>Bis-Tris</td>
<td>bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane</td>
</tr>
<tr>
<td>CDG</td>
<td>Congenital disorder of glycosylation</td>
</tr>
<tr>
<td>CDT</td>
<td>Carbohydrate deficient transferrin</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>COG</td>
<td>Conserved oligomeric Golgi complex</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers-Danlos syndrome</td>
</tr>
<tr>
<td>FCMD</td>
<td>Fukuyama-type congenital muscular dystrophy</td>
</tr>
<tr>
<td>FKRP</td>
<td>Fukutin-related protein</td>
</tr>
<tr>
<td>HEMPAS</td>
<td>Hereditary erythroblastic multinuclearity with positive acidified-serum test</td>
</tr>
<tr>
<td>HME</td>
<td>Hereditary multiple exostoses</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>LLO</td>
<td>Lipid-linked oligosaccharide</td>
</tr>
<tr>
<td>MEB</td>
<td>Muscle–eye–brain disease</td>
</tr>
<tr>
<td>MPa</td>
<td>Mega Pascal</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PMM</td>
<td>Phosphomannomutase</td>
</tr>
<tr>
<td>TBG</td>
<td>Thyroxin-binding globulin</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>WWS</td>
<td>Walker-Warburg syndrome</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Congenital disorders of glycosylation (CDG)

The cause of carbohydrate-deficient glycoprotein Syndrome, later renamed congenital disorders of glycosylation (CDG) lies in defective synthesis and processing of oligosaccharides of N-bound glycoproteins (Jaecken J 2003). CDG impairs all organs of the body; the severity level of clinical development and the presentation of the CDG types vary strongly from patient to patient. The disorders fall into two groups, CDG type I and type II, based on the localization of the defect; in cytoplasmic reticulum or Golgi apparatus. So far, it is possible to distinguish 18 subtypes (Ia - II), and (Ia - IIf).

CDG-Ia is the most frequent CDG type (over 85%) with more than 500 patients described worldwide; it is caused by a deficiency of phosphomannomutase (PMM), which converts mannose-6-phosphate to mannose-1-phosphate. Over 75 different mutations have been found at the corresponding gene of this type; the most frequent one, R141H, accounts for 40% of CDG Ia. Patients can be often diagnosed on the basis of typical clinical features, such as inverted nipples and fat pads, in addition to strabismus, muscular hypotonia, cerebellar hypoplasia and failure to thrive. The number of patients with a less typical presentation is increasing (Marquardt T 2003).

CDG-Ib is caused by a deficiency of phosphomannose isomerase, which affects the endogenous production of mannose-6-P. In contrast to in CDG-Ia, mental and motor development is normal. The predominant symptom is protein-losing enteropathy with chronic diarrhoea, commonly starting during the first year of life; some of patients present with congenital hepatic fibrosis. Cyclic vomiting, failure to thrive, hypoglycaemia, hypoalbuminemia, elevated aminotransferases and low antithrombin III activity are common findings in CDG-Ib. Over 20 patients have been known with this type (Marquardt T 2003).

CDG-Ic is a mainly neurological disorder that is in general milder than CDG-Ia, and without dysmorphism. More than 50 CDG-Ic patients have been described so far (Matthij G 2004).

Only a few cases, enlisted as the other CDG types, have been described; see an overview in Table 1.

CDG-x

About 20% of CDG patients remain untyped and are named CDG-x. Apart from the CDG typical clinical presentations, oligohydramnion, hydrops fetalis, absent of psychomotor retardation, severe thrombocytopenia, ascites, demineralization of distal bones, tubulopathy, and death in status epilepticus have also been reported (Marquardt T 2003, Grünwald S 2003).

Beside N-glycosylation of proteins, defects also occur in other pathways such as O-linked glycosylation, proteoglycan synthesis, and lipid glycosylation. Some disorders affecting membrane glycoproteins (e.g. dyserythropoietic anaemia, HEMPAS), and O-glycosylation defects presently wait for precise enlistment. Two main groups of O-glycosylation disorders can be identified so far: 1) defects in O-mannosylglycan synthesis, involving Walker-Warburg syndrome (WW) and Muscle-eye-brain (MEB) disease, and 2) defects in O-xyllosylglycan formation with clinical expression of hereditary multiple exostoses (HME) and the progeria variant of Ehlers-Danlos syndrome (EDS). O-hypoglycosylation abnormalities can also be found in Fukuyama-type congenital muscular dystrophy (FCMD) and Fukutin-related protein (FKRP) defect. The O-glycosylation defects are characterized mainly by muscle dystrophy, structural eye abnormalities, and brain malformation.

Abnormal glycosylation of α-dystroglycan was found in a congenital muscular dystrophy, limb-girdle muscular dystrophy and FKRP. This protein is modified by both
N- and O-linked glycosylation. There is evidence for a requirement of sialylated O-glycans on α-dystroglycan for binding to many of its ligands; these bindings are defective in MEB and FCMD (Grünewald S 2003).

Some patients diagnosed as the CDG type II have deficiencies that affect both N- and O-linked pathways, showing that the two synthetic processes are not completely separate.

1.2 CDG diagnostics

Abnormal liver function tests, low plasma cholesterol and proteinuria are common findings in patients with CDG type Ia. Frequently found hypoalbuminemia, hypoglycaemia with inadequately increased insulin production, and high activities of aminotransferases, are typical for CDG Ib (Keir G 1999).

The enzymatic activities or levels of plasma glycoproteins, including transport proteins, e.g. α1-antitrypsin (α1-AT), thyroxin-binding globulin (TBG), and transferrin (Tf), glycoprotein hormones, coagulation and anticoagulation factors (particularly the factors V, XI, II, X, antithrombin III), proteins C, S and heparin cofactor II are usually low, while the levels of fibrinogen and D-dimer are frequently raised (Jaeken J 2001).

A large number of serum glycoproteins have been shown to have abnormal isoelectric focusing (IEF) pattern. The common diagnostic test for CDG is IEF of serum Tf; its microheterogeneity on IEF has turned out to be a sensitive biochemical marker of most N-glycosylation defects (Stibler H 1998).

The isoforms of the Tf molecule have been differentiated as a result of three types of variations: 1) Sequence of amino acids of the polypeptide chain corresponding to its genetic polymorphism. 2) Degree of iron saturation (there exist two independent metal iron-binding sites, one within the N-terminal and the other within the C-terminal domain). 3) Composition of the carbohydrate chain (N-linked complex glycan chains).

The two carbohydrate chains vary in their degree of ramification, presenting with 2 or 3 external antennas. Each antenna finishes negatively with a sialic acid (N-acetylneuraminic acid), which contributes to the total load of the glycoprotein. The content of sialic acid can vary from zero to eight, and determines the heterogeneity of the Tf molecule. Deficient synthesis of N-glycans results in a deficient incorporation of sialic acid, the terminal negatively charged sugar; the molecules acquire a more positive charge, which causes a cathodal shift in the IEF pattern of Tf. Therefore; the proportions of glycoforms with zero (asialo-) and two (disialo-Tf) oligosaccharide branches are increased with a corresponding decrease in tetrasialo-Tf.

The CDG type I presents with partially (one chain) or totally decrease of N-glycans. In the CDG type II, the number of glycans is normal, but processing defects of the oligosaccharide lead to truncated carbohydrate structures.

One has to make sure that the abnormal pattern is not the result of a Tf protein variant; digestion of sialic acids with neuraminidase, or comparison of the IEF pattern in the patient and his parents (the same variant should be found) are possible means of how to differentiate between CDG and protein variants. Analysis of other serum glycoproteins, e.g. α1-AT may help in documentation of a generalized glycosylation defect in the patient.

CDG-IIB, CDG-IIc, CDG-IIf, MEB, WWS, FKRFP, FCMD, HME, and EDS are not detectable by Tf IEF, though there was a report in one patient with HEMPAS, having hypoglycosylation of serum Tf. HEMPAS can be detected by a loss of lectin binding to specific glycoconjugates. Even some CDG-Ia patients might be missed by the IEF Tf test.
Thin-layer chromatography of urine oligosaccharides is the method of choice in the case of CDG-IIb. Sialyl Lewis X antigen is absent on the neutrophils in CDG-Ilc and IIff, which also shows the Bombay blood group phenotype (Marquardt T 2003).

Analysis of LLO is indicated, if IEF have a CDG pattern, but enzyme assay of PMM and phosphomannno isomerase shows normal activities.

Analysis of glycan structure will be instrumental for the elucidation of CDG-x cases, by pinpointing candidate enzymes and genes responsible for the abnormal glycan synthesis.

Tf IEF method is not reliable for prenatal diagnosis (Clayton P 1993). Measurements of enzymes activities in cultured amniocytes or trophoblasts are useful, but may give inconclusive data (Matthijs G 1998). As a result, preference is given to direct mutation analysis in the foetus. Prenatal diagnosis is possible in all types of CDG for which the molecular defect is known, on the condition that the diagnosis has been confirmed in the index patient, or the mutations have been detected in the parents.

1.3 Treatment of CDG

Unfortunately, an efficient treatment is still not available for the CDG-Ia patients. The biguanide drug metformin experimentally corrected N-linked glycosylation: It stimulates AMP-activated protein kinase, a master regulator of cellular energy metabolism, and it activates a novel fibroblast mannose-selective transport system. This suggests that AMP-activated protein kinase may be a regulator of mannose metabolism, thus implying a therapy for CDG-Ia (Shang J 2004).

Results of quite recent experiments might be promising (Eklund EA 2005): two hydrophobic, membrane-permeable derivatives of mannose-1-phosphate were synthesized, and their ability to normalize LLO size and N-glycosylation in CDG-Ia fibroblasts tested: glycosylation has been restored to control cell levels.

CDG-IIb was the first disorder of glycosylation where a specific therapy was available. Symptoms can be effectively reduced with the oral mannose administration (Niehues R 1998). Mannose normalizes the hypoglycosylation of proteins, hypoproteinemia, blood coagulation, protein-losing enteropathy and hypoglycemia. While the clinical symptoms disappear rapidly, significant improvement of the Tf IEF pattern during mannose therapy takes several months of treatment to occur (Niehues R 1998).

Despite the successful correction of immunodeficiency-related defects in CDG-IIe, correcting the delayed psychomotor development is more difficult to achieve (Marquardt T 2003).

There are no causal therapeutic options for other CDG types, HME, EDS, HEMPAS, FCMD, FKRP, WWS and MEB.
<table>
<thead>
<tr>
<th>Type</th>
<th>Gene</th>
<th>Gene location</th>
<th>Deficient enzyme</th>
<th>Prevailing symptoms</th>
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<tbody>
<tr>
<td>Ia</td>
<td>PMM1, 2</td>
<td>16p13.3-13.2</td>
<td>Phosphomannomutase</td>
<td>Dysmorphism, hypotonia, cerebellar hypoplasia</td>
</tr>
<tr>
<td>Ib</td>
<td>MPI</td>
<td>15q22-qter</td>
<td>Phosphomanno isomerase</td>
<td>Hepatic fibrosis, enteropathy, coagulopathy</td>
</tr>
<tr>
<td>Ic</td>
<td>ALG6</td>
<td>1p22.3</td>
<td>Glucosyltransferase I</td>
<td>Moderate form of CDG-Ia</td>
</tr>
<tr>
<td>Id</td>
<td>ALG3</td>
<td>3q27</td>
<td>Mannosyltransferase VI</td>
<td>Profound form of CDG-Ia</td>
</tr>
<tr>
<td>Ie</td>
<td>DPM1</td>
<td>20q13.13</td>
<td>Dolichol-P-mannose synthase I</td>
<td>Similar to CDG-Ia, cortical blindness, microcephaly</td>
</tr>
<tr>
<td>If</td>
<td>MPDUI</td>
<td>17p13.1-p12</td>
<td>Dolichol-P-mannose I utilization</td>
<td>Typical CDG-Ia symptoms, ichthyosis</td>
</tr>
<tr>
<td>Ig</td>
<td>ALG12</td>
<td>Chr.22</td>
<td>Mannosyltransferase VIII</td>
<td>Common CDG-Ia symptoms, low IgG</td>
</tr>
<tr>
<td>Ih</td>
<td>ALG8</td>
<td>11pter-p15.5</td>
<td>Glucosyltransferase II</td>
<td>Similar to that of CDG-Ib</td>
</tr>
<tr>
<td>IIi</td>
<td>ALG2</td>
<td>9q22</td>
<td>α-1,3 Mannosyltransferase</td>
<td>Typical symptoms of CDG-Ia</td>
</tr>
<tr>
<td>IIj</td>
<td>DPAGT1</td>
<td>11q23.3</td>
<td>Dolichol-P-N-NAcGlc-1-P-transferase</td>
<td>Similar to that of CDG-Ia</td>
</tr>
<tr>
<td>Ik</td>
<td>ALG1</td>
<td>16p13.3</td>
<td>Mannosyltransferase I</td>
<td>Common CDG-Ia symptoms, ↓ B-cells, IgG</td>
</tr>
<tr>
<td>II</td>
<td>ALG9</td>
<td>11q23</td>
<td>α-1,2 - Mannosyltransferase</td>
<td>Microcephaly, hypotonia, seizures, hepatomegaly</td>
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<tr>
<td>IIa</td>
<td>MGAT2</td>
<td>14q21</td>
<td>N-Acetyl-glucosaminyltransferase II</td>
<td>Developmental delay, dysmorphism, seizures</td>
</tr>
<tr>
<td>IIb</td>
<td>GCSI</td>
<td>2p13-p12</td>
<td>Glucosidase I</td>
<td>Dysmorphism, hypotonia, seizures, hepatic fibrosis</td>
</tr>
<tr>
<td>IIc</td>
<td>FUCT1</td>
<td>11p11.2</td>
<td>GDP-fucose transporter</td>
<td>Recurrent infections, developmental delay</td>
</tr>
<tr>
<td>IIId</td>
<td>β4GALT1</td>
<td>9p13</td>
<td>β-1,4- Galactosyltransferase I</td>
<td>Myopathy, Dandy-Walker malformation, coagulopathy</td>
</tr>
<tr>
<td>IIe</td>
<td>COG7</td>
<td>16p</td>
<td>COG 7</td>
<td>Dysmorphism, hypotonia, recurrent infections</td>
</tr>
<tr>
<td>IIIf</td>
<td>CMP</td>
<td></td>
<td>CMP- sialic acid transporter</td>
<td>Thrombocytopenia, no neurologic symptoms</td>
</tr>
</tbody>
</table>
2 Aims of the study
The goals of this study were:
- Introduction of a screening method (IEF) for the diagnostics of CDG.
- Verification of the abnormal results by another method (HPLC).
- Introduction of an enzyme essay for the most common CDG type Ia.
- Determination of the CDG frequency in our set of patients under clinical suspicion of a congenital metabolic defect.
- Presentation of an algorithm design of CDG screening.
- Presentation of our experience in the CDG screening of a paediatric population suspected of a metabolic disease.

3 Materials and Methods
All chemicals were of analytical grade, supplied by Pharmacia, Merck, Sigma, DAKO, or Bio-Rad. Use of double-distilled water for all analytical steps is essential.

3.1 Samples
About 100 healthy individuals (blood-donors, friends and relatives) and over 1100 patients, both children and adults under clinical suspicion of a congenital metabolic defect of various ages (1 month – 62 years), were examined by IEF. Beside these, several groups of patients with isolated signs or clear diagnoses were screened. Also CDG-positive samples (3) obtained from other laboratories that served as pathological reference samples have also been checked out. Only surplus material from routine investigations was used for analyses. Serum, plasma, Guthrie card with serum or full blood (venous or umbilical cord), urine, amniotic and cerebrospinal fluid (CSF) were tested.

Apart from controls, CDG and the other patients with higher CDT, previously screened out by IEF, all with common and rare protein Tf variants were analysed by HPLC.

Blood samples for enzyme assay were obtained from 12 healthy individuals, age range 2 years – 60 years.

3.2 IEF
A common IEF method, adapted for analysis of Tf and α1-AT (Albahriz Z 2004), using Multiphor II apparatus and Immobiline DryPlate gels of pH 4-7, detected by immunofixation and Coomassie blue staining, was applied in this study; digestion with neuraminidase helped in identification of Tf variants.

The samples were saturated with Fe-citrate/NaHCO₃ solution, (alternatively, FeNTA and FeCl₃ solutions were also used) then incubated for 1 hour at room temperature, 6times diluted with water, and applied on the gel. For combined Tf and α1-AT analysis, the samples were prepared as previously, but with 6times dilution. When α1-AT alone was analysed, serum was pre-treated with cysteine solution. Samples eluted from dry spots were also tested.

CSF, amniotic fluid and urine were concentrated in vacuum, further saturated, and applied on the gel without dilution.

The peak areas or/and heights of the penta-, tetra-, tri-, di-, mono-, and asialo-Tf isoforms were used for calculation (the ratio in %); the hexa-, penta-, tetra-, tri-, di-, mono- and asialo- α1-AT were calculated similarly.
3.3 HPLC

The HPLC procedure (Jeppsson JO 1993), based on anion-exchange chromatographic separation of the individual Tf glycoforms using linear salt gradient, and followed by photometric detection of the Fe-Tf complex at 460 nm was used in this study.

Various HPLC systems were tested: ECOM system with LCD 2084 UV/Vis detector, Hewlett-Packard 1050, Shimadzu LC-10AD VP equipment with SPD-10A VP UV/Vis detector, and Agilent 1100 Series equipped with multiple wavelength UV/Vis detector.

Two types of strong anion-exchange columns, ResourceQ and MonoQ with 0.5 µm inline solvent filter, 0.2 µm filter for mobile phase filtration, and various types of 0.45 µm disposable sample filters, were used.

The mobile phases (Bis-Tris-NaCl) for gradient elution consisted of the following solutions: buffer A was 20 mmol/L Bis-Tris, pH 6.2, buffer B was buffer A, containing 0.35 mol/L NaCl, and solution C was 1 mol/L NaCl. Apart from those described by Jeppsson (Jeppsson JO1993), other programs of linear salt gradient were also tested. Finally we adopted that one used by Turpeinen (personal communication) with a total run time 49 min.

Saturated, delipidated, diluted and filtered samples were analysed; volume of 100 µL was injected. Apart from native serum, also serum dry spot, and samples digested with neuraminidase were analysed.

3.4 Enzyme essay

Screening of the most common CDG type Ia is completed by measurement of cellular PMM activity. A two-step procedure has been adopted for isolation of peripheral blood leucocytes; a whole blood heating-step was followed by centrifugation, and a saline washing of isolated leucocytes (Beutler E 1970). The cells were lysed mechanically by numerous passes through a syringe needle (Barnier A 2002), and PMM activity was assayed spectrophotometrically on the basis of NADP(+) reduction to NADPH (van Schaftingen 1995).

4 Results
4.1 IEF

The IEF conditions for Tf analysis which we have adopted are as follows: 1.5 µL of serum sample, previously iron-saturated and then diluted 6 times, is applied on a pre-focused Immobiline DryPlate gel of pH range 4-7, then focused at 2000 V, 4 mA and 8 watts for 4 h (8000 Vh); detection by direct immunofixation (RAHU anti-Tf, Coomassie blue) was evaluated by densitometry.

Beside the analysis of Tf, a method to separate α1-AT isoforms was introduced; similar analytical conditions can be used. Simultaneous analysis of Tf and α1-AT in the same gel was tested with good results, and successfully used since then. A maximum of 52 samples could be applied on the gel. Later on, the method was adapted for lower series, by use of smaller pieces of gel, which allows obtainment of results more quickly.

In serum of healthy individuals, we can identify a major Tf isoform tetrasialo- Tf at pI 5.4, and three additional Tf isoforms, pentasialo-, trisialo-, and asialo- at pI 5.3, 5.6 and 5.7 respectively. Higher di-, mono, or asialo-Tf (CDT) distribution is characteristic for CDG patients.

In control CSF, considerably higher mono-, and asialo-Tf were found, while pentasialo-, tetrasialo-, trisialo- and disialo-Tf were decreased, when compared to serum. Analysis of amniotic fluid resulted in an IEF pattern similar to that obtained with serum.

No useful results were obtained from urine, because of the high salt concentration in the samples.
The *in-vitro* iron-saturation step is necessary for reliable interpretation of results. For safe recognition of improperly saturated samples, the same native sample and iron-treated serum were analysed for comparison: sharp bands of Tf isoforms could be detected in the properly saturated sample, while tailing is noted in the native sample. Various Fe-solutions (citrate, chloride, and FeNTA) were tested, but no significant difference was noted.

Optimal conditions for dry spot samples were achieved, when 10 µL (eluate of blood dry spot), or 5 µL (eluate of serum dry spot) aliquots were applied on the gel; no distinct difference was noted, when compared to serum. Using this type of material, both glycoproteins (Tf and α₁-AT) could be analysed with nearly the same results, when compared to serum.

For complete Tf desialisation, 150 µg of neuraminidase is sufficient for 50 µL serum. Similar conditions are used for samples eluted from dry serum/blood spots.

Distribution of Tf and α₁-AT isoforms in our set of controls and some CDG patients has been established (Tables 2 - 5). No gender differences from the viewpoint of both aspects, hypoglycosylation and distribution of genetic variants (see Chapter 4.1.2), have been recognized.

Table 2 Reference values of serum Tf isoforms in children (n= 35)

<table>
<thead>
<tr>
<th>Tf isoforms</th>
<th>Mean (%)</th>
<th>SD</th>
<th>x - 2 SD</th>
<th>x + 2 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8</td>
<td>0.7</td>
<td>-0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
<td>0.9</td>
<td>-0.9</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>2.4</td>
<td>2.2</td>
<td>11.9</td>
</tr>
<tr>
<td>3</td>
<td>10.7</td>
<td>3.1</td>
<td>4.5</td>
<td>17.0</td>
</tr>
<tr>
<td>4</td>
<td>63.2</td>
<td>6.9</td>
<td>49.5</td>
<td>77.1</td>
</tr>
<tr>
<td>5</td>
<td>17.0</td>
<td>6.3</td>
<td>4.3</td>
<td>29.6</td>
</tr>
</tbody>
</table>

Table 3 Reference values of serum α₁-AT isoforms in children (n= 13)

<table>
<thead>
<tr>
<th>α₁-AT isoforms</th>
<th>Mean (%)</th>
<th>SD</th>
<th>x - 2 SD</th>
<th>x + 2 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
<td>0.3</td>
<td>-0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.3</td>
<td>-0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>9.5</td>
<td>4.9</td>
<td>-0.3</td>
<td>19.3</td>
</tr>
<tr>
<td>3 + 4</td>
<td>36.6</td>
<td>1.4</td>
<td>33.2</td>
<td>38.8</td>
</tr>
<tr>
<td>6</td>
<td>34.5</td>
<td>5.0</td>
<td>24.5</td>
<td>44.5</td>
</tr>
<tr>
<td>7</td>
<td>19.2</td>
<td>2.6</td>
<td>13.8</td>
<td>24.2</td>
</tr>
</tbody>
</table>

4.1.1 Glycosylation abnormalities

A patient with typical clinical symptoms and abnormal IEF pattern was finally identified as a CDG type IXa (see Chapter 4.4). The frequency of CDG (primary abnormality) in our set of patients has been calculated to be approximately 1 : 1 100.

Serum of three CDG 1a patients (kindly provided by another laboratory) served as pathological reference samples in this study; they showed a typical CDG I pattern with
lower tetrasialo-, and a marked increase of disialo-, and asialo-Tf. Our CDG patient showed higher disialo-, but only slight increase in asialo-Tf; see Table 4.

The IEF pattern of $\alpha_1$-AT in the three CDG Ia patients was characterized by increased asialo- and monosialo- isoforms, together with lower tetrasialo-$\alpha_1$-AT, when compared with controls. Our patient, however, showed only increase in monosialo-$\alpha_1$-AT (Table 5).

### Table 4 Distribution of Tf isoforms (%) in controls and CDG patients

<table>
<thead>
<tr>
<th>Samples</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDG Ia 1</td>
<td>7.91</td>
<td>4.07</td>
<td>30.58</td>
<td>11.44</td>
<td>34.86</td>
<td>11.14</td>
</tr>
<tr>
<td>CDG Ia 2</td>
<td>19.81</td>
<td>3.43</td>
<td>30.58</td>
<td>8.32</td>
<td>29.68</td>
<td>8.18</td>
</tr>
<tr>
<td>CDG Ia 3</td>
<td>11.89</td>
<td>5.35</td>
<td>28.53</td>
<td>12.44</td>
<td>31.48</td>
<td>10.31</td>
</tr>
<tr>
<td>Our patient</td>
<td>4.70</td>
<td>1.87</td>
<td>18.46</td>
<td>16.90</td>
<td>38.99</td>
<td>19.08</td>
</tr>
<tr>
<td>Controls (mean)</td>
<td>0.80</td>
<td>1.30</td>
<td>7.00</td>
<td>10.70</td>
<td>63.20</td>
<td>17.00</td>
</tr>
</tbody>
</table>

### Table 5 Distribution of $\alpha_1$-AT isoforms (%) in controls and CDG patients

<table>
<thead>
<tr>
<th>Samples</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3 + 4</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDG Ia 1</td>
<td>5.6</td>
<td>7.8</td>
<td>12.4</td>
<td>31</td>
<td>29.4</td>
<td>13.8</td>
</tr>
<tr>
<td>CDG Ia 2</td>
<td>6.2</td>
<td>6.1</td>
<td>11.9</td>
<td>30.5</td>
<td>32.0</td>
<td>13.3</td>
</tr>
<tr>
<td>CDG Ia 3</td>
<td>6.0</td>
<td>6.3</td>
<td>10.9</td>
<td>32.7</td>
<td>31.0</td>
<td>13.1</td>
</tr>
<tr>
<td>Our patient</td>
<td>0.1</td>
<td>4.8</td>
<td>9.6</td>
<td>32.3</td>
<td>39.2</td>
<td>14.0</td>
</tr>
<tr>
<td>Controls (mean)</td>
<td>0.1</td>
<td>0.1</td>
<td>9.5</td>
<td>36.6</td>
<td>34.5</td>
<td>19.2</td>
</tr>
</tbody>
</table>

![Isoforms of Tf C1](image1)

![Isoforms of $\alpha_1$-AT M](image2)

Fig. 1. IEF of serum $\alpha_1$-AT and Tf on Immobiline DryPlate gel with pH range of 4-7. See the increase of monosialo- $\alpha_1$-AT, and disialo-Tf with asialo-Tf in our CDG patient (CDG-IIx), compared to controls. F= fucosylated form of $\alpha_1$-AT, 0=asialo, 1=monosialo, 2=disialo 3=trisialo-, 4=tetrasialo-, 5=pentasialo-, 6=hexasialo-, 7=septasialo-, 8=octasialo-Tf, or $\alpha_1$-AT.
Secondary, non CDG abnormalities were detected in about 7.2 % (n= 85) of the total group of patients; they were mostly associated with other diagnoses, e.g. Hashimoto thyroiditis (in 2 of 2 patients diagnosed, 2/2), Budd-Chiari syndrome (1/3), systemic lupus erythematosus, (1/3), rheumatoid arthritis (4/11), Wilson disease (1/1), epilepsy (14/40), bronchial asthma (1/11), nephrotic syndrome (1/12), obesity (1/5), speech disorders (1/4), acute tubulo-interstitial nephritis (1/1), and sclerosis multiplex (1/1). Increased CDT was also noted in 18 of 38 newborns of age 1-3 weeks, and in 3 of 60 plasma samples. Other abnormalities were found in patients with hepatoopathy, hypotonia, psychomotor retardation, convulsions, failure to thrive, anaemia, and psychic abnormalities. Apart from that, a group of adults with severe hepatic disease (n=15, including hepatitis C, cirrhosis, fibrosis, and liver failure) were analysed; moderately increased CDT was found in 3 patients. Also several adults acutely intoxicated by alcohol and/or drugs intake (n=8) were screened for CDT; 6 patients had an abnormal Tf pattern. Due to reported influence of some common chronic diseases on protein glycosylation, a group of children with cystic fibrosis (n=40) and Crohn’s disease (n=24) were tested; an increase of CDT was detected in three patients with cystic fibrosis and one patient with Crohn’s disease. Serum Tf isoforms were also analysed in healthy newborns with gestational age between 36-38 weeks; among the 12 of the newborns tested, 7 showed higher CDT.

Effect of long term (over three months) treatment with cytostatics (methotrexaet, n=15), antibiotics (n=10), corticosteroids (n=10), antiepileptics (n=13), antirheumatics (salazopyrin, n=11; chlorochine, n=5) has been followed; only slight signs of hypoglycosylation were found in three children treated by methotrexaet, or Phenaemaletten, and in an adult with combined therapy of carbamazepine, primidone and valproate; no effects of corticosteroids, antirheumatics, or antibiotics (trimethoprim, penicillin, or amoclen) were noticed.

4.1.2 Tf and α₁-AT genetic variants

In this study, seven different phenotypes of Tf have been recognized. Only the variants C₁C₁ (in 86 %), and C₁C₂ (16 %) could be demonstrated among healthy subjects, while in a comparatively larger group of patients, apart from these two (in 78.7 % and 20 %, respectively), also the rare Tf C₂C₂ (0.6 %), and C₁C₃ (0.3 %), as well as heterozygous CB (0.2 % for Tf C₁B₁₂) and CD (0.1 % for both Tf C₁D₂ and Tf C₁D₄₅) phenotypes were found. Apparently higher incidence of the Tf C₁C₂ subtype, noted in the group of patients suspected of an inborn metabolic disease IMD (20 %; n=1100) and especially in two smaller groups of children with Crohn’s disease (29.2 %; n=24) and cystic fibrosis (27.5 %; n=40), when compared to healthy controls (16 %; n=100), was not in fact, significant (Ch square and Fisher's exact tests). No differences could be found in other subgroups of patients tested.

An abnormal IEF pattern was found in a 14-year old boy with the diagnosis of ankylosing spondylitis. IEF revealed a considerable, but isolated increase in the asiaglo-Tf band. This finding, not typical for the most common CDG types, together with a lack of CDG symptoms prompted us to rule out a slow Tf CD variant. However, just one band, reflecting asiaglo-Tf C₁, appeared after Tf desialization by neuraminidase, and thus conditions, more suitable for Tf D variant were used (pH range of 3.5-9.5). Then indeed, a double-band IEF pattern, typical for the presumed Tf C₁D₄₅ variant, was found. A finding of the same Tf phenotype in the patient’s father, and Tf C₁ with normal ratio of isoforms in the mother, together with a normal result of α₁-AT analysis in the boy and his parents supported the presence of the rare Tf variant.

α₁-AT was analysed in about 50 individuals. In addition to the most common phenotype MM, a variant MS has also been recognised in one patient.
4.2 HPLC

We introduced the HPLC of Tf procedure described by Jeppsson (Jeppsson JO 1993), using the ECOM system and MonoQ column, but our results were not satisfactory.

Taking into account the ECOM producer claims of the high standard of quality of the detector supplied, as well as our lack of experience with this type of HPLC column, we decided to check on the individual steps of the procedure. We started with the sample pretreatment, preparation of mobile phases, considering various programs of elution gradient and flow rate, and then comparing various HPLC systems, namely detectors with the optimal wavelength setting, weighing up the choice of chromatographic column.

First of all we focused our attention on sample pretreatment. Since the saturation step is evidently a critical point of the Tf analysis, various iron-reagents were tested, and working conditions such as temperature and time of saturation were optimised. To study the effect of incomplete iron saturation of Tf we prepared a native, “in vitro” non-saturated serum sample; this sample produced a different HPLC pattern with new peaks, due to the different charges of the incompletely saturated Tf molecule. Of the three different iron solutions tested, the serum sample saturated with FeCl₃ showed the best results. When the standard FeNTA reagent was tested, a peak with a retention time close to that of tetrasialo-Tf was noted; thus its use for Tf saturation may possibly result in false interpretation. We have also found that EDTA plasma showed a peak eluting at the position of trisialo-Tf.

Apart from serum and plasma, eluates of dry serum spot (Guthrie paper) were also tested on the Agilent 1100 system: the CDT isoforms were below the detection limit of the HPLC method.

Two anion-exchange chromatographic columns were used and compared; finally we chose the more economic ResourceQ, giving similar results.

We had been forewarned of the insufficiency of some systems with UV-Vis detectors (e.g. Hewlett-Packard 1090, Turpeinen U 2001) for this application; therefore we took the opportunity to test other HPLC systems available, such as Hewlett-Packard 1050, Shimadzu LC10AD VP, and Agilent 1100. Various systems showed different detection sensitivity of Tf isoforms.

Analysis of healthy controls by ECOM system resulted in separation of the main three peaks (tetra-, penta-, and trisialo-Tf), but the disialo- and lower-sialo-Tfs were under the detection limit. In our CDG patient, an increase in the disialo-Tf, but not the other CDT peaks (asialo-Tf), was found. Various samples (of newborns, patients with cystic fibrosis or those treated with antiepileptics), showing abnormalities by IEF method did not present any CDT-typical peaks with the ECOM HPLC system. We have been more successful in differentiating between the common TfC₃ and some other genetic variants of Tf.

Analogous separation, but with higher yield in controls as well as in our CDG patient could be obtained on the Shimadzu system (Fig. 2).
Fig. 2. Analysis of serum Tf isoforms by ECOM and Shimadzu HPLC systems on ResourceQ column with detection at 460 nm wavelength; three / four peaks corresponding to tetra-, penta-, tri-, and disialo-Tf (4, 5, 3, and 2, respectively) in control, and appearance / elevated disialo-Tf in our CDG patient, respectively.

When Agilent 1100 multiple wave lengths UV-Vis detector was used, satisfactory results were obtained, giving good resolution not only in individuals with increased CDT (alcoholics), but also in controls (physiological disialo-Tf was detected).

4.3 Enzyme essays
Parameters and results of PMM enzyme essay in our CDG patient and the other family members (controls) are presented in Table 6: volumes of the EDTA blood used for leucocytes isolation, volumes of supernatant gained after blood warming, protein concentration in the cells lysate, and enzyme activities are given.

Table 6 Results of enzyme activity in controls, our CDG patient, and his family

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Blood volume (mL)</th>
<th>Supernatant (mL)</th>
<th>Protein (g/L)</th>
<th>PMM activity U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Our CDG patient</td>
<td>9.6</td>
<td>2.2</td>
<td>1.84</td>
<td>0.67</td>
</tr>
<tr>
<td>Father</td>
<td>9.3</td>
<td>1.9</td>
<td>1.59</td>
<td>0.62</td>
</tr>
<tr>
<td>Mother</td>
<td>9.0</td>
<td>2.6</td>
<td>2.21</td>
<td>0.80</td>
</tr>
<tr>
<td>Brother</td>
<td>8.5</td>
<td>1.7</td>
<td>1.60</td>
<td>0.65</td>
</tr>
<tr>
<td>Controls (n=12)</td>
<td>6 – 10</td>
<td>1.2 – 2.3</td>
<td>1.30 – 2.23</td>
<td>0.82 ± 0.28</td>
</tr>
</tbody>
</table>

PMM results are expressed as mean ± SD U/g of total protein content. 1 U of enzyme activity was defined corresponding to the formation of 1 μmol of NADH. All members of the family, our patient included, had a normal PMM activity.
4.4 Our CDG patient

Our CDG patient is a 5.5-year-old boy. At age 3.5 years, the boy was admitted to hospital with severe laryngopharyngitis, and at that time a screening for CDG was performed. IEF and HPLC of serum Tf showed a marked increase of distalo-Tf and slightly increased asialo-Tf. Abnormalities of α₁-AT and TBG proved a generalized defect of N-glycosylation. Increased fucosylation (F) in many known subtypes of CDG was reported (Callewaert N 2003); our patient showed a trace band in cathodic side, possibly corresponding to fucosylated isofrom of α₁-AT (Fig. 1).

Secondary causes, such as galactosemia and fructosemia were ruled out.

CDG types Ia and Ib could be excluded on the basis of normal enzymatic activity of the relevant enzymes in leukocytes and fibroblasts. Lipid linked oligosaccharide analysis in fibroblasts showed an essentially normal result, thus making any defect of CDG type I improbable. A physiological pattern of urinary oligosaccharides excluded CDG IIb, while normal expression of sialy Lewis X antigen in neutrophils testifies against types IIc and IIIf. The patient was classified as having an unidentified form of CDG type II.

The finding of a hyposialylation IEF pattern of apolipoprotein C-III (apo C-III) with increased apo C-III₀, normal apo C-III₁ and decreased apo C-III₂ levels led to the suspicion of a combined N- and O-glycosylation biosynthesis defect. Further analyses of glycans for elucidation of the basic defect are pending.

At age 5.5 years, the clinical pictures of our patient is characterized mainly by microcephaly, oligophrenia, dyslasia, muscular hypotonia, nystagmus, severe myopia, wrinkled skin, hypertrichosis, kyphosis, bilateral hallux varus and brachymetatarsia. APTT is repeatedly abnormal, but without any clinical manifestations. Coagulation factors II, V, VIII, X, XI and antithrombin III are basically normal. IEF of Tf and α₁-AT permanently shows hyposialylation patterns; FMG is normal, EEG is only slightly abnormal.

Recently, three cutis laxa patients have been described with a combined defect in N- and O-glycosylation (Morava E 2005, Wopereis S 2005), where the underlying enzyme defect still has to be elucidated. Another sibling pair with a combined defect in the biosynthesis of N- and O-glycans has been described with a Golgi traffic defect and mutations in COG7 presenting with excessive, wrinkled skin around the neck (Wu X 2004). Our patient has some overlapping features with the so far described children with combined glycosylation defects, including microcephaly, congenital brain malformations, congenital pes equinovarus, hypotonia, heart malformations, urinary abnormalities and the loose, wrinkled skin, although the onset of the skin symptoms greatly differs: in all previously described cases the skin anomalies were the most prominent directly after birth and improved thereafter, while our patient had a belated appearance of mild cutis laxa.

The patient’s younger brother, age 4.5 years, is healthy. Without detailed classification, prenatal diagnosis was impossible, and thus the foetus of the third pregnancy in the family was monitored only by ultrasound. A third boy was born in 39th week of gestation, fortunately without any apparent abnormalities (now at the age of 9 months). IEF of Tf was performed the first day of life from umbilical-blood spot, showing a slight increase of distalo-Tf (physiological in newborns); when repeated at the end of the first month of life the tests for Tf and α₁-AT were normal.
5 Discussion

5.1 Preanalysis

In this study, various types of material were analysed. Apart from serum, suitability of plasma was tested. We noted higher CDT values in a small number of plasmas heparin samples analysed by IEF. Generally, serum is preferred over the plasma samples for both IEF and HPLC application, since the heparin and EDTA reportedly (Arndt T 2001) disturb the Tf saturation, and they present additional peaks/bands on HPLC/IEF records.

In amniotic fluid, well-separated Tf isoforms by IEF could be detected in controls; it was thus supposed that the prenatal diagnosis of CDG would be possible (Stibler H 1991). However, Clayton et al (Clayton P 1993) was not able to find any abnormalities of Tf or α₁-At in a CDG patient until to third week after birth. The prenatal diagnostic failure may be due to maternal metabolism, compensating for the defect in the foetus.

In CSF, physiologically higher CDT was found when compared to serum, which is caused by higher activity of neuraminidase in the brain. Significantly increased levels of CDT were found in CDG Ia patients (Keir G 1999). Unfortunately, CSF was not available in our CDG patient; moreover, there were no reports on validity of this material in other CDG types.

The urine is an easily obtained material, widely used in the screening of inborn metabolic diseases IMD; Kishi (1990) referred, that Tf isoforms could be detected by immunoblotting in urine. Our failure when analysing this material could be possibly avoided by sample desalting, or by more sensitive detection; however, it makes the procedure rather complicated.

Dry spots are used in the screening of metabolic diseases; it simplifies sampling, storage, and transportation of specimens, thus making larger population studies available. It was shown that neonatal diagnosis was possible by IEF of Tf eluted from 14-years-old dry spot samples from newborn screening. IEF thus provide a means for early diagnosis of the CDG in micro litre volumes of capillary blood (Stibler H 1991).
We analysed dry spot samples of three CDG patients, which showed distinct abnormalities of both Tf and α₁-AT. The sample of our CDG patient was also analysed as a dry spot sample, and similar results as in serum were obtained. To our knowledge, no information has been reported on the usability of dry spot samples for Tf analysis by HPLC; our preliminary results do not testify to its suitability even when a good HPLC detector is used.

For correct identification of Tf polymorphism by IEF or HPLC, in vitro treatment of serum Tf with neuraminidase (sialidase) is used. Elimination of the sialic acid residues reduces all glycoforms to the asialo-Tf; after digestion, all homozygote Tf variants present with just one peak/band at the asialo-position of the type, shifted either to the cathode (Tf D) or to the anode (Tf B) with respect to Tf C, while in heterozygotes the variants always present as two peaks/bands, related to the particular subtype. Thus a CDG versus a variant differentiation, based on the abnormal quantities or abnormal positions of bands, respectively, can be made (Helander A 2001).

We were further looking for optimal working conditions including various iron-reagents, temperature and time of saturation: When we tested native samples, a tailing of Tf isoforms bands was noted on IEF, and similarly small and not well-separated peaks appeared on HPLC. No significant differences among the three different iron solutions were found in our IEF experiments. In HPLC analysis, the FeCl₃ resulted in the highest peaks of Tf isoforms, when compared with the Fe-citrate and the FeNTA reagent, otherwise the well-known and generally recommended Tf iron donor, giving almost instant saturation (Helander A 2003). However, we noted an extra peak (matching the standard FeNTA) close to the position of the tetrasialo-Tf on HPLC chromatogram, obtained from FeNTA-treated serum (not previously reported).

5.2 IEF

IEF as an analytical tool is simple, rapid, and highly sensitive; various types of equipment are available. The Multiphor II system used in this study provides excellent resolution and relatively rapid separations in a large-format gel and thus larger number of samples could be analysed in one run.

Both polyacrylamide and agarose gels are suitable for IEF (Petrén S 1989). In the present study, as in most of the reports on IEF of Tf, polyacrylamide gels were used. Apart from hydrated gels ready to use, dry gels are available; they require rehydration with the water-glycerol, which improves the solubility of the hydrophobic proteins; ampholytes are immobilised within the polyacrylamide producing an immobilised pH gradient. They are more stable, and the pH gradient is fixed in place, this leads to improvements in reproducibility, as they are mechanically strong and the pH gradient cannot drift.

Immunoprecipitation is a quick method since only one antibody is required, and the cross-reactivity of secondary antibody used in immunoblotting is eliminated; it was thus chosen for the present IEF screening method. However, direct immunoprecipitation of the protein in the gel is sometimes not practical or impossible (insufficient antigen concentration in the gel, antigen of interest is insoluble or readily degraded and cannot be easily immunoprecipitated) and thus a blotting technique is preferred. As only small amounts of the antigens are required for this method, it is useful for the detection of Tf isoforms in CSF and urine samples (Keir G 1999).

Quick and simple Coomassie blue staining was selected in this study because the concentration of Tf and α₁-AT in serum is high enough; a 50x more sensitive, but rather complicated silver staining is essential for detection of low levels of glycoproteins, and may be used for CSF or urine, instead of sample concentration (Keir G 1999).
5.3 HPLC

We introduced the screening of CDG by IEF of serum Tf; suspicious and pathological results should be further verified by another method. Our experiences with HPLC method are described, assessing its applicability in CDG diagnostics and pointing out pitfalls and critical moments, which may affect results.

Various anion-exchange HPLC columns have been successfully used for separation of Tf isoforms, e.g. ResourceQ, MonoQ, or Source15Q (Amersham Bioscience). For routine analysis we have chosen the more economic ResourceQ column, which gave similar results to those observed on the more expansive MonoQ (Turpeinen U 2001).

We compared various types of the Bis-Tris-NaCl buffer gradient: to conclude, for each HPLC system the appropriate gradient profile should be adapted to achieve an optimal separation of Tf isoforms.

High detector sensitivity is essential for the analysis of Tf isoforms, due to the low concentration of CDT glycoforms in serum, and low molar absorbency (despite high specificity) of the iron-Tf complex at 460 nm. Various types of detectors were compared: Agilent 1100, Shimadzu 10AD VP, ECOM 2084, Agilent 1090 and Hewlett-Packard 1050 system. The last three types have not been found sensitive enough (high baseline noise). Many other types have been referred to be suitable for the analysis of Tf isoforms, e.g. Jasco 870 (Jepsson O 1993), LKB 2141 (Bean P 1997), Merck L4250 (Renner F 1997), Beckman Gold (Werle E 1997), VWR (Arndt T 2004), Waters 996 (De la Calle Guntinas MB 2004), and Spectraflow 783 (Turpeinen U 2001), commonly used in sensitive forensic medical applications (follow-up of alcohol abuse).

The measured iron-Tf complex shows a maximum at 460 nm; even if the absorbance at this wavelength makes only about 10% of that recorded at 280 nm, it is highly specific for the Tf fractions. Helander et al. (2003) reported an improvement of the method by the detection of Tf at 470 nm; in our experiments there was no significant difference between both wavelengths on the ECOM system, which may be explained by the low detector sensitivity.

It is generally assumed that the HPLC analysis of Tf provides reproducible separation and relative quantification (Helander A 2003).

5.4 Enzyme essay

CDG type Ia represents about 85% of the all CDG subtypes so far known. PMM and phosphomannno isomerase essay, preferably in leucocytes, can confirm the diagnosis of CDG Ia and CDG Ib respectively, while only fibroblasts are suitable for analysis of the other enzymes. Various experiments were performed to achieve the highest yield of leucocytes; the best results were obtained by combination of two methods described by Beutler (Beutler E 1970).

Leucocytes were isolated from blood samples. Erythrocytes are more susceptible than leucocytes to hypotonic shock and burst rapidly in the presence of a hypotonic buffer. After lysing erythrocytes using different concentrations of NaCl, intact leucocytes are then collected by centrifugation. All sugar substances, the enzyme cocktail and NADP must be freshly prepared, and all steps should be performed as quickly as possible.

The reference values of PMM activities in our group of controls are similar to those reported in the literature (van Schaftingen E 1995, Barnier A 2002); enzyme activities in our CDG patient and his family were normal, and thus the most common CDG type Ia can be excluded.
5.5 Interpretation of results

IEF of Tf in a pH range of 4.0-7.0 reveals eight isoforms: the tetra- and pentasialo-Tf prevail in healthy controls, while the serum of CDG patients shows a marked elevation of hyposialylated forms, such as disialo-, monosialo-, and asialo-Tf.

The secondary abnormalities of glycosylation in our set of patients presented with increases in asialo- and/or mono sialo-Tf (1.9 – 5%, and 3.3– 4%, when compared to controls, 0.0 – 1.9, and 0.0 – 3.3, respectively), with normal range of disialo-Tf.

CDG screening should not be performed during the first 3 weeks of life due to a danger of false results in newborns, caused by liver immaturity, or circulating Tf of maternal origin. However, positive serum samples have already been referred at the first week of life (Güthrie paper from neonatal screening) in many CDG patients (Stibler H 1991). Increased CDT was found in the brother of our CDG patient when the test was performed directly after birth; repeated analyses in the 1st and the 6th months of age for both Tf and α1-AT were normal.

Some other conditions such as galactosemia, fructosuria, or severe liver impairment should be first excluded before the diagnosis of CDG is made: direct inhibition of galactosyltransferase (an enzyme important for the correct processing of glycans) is attributable to galactose-1-phosphat, which is increased in galactosemia patients; analogous pathogenesis is assumed in the congenital fructosuria (Keir G 1999).

Changes in the amino acid sequence of the Tf polypeptide chain (namely the D genetic variants) should be always considered; a test with neuraminidase gives an unambiguous result (Keir G 1999).

Common chronic diseases, such as hypertension, asthma or bronchitis, diabetes mellitus, abnormal lipid metabolism, digestive tract disorders, angina pectoris, or depression do not influence the physiological proportion of Tf isoforms (Meerkerk GJ 1998).

The CDT level is usually increased in CDG patients, but tends to decrease with age. The normalization of the glycoprotein glycan content could reflect an adaptation to the metabolic abnormalities (Stibler H 1991). In one patient, enzymatically and genetically well diagnosed as CDG type Ia, signs of hypoglycosylation disappeared after 4 years of life (Marquardt T 2003); after two years of follow-up of Tf IEF pattern in our CDG patient, no distinct changes have been noted.

There are several conditions, resulting in abnormal levels of hypoglycosylated glycoproteins (usually Tf), thus reducing their specificity as a CDG marker.

In our group of patients with liver diseases, false positive result was noted only in few patients (3/15); the CDT changes are related to severity of the disease, caused by degree of enzyme activity and inhibited sialylation of Tf (de Jong G 1990).

Higher CDT was also reported in rheumatoid arthritis, hemochromatosis (Arndt T 2001), cystic fibrosis (Larsson A 1998), and pregnancy (Stauber RE 1996), distinct changes in total serum Tf may be caused by Fe-deficit or low serum ferritin (Arndt T 2001). Malignancy, demyelinating diseases, pancreatic, neuropsychiatric and some chronic lung disorders may also lead to protein hypoglycosylation (Van Eijk HG 1987, Reif A 2001, Arndt T 2001). In the present study, higher CDT was noted only in 3 of 40 studied patients with cystic fibrosis; this may be explained by various degrees of this disease, and to the secondary affects of liver function. Lowe reported that specific alterations in N-glycan structure could disable processes in the immune system, which keeps pathogenic self-recognition at bay (Lowe JB 2001).

Alcohol abuse has an inhibitory influence over glycosylation pathway, possibly due to altered biosynthesis or transfer of dolichol-oligosaccharide intermediates (diminished mRNA level and the glycoprotein glycosyltransferase activities), or by enhanced neuraminidase activity.
CDT seems to behave like a glycosylated haemoglobin test: it generally decreases within 2 weeks of abstinence, and will increase if the person resumes drinking (Fleming M 2004). We noted that the chronic alcohol abuse has a greater impact than acute alcohol abuse (with the exception of the acute intoxication) on Tf glycosylation, which is more likely a result of the liver function failure, and not an effect of alcohol itself.

A higher level of circulating neuraminidase produced by certain microbes (e.g. Streptococcus pneumonia) in the acute phase of a disease may also result in hypoglycosylation (de Loos F 2002).

Estrogens, antiepileptics, and β-blockers have been reported to falsely increase the hypoglycosylated isoforms of Tf (Musshoff F 2002), effect of all other drugs should be further verified. Our experience has shown that treatment with phenobarbital and methotrexate may also result in CDT increase.

The plasma half-life of CDT is \(\sim 14\) days (Arndt T 2001). Therefore, repeating the test after 3 weeks helps in the differentiation between a secondary and primary CDT increasing.

**5.6 Tf and α\(_1\)-AT genetic variants**

At least 36 Tf protein variants are known (Arndt T 2001). The most common is Tf C (\(>99\%\); subtypes C\(_1\) - C\(_{10}\)), whereas Tf B (B\(_0\) - B\(_2\)) and D (D\(_1\) - D\(_{4,3}\)) variants are rare (Kamboch KM 1987). For the differentiation of CDT positively and possible of Tf variants, comparison of results before and after Tf digestion by neuraminidase, in addition to analysis of serum Tf in parents (carriers of the same genetic variant) may help in suspicious cases.

Particularly, the D Tf allele may simulate a CDG-positive result, as the prominent tetrasialo-Tf D band shifts cathodally to the position of the hypoglycosylated Tf isoforms. In the serum of our patient with a rare Tf C\(_1\) D\(_{4,5}\) variant, digestion with neuraminidase, analysis of a new sample of the patient and his parents, and use of a gel of larger pH range, all helped in the final diagnosis of this Tf variant.

An association of certain Tf genetic variants with some pathological conditions has been reported, e.g. higher frequency of Tf C\(_2\) variant in spontaneous abortion, prematurity, rheumatoid arthritis, Alzheimer's disease, and lower Fe-binding capacity. Tf C\(_3\) is suggested to show certain protection against some types of smoking-derived lung-carcinoma. On the other hand, comparably lower occurrence of Tf C\(_1\) C\(_2\) is reported in patients with cystic fibrosis (Petrén S 1989).

Distribution of Tf variants in our set of controls and patients has been described. Suggested higher frequency of C\(_1\)C\(_2\) Tf in a few rather small groups of children was not significant; studies on an extended number of patients are necessary.

Distribution of Tf variants in our set of controls and patients has been described; to my knowledge, this is the first attempt at presenting such results in Czech Republic.

The gene for α\(_1\)-AT is highly polymorphic, with more than 70 different alleles described in the European population: The most common M allele has a frequency of 0.95, as much as 90% of white Europeans have the MM genotype. Two mutant alleles, S and namely Z, account for most of the diseases associated with α\(_1\)-AT deficiency. These variants should be properly recognised for a reliable interpretation of results. Thus, sample pre-treatment with neuraminidase, and the testing of parents for the identification of their α\(_1\)-AT phenotypes, in addition to Tf analysis, may be help in the result interpretation. IEF of plasma α\(_1\)-AT leads to the detection of eight bands. Physiological, the bands hexasialo- and tetrasialo-α\(_1\)-AT are the most abundant isoforms, making up 40% and 34% of the total plasma α\(_1\)-AT, respectively. In this study, we have analysed α\(_1\)-AT in a relative small group of patients, and thus possible relation of α\(_1\)-AT variants and disease could not be followed.
5.7 Comparison of methods used in CDG screening

Tf isoforms may be analysed by various electrophoretic techniques. Besides these, commercial CDTect assays, which measure the CDT and non-CDT portions of Tf isoforms, have been developed (Musshoff F 2002), and can be applied to CDG screening.

The CDT assays are still used as screening tools, since they are commonly available in clinical laboratories, but the diagnostic value of the CDT assays is often low, its clinical utility is considered questionable and the tests should be replaced by other methods (Keir G 1999, Arndt T 2003).

IEF is suitable as a qualitative confirmatory method for small sample volumes. This procedure provides a good separation of the Tf isoforms, and it is considered also a reference method for detection of all Tf genetic variants.

Apart from analyses of Tf isoforms, investigation of other glycoproteins, e.g. TBG, antithrombin III, and α1-AT may be also used to confirm the general defect of glycosylation in CDG.

IEF of serum Tf remains the most powerful test for first step of CDG screening. However, not all rare forms (such as CDG type IIb, IIc, IIif and a group of O-glycosylation defects - WWS, MEB, HEMPAS, HME, and the progeria variant of EDS) can be detected by this assay. Apo C-III, apo E, and α-DG characteristic markers of some O-glycosylation defects (e.g. MEB), may be determined.

Most of Tf variants can be also detected by HPLC procedure. HPLC/UV-Vis of Tf isoforms in CDG screening, based on specific 460 –470 nm absorbency, give reproducible results and can be automated for large sample series. However, there are disadvantages of HPLC method, a need for frequent and complex column regeneration, and high cost of columns employed (Jeppsson JO 1993).

Determination of glycoproteins by capillary electrophoresis (CE) has been demonstrated as reliable, due to its rapidity, sensitivity and good precision, without any requirement of sample pre-treatment. The main problem with these techniques is a prevention of protein adsorption. Using commercial kit with double-coated capillary surface can solve this problem. Quantitative HPLC and CE have equal sensitivity, and both demonstrate higher specificity in comparison to the qualitative CDTect screening test.

HPLC is probably more common in clinical laboratories when compared to CE. Comparison of commercial kits for common screening methods of Tf analysis is demonstrated in Table 7.

Many commercial kits, which might facilitate Tf analysis, are now available.
Table 7 Comparison of commercial kits for common screening methods of Tf analysis

<table>
<thead>
<tr>
<th>Method</th>
<th>Company</th>
<th>Kit price (Euro)</th>
<th>Number of tests per kit</th>
<th>Reference CDT values (%)</th>
<th>Time of analysis (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEF</td>
<td>Serva</td>
<td>250</td>
<td>288</td>
<td>4.4</td>
<td>360</td>
</tr>
<tr>
<td>HPLC</td>
<td>Recipe</td>
<td>500</td>
<td>100</td>
<td>1.7</td>
<td>50</td>
</tr>
<tr>
<td>CE</td>
<td>Analis</td>
<td>320</td>
<td>40</td>
<td>1.7</td>
<td>50</td>
</tr>
<tr>
<td>CDTect</td>
<td>Axis-Shield</td>
<td>500</td>
<td>50</td>
<td>5 – 6</td>
<td>120</td>
</tr>
</tbody>
</table>

Analyses of glycan by sensitive Matrix-assisted laser desorption - time of flight - mass spectrometry is complementary to IEF for the diagnosis of unclear CDG types. A protocol using this method to profile molecular weights of enzymatically released unmodified glycans has been developed as a preliminary procedure. However, since the positions of individual sugar linkage within a glycan cannot be correctly localized by this method, its ability for detailed structural analysis may be limited; for this, electrospray ionization mass spectrometry is the best technique. Currently, a general strategy combining HPLC, CE, Mass spectrometry and exoglycosidase sequencing represents the most effective way to approach the analysis of glycans.
Algorithms of CDG screening

Suspicion of CDG (clinical symptoms, biochemistry)

IEF of serum Tf (+ α₁-AT, TBG, ...)

Secondary causes
- Positive
- Negative

Other diagnoses

N-GLYCOSYLATION defects (confirm by CE, HPLC)
2-3 diff. Tf IEF/CE/HPLC patterns:

- CDG type I (different for Ia, Ie)
  - S-α₁-antichymotrypsin, CSF, β.trace protein in dif dg. Ia/Ie

- CDG type II
  - Ila, IID, Ile
  - ↓ IgG, Ery band III + glycerophorine A (Ig)

- Enzymes of CDG subtypes Ia, Ib, Ic
  - Decreased
  - Normal

Analysis of LLO (Lipid-linked oligosaccharide)

- Abnormal
- Normal

Structure of glycans (MALDI-TOF MS)

- Abnormal
- Normal

Enzymes of subtypes CDG IId/If/Ig/Ih/... /It

- Abnormal
- Normal

Abnormal

Enzymes of subtypes CDG IIa/IIb/- III

- Normal
- Abnormal

Abnormal

Mutation analysis
- most frequent R141H - (Ia), R219Q (Ib), A333V (Ic)

Abnormal

Urine oligosaccharides (IIb)
Bombay blood phenotype (IIc)
Sialy Lewis X (IIc, III)

Abnormal

Normal

Other diagnoses
6 Conclusions

Clinical diagnosis of CDG is complicated by heterogeneous, usually age-related features. Many congenital diseases of unknown aetiology might turn out to be CDG, which indicates a need to widen the screening criteria. No single screening test is available for all of 19 CDG subtypes recognized so far.

The aim of this work was to introduce screening methods, determine a diagnostic algorithm, and report our results.

I have introduced IEF method of serum Tf with direct immunofixation. Apart from the Tf, I analysed also α1-AT, either separately or simultaneously on the same IEF gel. The method was adapted for lower series of samples, by use of smaller pieces of gel, which allows obtaining of results more quickly. Besides serum, also plasma, amniotic fluid, CSF, and serum/plasma/whole blood-dry spots have been checked out by IEF, with good results.

I have introduced a HPLC procedure in our laboratory, and my experiences are described; first of all, HPLC system equipped with detector of high sensitivity should be used, since not all detectors are suitable for this specific procedure. This HPLC procedure is reliably used in sensitive forensic medical applications (follow-up of alcohol abusing), in follow-up studies of Tf glycosylation under physiological and pathological conditions, and for detection of Tf protein variants in the genetic field.

The diagnostic procedure is accomplished by testing PMM activity in isolated leucocytes of CDG type Ia, which forms about 85% of the all CDG subtypes so far known; I have established PMM enzyme assay, and compared results in the control group and our CDG patient.

In all cases suspicious of other CDG types, further investigations, such as LLO analysis, glycan structures, and enzyme assays or molecular studies to identify the specific mutation involved, should be performed in specialized laboratories.

Practical knowledge and hands on experience have been acquired during each of the particular steps of investigation. Some drawbacks connected with those methods have been commented upon.

About 100 healthy individuals of various ages and over 1100 children/adults with signs of a congenital metabolic defect have been examined. Beside these we screened out several groups of patients with various chronic diseases. Mild abnormalities of glycosylation, detected in 7.2% of our patients group have been associated with various, mostly pathological conditions.

I have identified the Tf genetic variants in the studied groups of patients and controls, using the neuraminidase enzyme treatment for differentiation of rare variants, which can interfere with the diagnostics of CDG. Besides that, I studied a possible connection of various diseases with the Tf variants, recognized in our study.

I examined a patient showing a CDG-suspicious IEF pattern, who appeared to have a rare Tf C,D1,D4,5 protein variant, as proved by neuraminidase treatment, analysis of serum α1-AT, and by investigation in the family of the affected adolescent boy.

The most prominent IEF abnormality found in a 5.5-year old child might correspond to CDG type IIX; also α1-AT and TBG analysis revealed abnormal IEF-profile. The PMM activity in leucocytes shows normal results. The finding of a hyposialylation IEF pattern of apoo C-III levels led to the suspicion of a combined N- and O-glycosylation biosynthesis defect. Further analyses of glycans for elucidation of the basic defect are pending.

The patient's younger brother, aged 4.5 years, is healthy. Without detailed classification, prenatal diagnosis was impossible, and thus the foetus from the third
pregnancy in the family was monitored only by ultrasound. A third boy was born, fortunately without any apparent disease, and showed normal tests for Tf and α₁-AT.

7 References


8 Publications of the author

9 Summary
Congenital disorders of glycosylation (CDG) constitute a rapidly growing group of inherited diseases caused by defects in either synthesis (CDG type I, subtypes a-l), or processing (CDG type II, subtypes a-f) of the N-linked glycans of glycoproteins, resulting in proteins hypoglycosylation; the most common type is CDG Ia (>85 %). The clinical and biochemical picture of CDG is characterized by great variation in expression.

The diagnosis of CDG can be made by examination of N-glycans on any serum glycoprotein; most commonly used is transferrin (Tf). Carbohydrate deficient Tf (CDT) is relatively increased in CDG patients, and it thus serves as an indicator of this disease. Various methods are available for quantification of serum Tf isoforms. Decreased activity of appropriate enzymes in blood cells or tissues, topped by the identification of specific gene mutations, helps the CDG typing.

Aims of the study
The goals of this study were:
- Introduction of a screening method (IEF) for the diagnostics of CDG.
- Verification of the abnormal results by another method (e.g. HPLC).
- Introduction of an enzyme essay for phosphomannomutase (PMM), which is deficient in the most common type CDG Ia.
- Determination of the CDG frequency in our set of patients under clinical suspicion of a congenital metabolic defect.
- Presentation of an algorithm design of CDG screening.
- Presentation of my experience in the CDG screening of the paediatric population, suspected of having metabolic disease.

Materials and methods
A group of about 100 healthy individuals, and over 1100 patients, mostly with signs of a metabolic disease were examined. Beside these, several groups of patients with various chronic diseases have been screened out. Three CDG-positive sera that served as pathological reference samples obtained from other laboratories have also been checked out.

A common screening method based on IEF with direct immunofixation and Coomassie blue staining of serum Tf and α₁-antitrypsin (α₁-AT) has been selected. The procedure was accomplished by HPLC analysis (based on anion-exchange chromatographic separation of the individual Tf glycoforms on MonoQ/ResourceQ columns using gradient of Bis-Tris/NaCl buffers, and followed by photometric detection of the Fe-Tf complex at 460 nm), and by enzyme assay of PMM activity (followed spectrophotometrically, based on NADP(+) reduction to NADPH at 340 mm absorbency) in isolated leucocytes.

Results
I have introduced an IEF method of serum Tf. Apart from the Tf, also α₁-AT was analysed, either separately or simultaneously on the same IEF gel. The method (originally intended for 52 samples) was adapted for lower series by use of smaller pieces of gel, thus allowing obtaining of results more quickly. Besides serum, also plasma, amniotic fluid, CSF, and serum/plasma/whole blood-dry spots have been checked out by IEF with good results. Distribution of isoforms of both glycoproteins in the controls and patient groups has been established.

I have introduced a HPLC procedure for verification of abnormal results obtained by IEF, and my experiences are described; in particular, HPLC system equipped with
detector of high sensitivity should be used, since not all detectors are suitable for this specific procedure.

I have established an enzyme assay in isolated leucocytes, and obtained the references values of PMM enzyme activity in controls and the family of our CDG patient.

Mild abnormalities of glycosylation (secondary, non CDG), detected in 7.2 % of our patients group have been associated with various, mostly pathological conditions, e.g. Hashimoto thyroiditis, systemic lupus erythematosus, epilepsy, hepatopathy, and cystic fibrosis.

Effect of long term treatment were found in two children treated by methotrexate or Phenaemaletten, and in an adult with combined therapy of carbamazepine, primidone and valproate; no effect of corticosteroids, antimalarics, or antibiotics, such as penicillin, or amoclen (trimethoprim) was noticed.

In this study, seven different phenotypes of Tf have been recognized. Only the variants C1C1 (in 86 %), and C1C2 (16 %) could be demonstrated among healthy subjects, while in a comparatively larger group of patients, apart from these two (in 78.7 % and 20 %, respectively), also the rare Tf C2C2 (0.6 %), and C1C3 (0.3 %), as well as homozygous CB (0.2 % for Tf C1B1,2) and CD (0.1 % for both Tf C1D2 and Tf C1D4,5) phenotypes were found.

Apparently higher incidence of the Tf C1C2 subtype, noted in the group of patients suspected of an inherited metabolic disease (20 %; n=1100), and especially in two smaller groups of children with Crohn’s disease (29.2 %; n=24) and cystic fibrosis (27.5 %; n=40), when compared to healthy controls (16 %; n=100), was not in fact, significant. No differences could be found in other subgroups of patients tested.

Since the Tf variants CB and Tf CD interfere with the usual IEF and also HPLC chromatography pattern, it is necessary to differentiate between the CDT and Tf variants by neuraminidase test, in addition to analysis of serum Tf in parents (carriers of the same genetic variant), which may help in suspicious cases.

I examined a 12-years patient showing a CDG-suspicious IEF pattern, who appeared to have a rare Tf C1D4,5 protein variant, as proved by neuraminidase treatment, analysis of serum α1-AT, and by investigation in the family of the affected adolescent boy.

α1-AT was analysed in about 50 individuals. In addition to the most common phenotype MM, a variant MS has also been recognised in one patient.

I found out a 5.5-year old child that might correspond to CDG type IX; he presented with dysmorphic features, mental retardation, partial agenesis of corpus callosum, and Ladd syndrome. Apart from Tf, also α1-AT and TBG analysis revealed abnormal IEF-profile. The PMM activity in leucocytes showed normal results. The finding of an abnormal IEF pattern of apo C-III led to the suspicion of a combined N- and O-glycosylation defect. Further analyses of glycans for elucidation of the basic defect are pending.

Conclusion

CDG is a newly discovered metabolic disorder characterized by great diversity. So far, it is possible to distinguish 18 subtypes (some of them are treatable) with more than 600 patients described worldwide. Common CDG diagnostic methods are based on examination of N-glycans on any serum glycoprotein. Prenatal diagnosis is possible in all types of CDG for which the molecular defect is known.

This study describe 1) screening methods and algorithm of CDG diagnostic, 2) an overview of hypoglycosylation-findings in our set of investigated subjects, 3) a CDG patient with a rare combination of glycosylation defects, 4) distribution of Tf protein
variants, and their distribution in various diseases, 5) misleading rare Tf variants detected, 6) observed association of some diseases / symptoms with increased CDT, 7) influence of some drugs by long-term treatment, 8) general pitfalls in CDG diagnostics, and 9) my practical experience having been acquired by 4years screening.
10 Souhrn

Kongenitální poruchy glykosylace (CDG) představují rychle narůstající skupinu dědičných chorob, způsobených defektem biosyntézy glykoproteinů (CDG typ I, podtypy a-l) nebo jejich dalšího zpracování (CDG typ II, podtypy a-f) v procesu N-glykosylace, vedoucí k hypoglykosylaci proteinů; nejčastější je typ CDG Ia (>85 %). Klinické a biochemické nálezy CDG jsou velmi rozmanité.

Diagnostika CDG je založena na analýze N-glykanu různých sérových glykoproteinů, nejčastěji transféruinu (Tf). Relativní zvýšení hypoglykosylovaného Tf (označovaného jako „carbohydrate deficient Tf“, CDT) u CDG pacientů je využíváno jako indikátor při screeningu choroby. Pro kvantifikaci isofoarem sérového Tf byla vypracována řada analytických postupů. Nález snížené aktivity příslušných enzymů ve tkáních nebo buňkách periferní krve spolu s identifikací genových mutací umožní určení typu CDG.

Cíle

Cílem disertační práce bylo:
- Zavést screeningovou metodu (IEF) pro diagnostiku CDG.
- Abnormální výsledky screeningu ověřit pomocí další metody, např. HPLC.
- Zavést metodu stanovení aktivity enzymu fosfomanomutázy (PMM) pro diagnostiku nejčastějišlého typu CDG Ia.
- Stanovit frekvenci výskytu CDG ve skupině nemocných s příznaky dědičné metabolické poruchy.
- Vypracovat algoritmus screeningu a diagnostiky CDG.
- Prezentovat vlastní zkušenosti s diagnostikou CDG mezi pacienty s podezřením na metabolickou poruchu.

Soubor a metodika

Bylo vyšetřeno téměř 100 zdravých jedinců a více než 1100 nemocných, většinou s podezřením na dědičnou metabolickou poruchu; další skupiny tvořili pacienti s různými typy chronického onemocnění. Krevní séra tří pacientů s CDG typem Ia, získaná z jiné laboratoře, sloužila jako patologické referenční vzorky.

Zvolili jsme screeningovou metodu, založenou na IEF a přímé imunofixaci Tf a α1-antitrypsinu (α1-AT) s následným barvením pomocí Coomassie modře. Tento postup byl doplněn HPLC analyzou Tf (založenou na chromatografické separaci glykofoarem Tf na anexových kolonách MonoQ / ResourceQ s gradientovou elucí pomocí Bis-Tris/NaCl pufů a fotometrickou detekcí Fe-Tf komplexu při 460 nm), a spektrofotometrickým stanovením aktivity enzymu PMM (monitorována změna absorbance NADP(+) na NADPH při 340 nm) v izolovaných leukocytech.

Výsledky

Zavedl jsem metodu IEF Tf a α1-AT při současné analýze 52 vzorků; postup jsem dále upravil pro paralelní analýzu obou glykoproteinů na stejném gelu, a pro menší série pacientů z důvodu urychljení odevzdu vzhledem k frekvenci indikací tohoto vyšetření. Sérum, plasma, suchá kapka (krve/ sera/ plasmy), plodová voda a mozkomišní mok byly testovány s dobrým výsledkem; stanovil jsem referenční hodnoty pro jednotlivé isoformy.

Zavedl jsem HPLC metodu pro kvantitativní ověření patologických náležů při screeningu IEF. Uvádím vlastní zkušenosti s touto aplikací; důležitá je především volba HPLC systému s dostatečně citlivým detektem.

Zavedl jsem metodu stanovení aktivity enzymu PMM v leukocytech a stanovil referenční hodnoty u zdravých osob a členů rodiny našeho CDG pacienta.
Lehké sekundární abnormality glykosylace byly nalezeny v 7,2 % vyšetřených pacientů s různými symptomy a diagnózami (např. Hashimotova thyroiditiis, systémový lupus erythematosus a epilepsie), kromě jaterních chorob, a cystické fibrózy.

Mírné známky hypoglykosylace jsme zaznamenali ve třech případech při dlouhodobé terapii: u chlapce užívajícího metotreksát, v případě lěčby Phenemaletenem, a u dospělého pacienta při kombinované léčbě antiepileptiky (carbamazepin, pridomonum, valproát). Při léčbě kortikoidy, antimalariky, ani antibiotiky, jako jsou amoclen, penicilin (trimethoprím) nebyly změny fyziologického zastoupení jednotlivých isoform TF nalezeny.

V této studii jsem identifikoval 7 různých variant TF; u kontrol byly zjištěny pouze (v celé populaci nejčastější) varianty C1C1 (v 86 %) a C1C2 (v 16 %), zatímco ve skupině pacientů jsem zaznal kromě těchto dvou, C1C1 (78,7 %), a C1C2 (20 %), i vzácnější varianta C1C3 (0,3 %), C1B12 (0,2 %), C1D2 a C1D4,5 (0,1 %). Nejvyšší incidence varianty TF C1C2 byla zjištěna ve skupině dětí s Crohnovou chorobou (29,2 %) a cystickou fibrózou (27,5 %); na první pohled patrný rozdíl proti kontrolám (16%) však nebyl signifikantní.

Genetické varianty TF B a TF D mohou interferovat s výsledky IEF a HPLC, k jejich odlišení od pozitivních nálezů používáme testu s neuraminidázou. Analýza isoform TF u rodičů, nositelů stejné varianty, může přispět v diferenciální diagnóze podezřelých výsledků.

Na případu 12letého chlapce s ankylosující spondyilitidou lze demonstrovat riziko chybné interpretace výsledku IEF; analýza pomocí neuraminidázy a vyšetření rodičů ukázalo, že pacient má vzácnou genetickou variantu TF C1D4,5.

Další glykoprotein α1-AT byl analizován u 50 osob; kromě obecně nejčastější varianty MM (85 %), byl u jednoho pacienta nalezen typ MS.

Zachytěl jsem 3,5letého pacienta s CDG typem IIx (chlapce zdravých rodičů, nyní 5,5letý, mentálně retardovaný, dysmorfický, s částečnou agenezi corporis callosi a Laddovým syndromem); porucha glykosylace je patrná při IEF TF, α1-AT a TBG. Aktivita enzymu PMM v leukocytech byla normální. Nález abnormálního apo C-III při IEF vedl k podezření na kombinaci poruchy N- a O-glykosylace; analýza mutací a detailní typizace dosud probationá v zahraničí.

Závěry

CDG je nová skupina dědičných metabolických chorob charakterizovaná značnou pestrostí příznaků. Dosud je známo 18 podtypů (některé z nich jsou léčitelné) u více než 600 pacientů v celém světě. Diagnostika CDG je založena na analýze N-glykanu různých sérových glykoproteinů; prenatální diagnostika je možná u všech typů se známým molekulárním defektem.

Tato studie prezentuje 1) screeningové metody a algoritmus CDG diagnostiky, 2) přehled nálezů hypoglykosylace v našem souboru vyšetřených jedinců, 3) popis CDG pacienta se vzácnou kombinovanou poruchou glykosylace, 4) frekvence proteinových variant TF a jejich distribuce u různých chorob, 5) snadno zaměnitelné proteinové varianty, detekované v našem souboru, 6) asociace pozorovaného zvýšení CDT a u některých chorob/symptomů, 7) vliv dlouhodobé terapie některými léky na zvýšení CDT, 8) úskalí při CDG diagnostice, 9) praktické zkušenosti získané v průběhu 4 let provádění screeningu CDG.