SCREENING OF CONGENITAL DISORDERS OF GLYCOSYLATION

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DISSERTATION
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PAEDIATRICS

CHARLES UNIVERSITY IN PRAGUE
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SEPTEMBER 2005

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List of used abbreviations

α_1-AT	α_1-Antitrypsin
α-DG	α-Dystroglycan
ALG	Asparagine-linked glycosylation
ALT	Alanine aminotransferase
Apo C-III	Apolipoprotein C-III
APTT	Activated Partial Thromboplastin Time
AST	Aspartate aminotransferase
AT III	Antithrombin III
Bis-Tris	bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane
CDG	Congenital disorder of glycosylation
CDT	Carbohydrate deficient transferrin
CE	Capillary electrophoresis
CF	Cystic fibrosis
CMP	Cytidine monophosphate
CNS	Central nervous system
COG	Conserved oligomeric Golgi complex
CSF	Cerebrospinal fluid
CV	Coefficient of variation
EDS	Ehlers-Danlos syndrome
EEG	Electroencephalogram
EMG	Electromyography
EXT1	Exostosin-1
EXT2	Exostosin-2
FCMD	Fukuyama-type congenital muscular dystrophy
FKRP	Fukutin-related protein
FR	Flow rate
FSH	Follicle-stimulating hormone
GA	Golgi apparatus
Gal	Galactose
GDP	Guanosine diphosphate
GDP-Man	Guanosine diphosphomannose
Glc	Glucose
GlcNAc	N-acetylglucosamine
Glut1	Glucose transporter 1
HEMPAS	Hereditary erythroblastic multinuclearity with positive acidified-serum test
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HME	Hereditary multiple exostoses
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IMD	Inborn metabolic disease
pI	Isoelectric point
LAD II	Leucocyte adhesion deficiency type II
LH	Luteinizing hormone
LLO	Lipid-linked oligosaccharide

kDa	Kilo Dalton
MALDI-TOF	Matrix-assisted laser desorption - time of flight
Man	Mannose
MEB	Muscle–eye–brain disease
MIT	2-methyl-4-isothiazolin-3-one hydrochloride
MPa	Mega Pascal
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NADP	Nicotinamide adenine dinucleotide phosphate
NTA	Nitrilotriacetic
P	Phosphate
PGI	Phosphoglucose isomerase
PMI	Phosphomannose isomerase
PMM	Phosphomannomutase
PT	Prothrombin time
PTT	Partial thromboplastin time
PP	Pyrophosphate
rER	Rough endoplasmic reticulum
RT	Retention time
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBG	Thyroxin-binding globulin
TCA	Trichloroacetic acid
Tf	Transferrin
TLC	Thin layer chromatography
TP	Total protein
UDP	Uridine diphosphate
US	Ultrasound
WWS	Walker-Warburg syndrome

1 Introduction

Glycosylation is one of the most frequent and important post-translation modifications. In this process carbohydrates are attached covalently to asparagine (N-glycans), or serine/threonine (O-glycans) residues of proteins. Involving 40-50 enzymatic and transport steps, N-glycosylation runs through cystol, rough endoplasmic reticulum (rER) and Golgi apparatus (GA).

There are numerous different glycoproteins, exist abundant in living organisms, appearing in nearly every biological process. Their functions span the entire spectrum of protein activities, including those of enzymes, transport proteins, receptors, hormones and structural proteins. Carbohydrates serve as cell surface receptors, signals for protein targeting, mediators of cell-to-cell interaction, and protectors of polypeptides from proteases (Varki A 1998).

Protein glycosylation includes four important steps: synthesis of the carrier lipid dolichyl diphosphate, assembly of oligosaccharide-lipid intermediate, transfer of the oligosaccharide precursor from the dolichol to an aspargine residue on the nascent polypeptide, and finally, oligosaccharide modification in rER and GA.

In the first part of this pathway, phosphomannomutase (PMM) and phosphomannose isomerase (PMI) participate in the synthesis of guanosine diphosphomannose (GDP-mannose) from mannose-6-phosphate (Man-6-P). The nucleotide sugars attached to the dolichol-pyrophosphate form the lipid-linked oligosaccharide (LLO) precursor. Two N-acetylglucosamines, and five mannoses are added to the LLO precursor, which is anchored to the cytosolic membrane of rER. Subsequently, the LLO precursor is flipped to the luminal side of rER, where more sugars are added sequentially using dolichol-P-Man/glucose as donors (Fig. 1). Then, the glycoprotein is transferred to the GA for final modification. Biosynthesis of the oligosaccharide branches is terminated by the addition of sialic acid residues. Certain glycoproteins are further modified by fucosylation or phosphorylation. Finally, the glycoproteins are secreted from the GA to their sites of action. Secretory glycoproteins are secreted into the body fluids, membrane glycoproteins are incorporated in cell membranes, and lysosomal enzymes are taken up by lysosomes (Varki A 1998, Jaeken J 2001).

1.1 Congenital disorders of glycosylation (CDG)

Defects, first described as "Carbohydrate Deficient Glycoprotein Syndrome (CDGS)" (Jaeken J 1980), were later renamed "Congenital Disorders of Glycosylation (CDG) (Aebi M 1999). The cause of CDG lies in a defective synthesis of oligosaccharides of N-bound glycoproteins. 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 (Schachter H 2001, Marquardt T 2003). The disorders fall into two groups, CDG type I and type II, based on the localization of the enzymatic defect, see Fig. 1. Currently known CDG subtypes, corresponding defects, and genes are listed in Table 1. The following chapter offers an overview of the CDG types, symptoms, diagnostics, and possibilities of therapy. Some patients and main symptoms of CDG are shown in Fig. 2.

1.1.1 CDG I

So far, it is possible to distinguish 12 subtypes (Ia to Il), caused by defects in the assembly of N-linked protein glycans. CDG I is characterized by partial or total absence of the glycan chains of serum N-glycoproteins.

CDG-Ia was first observed in homozygous twin sisters (Jaeken J 1980). It is the most frequent CDG type (over 85%) with more than 500 patients described worldwide; it is caused by a deficiency of PMM, which converts Man-6-P to Man-1-P (Van Schaftingen E 1995). Over 75 different mutations have been found at the corresponding gene of CDG Ia. The most frequent one, R141H, accounts for 40% of the disease alleles of CDG Ia patients (de Zegher F 1995, Marquardt T 2003).

Patients can be often diagnosed in the neonatal or early infantile period on the basis of typical clinical features, such as inverted nipples and fat pads, in addition to strabismus, muscular hypotonia, failure to thrive, and elevated transaminases (Jaeken J 1980, Imtiaz F 2000). A very common sign is cerebellar hypoplasia, which can usually be documented at, or shortly after birth. There is a substantial childhood mortality of approximately 20%, owing to severe infections or organ failure (Jaeken J 1991, 2001). At a later age, the impairment of the nervous system becomes more evident, presenting by a variable degree of mental retardation, cerebellar dysfunction, pigmentary retinopathy, and peripheral neuropathy. Tendon reflexes commonly disappear by the 1st year of age. Hypertrophic obstructive cardiomyopathy can develop (Keir G 1999, Jaeken J 2003, Marquardt T 2003).

Fever might endanger CDG children by decreasing the low PMM activity in their cells, so that some children experience seizures or exhibit stroke-like episodes with

complete recovery, which can occur mainly during feverish infections. Adult female patients can present with hypergonadotrophic hypogonadism (Jaeken J 1989, Keir G 1999).

The number of patients with a less typical presentation is increasing, many children present with nearly normal psychomotor development (Marquardt T 2003, Pancho C 2005).

CDG-Ib is caused by a deficiency of PMI, which affects the endogenous productions of Man- 6-P. Approximately 20 patients have been known to date. In contrast to CDG-Ia, mental and motor development is normal (De Koning TJ 1998, Niehues R 1998). The predominant symptom of CDG-Ib is chronic diarrhoea, commonly starting during the first year of life (Jaeken J 1998). Cyclic vomiting can be the leading symptom (De Koning TJ 1998). Failure to thrive and protein-losing enteropathy can occur (Niehues R 1998). Partial villus atrophy can be present in duodenal biopsies and might lead to suspicion of celiac disease (Jaeken J 1998, Niehues R 1998). Hypoglycaemia occurs frequently; some patients present with congenital hepatic fibrosis (Babovic-Vuksanovic D 1999). Hypoalbuminemia, elevated aminotransferases and low antithrombin III (AT III) activity are common findings in CDG-Ib patients. Thrombotic episodes and severe bleeding may complicate the course (Niehues R 1998).

Defect of the α–1,3-glucosyltransferase causes **CDG type Ic**. The enzyme catalyses attachment of the first glucose to the LLO intermediate Man_9 -N-acetyl-glucosamine($GlcNAc_2$)-PP-dolichol in the rER. CDG-Ic is mainly a neurological disorder that is in general milder than CDG-Ia. The features of CDG-Ia such as cerebellar hypoplasia, polyneuropathy, fat pads, and inverted nipples, are missing (Grünewald S 2000, Hanefeld F 2000). Psychomotor retardation is the main clinical symptom of the more than 50 CDG-Ic patients known so far (Matthijs G 2004). Muscular hypotonia is common, and nearly all patients have recurrent seizures. In contrast to CDG-Ia, there are no characteristic physical stigmata. Brainstem auditory-evoked potentials, tendon reflexes and nerve conduction velocities are normal, and there are no obvious cerebral abnormalities in magnetic resonance imaging (MRI). In early childhood, internal strabismus (as observed in CDG-Ia) may occur (Grünewald S 2000).

Activities of AT III and the factor XI are considerably reduced. The hypoglycosylation of β-trace protein, a cerebrospinal fluid (CSF) glycoprotein, is less severe than in CDG-Ia patients (Grünewald S 1999).

In **CDG-Id**, the defect is caused by a deficiency of the α-1,3-mannosyltransferase, which transfers mannose from dolichol-P-Man to the growing LLO in the rER. Dysplastic ears, abnormalities of the uvula, high-arched palate, coloboma iridis, adducted thumbs, hypotonia, microcephaly, epilepsy, atrophy of the optic nerve, hypoplasia of the cerebellum, and nearly absent psychomotor development were found in the three patients with this type described so far (Stibler H 1995, Denecke J 2004, Sun L 2005).

CDG-Ie is caused by a defect in the dolichol-P-Man synthase 1, which is required to generate the dolichol-P-Man, a donor of mannose for the growing LLO on the luminal side of the rER. Mutations causing a complete loss of enzymatic activity might be lethal.

Four patients have been diagnosed with this defect so far; clinical manifestations include severe psychomotor retardation, hypotonia, cerebral atrophy, epilepsy, cortical blindness, hepatosplenomegaly, coagulopathy, and dysmorphic features (gothic palate, hypertelorism, dysplastic nails and knee contractures). Liver transaminases are raised. Body weight, length and head circumference might be normal at birth, but later on microcephaly is typical of CDG-Ie (Imbach T 2000, Kim S 2000).

CDG-If is based on the alterations in a pre-located transmembrane protein (MPDU1/Lec3). The N-terminal half of the protein is predicted to have a cytosolic orientation. This protein seems to play a crucial role in the translocation of mannose and glucose into the lumen of the rER.

This defect was identified in four patients with psychomotor retardation, muscular hypotonia, seizures, and absence of speech development, short stature, failure to thrive, feeding problems, impaired vision and pigmentary retinopathy. Two of them have shown ichthyosis (Kranz C 2001, Schenk B 2001).

The defect in the **CDG type Ig** is located in the ALG12 mannosyltransferase. This enzyme adds the eighth mannose to the growing LLO in the rER (Chantret I 2002).

Thiel described the first patient with CDG-Ig (Thiel C 2002); a girl of Indian origin suffered from delayed psychomotor development, convulsions, microcephaly, muscular hypotonia, supragluteal fat pads, dysplastic ears and a short filtrum, prolonged partial thromboplastin time and enlarged lateral ventricles of the brain. One Danish and two British patients presented with low immunoglobulin G (IgG) in addition to common CDG symptoms (Grubenmann CE 2002, Eklund EA 2005).

CDG type Ih is due to the deficiency of the glycosyltransferase adding the second glucose onto the growing LLO in the rER. Three patients have been reported in the literature. Clinical presentation is similar to that of CDG-Ib: hypoalbuminaemia, protein-

losing enteropathy, hepatomegaly and coagulopathy, but without central nervous system (CNS) involvement (Chantret I 2003, Schollen E 2004).

CDG-Ii is caused by the deficiency of α-1,3- mannosyltransferase, which catalyses the transfer of mannosyl residues from GDP-Man to Man (1)GlcNAc(2)-PP-dolichol; it was the first defect of a glycosyltransferase discovered to be localised at the cytosolic side of the endoplasmic reticulum.

Only one patient with this type was reported; he had mental and motor retardation, colobomas, and cataract, nystagmus, seizures, hepatomegaly, and coagulation abnormalities. Cranial MRI showed a severely retarded myelinization (Thiel C 2003).

The **CDG-Ij** results from deficiency in UDP-GlcNAc: dolichol phosphate N-acetyl-glucosamine-1-phosphate transferase. The patient presents with severe hypotonia, medically intractable seizures, mental retardation, microcephaly, arched palate, micrognathia, strabismus, fifth finger clinodactyly, single flexion creases, and skin dimples on the upper thighs.

The defect in the **CDG-Ik** patients affects the mannosyltransferase I, an enzyme necessary for the elongation of dolichol-linked chitobiose during N-glycan biosynthesis. Reduced enzyme activity in two patients led to severe disease and death in early infancy. Grubenmann reported a patient without dysmorphic features and normal MRI scan of the brain; he suffered from multiple intractable seizures, generalized muscular hypotonia, blindness, liver dysfunction and coagulation problems related to low AT III (Grubenmann CE 2004).

Kranz described a CDG patient with seizures, severe muscular hypotonia, cerebral atrophy, nephrotic syndrome and a severe decrease of circulating B-cells with a complete absence of IgG, the boy died from respiratory failure at 11 weeks of age (Kranz C 2004).

De Koning also described two patients; in the first, ultrasound analysis at the 30th week of pregnancy revealed foetal hydrops and hepatosplenomegaly. The boy showed multiple dysmorphic features with a large fontanelle, hypertelorism, micrognathia, hypogonadism, contractures, areflexia, cardiomyopathy, and multifocal epileptic activity. The patient died at 2 weeks of age. The clinical features of the second patient included facial dysmorphism with hypertelorism, micrognathia, low-set ears, coloboma iridis, multiple contractures, and genital abnormalities. The boy died on the second day of life because of severe septicaemia (De Koning TJ 1998).

CDG-IL is caused by a deficiency of the enzyme α-1,2- mannosyltransferase, which causes an accumulation of lipid-linked-$GlcNAc_2Man_6$ and -$GlcNAc_2Man_8$

structures. Only one patient with this type has been reported so far; the clinical features include developmental delay, hypotonia, seizures and hepatomegaly (Frank CG 2004).

1.1.2 CDG-II

CDG type II refers to defects in the processing of the protein-bound glycans.

CDG-IIa is caused by a deficiency of the N-acetylglucosaminyl transferase II (GnT II). Only five patients have been known to have this disorder so far. Compared to CDG-Ia children, more profound psychomotor retardation, but no peripheral neuropathy, and normal deep-tendon reflexes are present in CDG-IIa. A dysmorphic coarse face, large low-set ears and widely spaced nipples, cortical atrophy, delayed myelinization, but no cerebellar atrophy has been described (Ramaekers VT 1991). CDG-IIa patients show generalized hypotonia, limb weakness, and stereotypical behaviour. Epilepsy developed in one patient. Raised liver transaminases, decreased activities of AT III, factors IX and XII were present (Jaeken J 1993).

In animal experiments over 60% mouse embryos lacking the gene encoding GnT II develop fully, but 99% of newborns die during the first week of postnatal development. It is suggested that the majority of humans with CDG-IIa die during gestation or shortly after birth (Freeze H 2001).

CDG-IIb is caused by a deficiency of glucosidase I, an enzyme removing the terminal glucose from the oligosaccharide, after its transfer to the polypeptide in the rER.

Only one patient with CDG-IIb has been identified so far (De Praeter CM 2000). He presented with severe developmental delay, muscular hypotonia, recurrent oedema, seizures, hypoventilation, apnoea, hepatomegaly and peculiar dysmorphy, including retrognathia, high arched palate, broad nose, and overlapping fingers. Motor nerve conduction velocity was reduced. Following a rapid decline and a stuporous state, the patient died at 2.5 months of age.

CDG-IIc was discovered by Etzioni in 1992, and named leucocyte adhesion deficiency type II (LAD II) (Etzioni A 1992); latter on it was enlisted to CDG. Fucosylated glycoconjugates are severely diminished in this disorder, due to a defect of GDP-fucose import into the GA (Lübke T 2001).

Dysmorphic features of the five known patients include short limbs and stature, a flat face with a broad and depressed nasal bridge, long eyelashes and broad palms (Etzioni A 1992, Marquardt T 1999). Moderate to severe psychomotor retardation, hypotonia and increased peripheral leucocyte counts are the predominant findings already

present in newborns. Leucocytosis and immune deficiency are due to the absence of fucosylated selectin ligands, decreasing the adhesion of leucocytes to endothelial cells, and migration of neutrophils to infection focuses (Etzioni A 1992, Marquardt T 1999).

CDG-IId is caused by a deficiency of β-1,4-galactosyltransferase, an enzyme adding galactose to the oligosaccharide of the newly synthesized glycoprotein in the GA. Only one patient with this disorder is known to date; in addition to muscular hypotony, severe psychomotor and mental retardation, blood coagulation abnormalities, and myopathy with elevated creatine kinase levels, he presented with a Dandy-Walker malformation with macrocephalus at birth, and progressive hydrocephalus later on (Peters V 2002, Hansske B 2002).

In the type **CDG-IIe**, the alteration of glycosylation is secondary to the alteration of a GA protein, not primarily involved in glycosylation. CDG IIe is caused by a mutation that impairs the integrity of the conserved oligomeric Golgi complex (COG7) and alters Golgi trafficking, resulting in the disruption of multiple glycosylation pathways. The only one CDG IIe patient presented with acute renal failure, slight hypotonia, macrocephaly, plagiocephaly, dry skin, haemolytic uraemia syndrome, thrombocytopenia, anaemia, hypoproteinemia, proteinuria, increased aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase.

COG7 deficiency is comparable to diseases such as Chediak-Higashi or Hermansky-Pudlak disease; this group of disorders affects different coat proteins later on in the secretory pathway. It is to be expected that this new group of 'coat' diseases, which includes COG7 deficiency, will grow just as rapidly as the CDG (Wu X 2004).

CDG-IIf is caused by altered transport of cytidine monophosphate (CMP) -sialic acid into the GA. Only one patient with this type was reported so far; the clinical features included a spontaneous massive bleeding in the posterior chamber of right eye, and cutaneous haemorrhage, severe thrombocytopenia, respiratory distress syndrome and opportunistic infections. Pulmonary viral infection and massive pulmonary haemorrhage with refractory respiratory failure led to death at the age of 3 years (Martinez-Duncker I 2005).

About 20% of CDG patients remain untyped and are named **CDG-x**. Apart from the CDG typical clinical presentations, oligohydramnion, hydrops fetalis, absent psychomotor development, severe thrombocytopenia, ascites, demineralisation of distal bones, tubulopathia, and death in status epilepticus have been reported (Charlwood J 1997, Acarregui MJ 1998, Eyskens F 1994, Skladal D 1996).

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1.2 Diagnostics of CDG

1.2.1 Basic laboratory investigations

Pathological changes of the common biochemical tests may be found as a consequence of defective pathways of protein glycosylation. Abnormal liver function tests, low plasma cholesterol and cholinesterase activity with proteinuria are common findings in patients with CDG type Ia. Frequently found hypoalbuminaemia, hypoglycaemia with inadequately increased insulin production, and high activities of aminotransferases, are typical for CDG Ib. Conversely, proteinuria is absent in CDG type II (Keir G 1999).

1.2.2 Total serum glycoproteins

The enzymatic activities or levels of plasma glycoproteins, including transport proteins, e.g. α_1-antitrypsin (α_1-AT), thyroxin-binding globulin (TBG), transferrin (Tf), glycoprotein hormones, coagulation and anticoagulation factors (particularly the factors II, V, IX, X, XI, AT III), proteins C, S and heparin cofactor II are usually low, while the level of fibrinogen and D-dimer protein are frequently raised (Jaeken J 2001). Thyroid hormones T3, T4, and rT3 are mostly subnormal (Keir G 1999). Adolescent females have elevated levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (De Zegher F 1995).

Lysosomal enzyme activities in serum may be elevated (e.g. arylsulphatase A, β-galactosidase, β-glucuronidase and β-hexosaminidase). In contrast to that, lysosomal enzyme activities are frequently low in leucocytes (e.g. α-fucosidase. β-glucuronidase, α-iduronidase, α- and β-mannosidase) (Barone R 1998).

1.2.3 Screening tests

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).

Tf is the major iron-carrier protein in human plasma and extra cellular space in tissues. It binds two ferric-ions with high affinity, and delivers iron to dividing cells, particularly to the erythroid progenitor cells in the bone marrow. Tf keeps the iron inaccessible from most bacteria and fungi. Therefore, Tf is an important antioxidant and anti-microbial protein in human plasma (de Jong G1990).

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Human Tf is an 80 kilo Dalton (kDa) glycoprotein synthesized in the liver; it is a polypeptide chain of 679 amino acid residues encoded by a gene on chromosome 3q21 (Baranov VS 1987). There are two homologous iron-binding domains, the N-terminal (residues 1–336) and the C-terminal (residues 337–679) domains, with carbohydrate moieties attached to the C-terminal domain at positions 413 and 611 (Bowman B.H 1988, de Jong G 1990), see Fig. 3. Polymorphism in human serum Tf was first detected by gel electrophoresis (Smithies O 1957).

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) Composition of the carbohydrate chain (N-linked complex glycan chains). 3) Degree of iron saturation.

The two carbohydrate chains vary in their degree of ramification, presenting structures with 2 or 3 external antennas. Each antenna finishes negatively with a sialic acid (N-acethylneuraminic acid) that 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.

In the CDG type II, the number of glycans is normal, but processing defects of the oligosaccharide lead to truncated carbohydrate structures.

CDG-IIb, CDG-IIc, and CDG-IIf, are not detectable by Tf IEF. Even some CDG-Ia patients might be missed by the IEF Tf test (Marquardt T 2003).

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 how to differentiate between CDG and protein variants.

Analysis of other serum glycoproteins, e.g. α_1-AT may help in documentation of generalized glycosylation defect in the patient (Knopf C 2000). α_1-AT is a 52-kDa glycoprotein, synthesized in the liver and macrophages, consists of a single polypeptide chain of 394 amino acids, and is 12% carbohydrate by weight. Concentration of plasma α_1-AT is 1.3 g/L and a half-life of 4–5 days. It is the main plasma protease inhibitor,

affecting a broad range of substrates, including trypsin, chymotrypsin, thrombin, kallikrein and plasmin. However, its primary target is neutrophil elastase, thus protecting the lower respiratory tract from proteolytic destruction.

α_1-AT has three N-glycosylation sites at asparagine residues 46, 83 and 247, which are occupied by mixtures of complex bi- and triantennary glycans. This gives rise to multiple isoforms with different isoelectric points (pI) and mass.

Thin-layer chromatography (TLC) of urine oligosaccharides is the method of choice in the screening of CDG-IIb.

Sialyl-Lewis X antigen is absent on the neutrophils in CDG IIf and IIc, which also shows the Bombay blood group phenotype (Lübke T 2001, Marquardt T 2003).

1.2.4 Analysis of glycan structure

Electrospray mass spectrometry of protein-bound oligosaccharide chains (glycoproteins) can separate different glycoforms (Yamashita K 1993, Ohkura T1993), and nuclear magnetic resonance spectroscopy can determine the glycan structures and molecular mass of the glycovariants (Coddeville B 1998). Such glycan structure analysis may be instrumental for the elucidation of CDG-x cases, by pinpointing candidate enzymes and genes responsible for the abnormal glycan synthesis.

1.2.5 Enzymes measurements

PMM and MPI activity are most readily measured in fibroblasts or leucocytes (van Schaftingen E 1995). Regarding PMM activity, leucocytes seem to be more reliable than fibroblasts, because a high residual activity has been observed in the fibroblasts of some CDG-Ia patients, whereas the leucocyte values are always seen in the clearly abnormal range (Grünewald S 2001). Thus, patients with slightly decreased of PMM in fibroblasts might still harbour mutations in PMM. Especially in the case when a clinical picture strongly suggests CDG-Ia, it is worthwhile to look for PMM mutations.

The enzymes deficiency responsible for other CDG types has been measured in fibroblasts. They are preferably assayed after analysis of LLO and glycan structure of serum glycoprotein, which leads to identification the locality of suspected defect.

1.2.6 Mutation studies

About 75 different mutations of the PMM gene have been reported in CDG Ia. Most patients are compound heterozygous for two different mutations. The R141H mutation is found in approximately 40% of all patients, homozygosity has not been observed; F119L is frequent in the Northern European countries owing to a founder effect, whereas V231M and P113L are frequent all over Europe (Matthijs G 2000, Le Bizec C 2005).

Combinations of D56Y with R141H, R123Q, or F157S result in severe phenotype with a death rate of four in five (Matthijs G 2000). High mortality was observed in the patients with the D188G/R141H genotype, four of five patients died before the age of 2 years (Matthijs G 1998). Genetic studies in CDG-Ic patients revealed a prevalence of the A333V mutation in ALG 6, probably related to a founder effect in the Dutch population.

Because a very limited number of patients are known, only a few of other mutations have been described to date (Grünewald S 2000, Hanefeld F 2000).

1.2.7 Prenatal diagnosis

Early attempts of prenatal diagnosis on the basis of Tf IEF in foetal blood have failed, and thus revealed that this method is not reliable (Clayton P 1993, Stibler H 1994). Enzymatic measurements activities in cultured amniocytes or trophoblasts are useful, but may give inconclusive data. 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. Potentially, pre-implantation genetic diagnosis (using four-to-eight-cell stage embryos could be an option for CDG, and this has already been offered in Europe for CDG-Ia and CDG-Ic. (Matthijs G1998).

Fietz M (2005) referred a 27 weeks gestation foetus, presented with hydrops fetalis, pericardial effusion and marked ascites. These futures called out a CDG suspicion, and therefore Tf isoforms was analysed in blood sample collected by cordocentesis. The results showed an abnormal pattern with low but significant levels of disialo- and asialo- Tf, strongly suggesting CDG. Measurement of PMM activity in cultured aminocytes proved the diagnosis of CDG type Ia. Thus, analysis of foetus blood obtained by cordocentesis may prove beneficial in case where there is a strong suspicion of CDG.

LLO assay in amniocytes or trophoblasts is too complex for use in a prenatal diagnostic setting. It is certainly not advisable to offer prenatal diagnosis solely on the

basis of such an analysis, especially if the enzymatic and molecular basis of the disease is not known (Matthijs G 2004). Otherwise, LLO analysis was performed in chorion cells in mother of affected patient with CDG Id, showing the same characteristic pattern as in the described CDG patient; however, the foetus had a normal glycosylation of several plasma proteins (Denecke J 2005).

1.3 Other glycosylation defects

Beside N-glycosylation of proteins, defects also occur in other pathways such as O-linked glycosylation, proteoglycan synthesis, and lipid glycosylation (Marquardt T 2003).

Muscle–eye–brain (MEB) disease. A defect in the gene of the protein O-mannosyl b-1,2-N-acetylglucosaminyl transferase (POMGnT1) causes a disease that has been referred to as muscle–eye–brain disease. The POMGnT1 catalyses the b-1,2 linkage of GlcNAc to O-linked mannose using UDP-GlcNAc as a donor substrate. The gene had previously been mapped to chromosome 1p32–34. In Finland, the incidence of MEB was reported to be 1:52 000.

The clinical presentation of MEB disease consists of congenital glaucoma and myopia, retinal hypoplasia, pallor of the optic discus, congenital muscular dystrophy, severe muscular hypotonia, myoclonic jerks, mental retardation, hydrocephalus, and electroencephalogram (EEG) abnormalities. MRI shows pachygyria, fatty brain stem and cerebellar hypoplasia (Cormand B 1999, Marquardt T 2003).

Walker-Warburg syndrome (WWS) is a very severe recessive form of congenital muscular dystrophy. This disorder is caused by mutation in the first step in O-mannosylation of proteins. WWS maps to chromosome 9q34, POMT1 gene. The clinical presentations include various ocular and retinal abnormalities, as well as a brain defects, and death within the first few months of life (Beltran-Valero De Bernabe D 2002, Cormand B 2001).

Progeria variant of Ehlers-Danlos syndrome (EDS) is another disease involving multiple glycosylation abnormalities of the connective tissues, including short stature, osteopenia of all bones, hypermobile joints, aged appearance, scanty scalp hair, and mental retardation. EDS is a relatively rare disorder, occurring in fewer than 1 in 20,000 people. The mutated gene encodes for an enzyme involved in the formation of the glycosaminoglycans link regions, yet another type of glycosylation pathway (Kresse H 1987, Jaeken J 2003).

Hereditary multiple exostoses (HME) are the most frequent type of benign bone tumours. The prevalence HME has been reported to be 1:100000 in European populations. Patients suffer from multiple cartilage-capped tumours located primarily at the long bones, often a short stature and various orthopaedic deformities (Solomon L 1963). The risk of developing malignancies of the cartilage or bone is significantly increased.

The vast majority of patients are linked to chromosome 8q24.1 (Ludecke HJ 1995) and chromosome 11p11-p12 (Wuyts W 1995). Subsequently candidate genes for the defect could be cloned and were found to be exostosin-1 (EXT1) and exostosin-2 (EXT2), (Wuyts W 1996). Biochemical studies confirmed that EXT1 and EXT2 possess glycosyltransferase activity, which is necessary for the formation of heparin sulphate by adding single D-glucoronic acid and N-acetylglucosamine.

The development of exostoses is proposed to be mediated by a lack of heparin sulphate proteoglycans, which play a crucial role in the negative feedback loop regulating chondrocyte proliferation and maturation (Duncan G 2001).

HEMPAS is known as a hereditary erythroblastic multinuclearity with positive acidifed-serum test (HEMPAS); over 130 cases have been reported. Mild to severe normocytic anaemia is the predominant symptom; jaundice, splenomegaly and gallbladder disease are common. Hemochromatosis and liver cirrhosis rarely complicate the course of the disease (Heimpel H 1963, Wickramasinghe SN 1997).

The finding that band 3 (anion exchange protein 1) and band 4.5 (Glut1), two prevailing membrane proteins of red blood cells, show abnormal migration in the sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) revealed that a defect in the glycosylation of the erythrocyte membrane proteins is a common biochemical characteristic of all patients; the abnormalities are limited to erythroblasts (Kameh H 1998). Linkage analysis of Italy cases using sets of microsatellite markers identified the HEMPAS causative gene (CDAN2) in chromosome 20q11.2 (Gasparini P 1997).

Muscular dystrophies. Abnormal glycosylation of α-dystroglycan (α-DG) was found in a Fukujama congenital muscular dystrophy (FCMD), limb-girdle muscular dystrophy and fukutin-related protein deficiency (FKRP) (Muntoni F 2003, Grünewald S 2002).

α-DG is modified by both N- and O-linked glycosylation. The protein is a dumbbell-shaped molecule with two globular domains separated by a central rod thought to be the O-glycosylated mucin-like domain. There is evidence for a requirement of

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sialylated O-glycans on α-DG for binding to many of its ligands, including laminin, agrin, neurexin and perlecan; these bindings are defective in MEB and FCMD (Marquardt T 2003, Grünewald S 2003). In Japan, FCMD is fairly common; the incidence is estimated to be about 7-12 per 100000 children (Taniguchi K 2003).

For diagnostics of these glycosylation defects, in addition to a careful personal, family history and physical examination, a number of tests in serum (creatine phosphokinase, aldolase, glutamic-oxaloacetic transaminase and glutamic pyruvic transaminase) point to the evidence of muscle damage. An EMG shows abnormal muscle function. Muscle biopsy is very important to establish the diagnosis of MEB and WWS.

MEB, WWS, HME, FCMD, FKRP and progeria variant of EDS are not detectable by Tf IEF, though there was a report in one patient with HEMPAS, having hypoglycosylation of serum Tf (Fukuda MN 1992).

Moreover, electrophoretic analysis of serum apolipoprotein C-III (apo C-III) and apo E, as well as α-DG in skeletal muscles may be helpful for detection of O-glycosylation defects (Marquardt T 2003).

In HEMPAS, 10 – 40% of erythroblasts are bi- and multinucleated, which serves as a major diagnostic criterion. Also, it can be detected by a loss of lectin binding to specific glycoconjugates.

The diagnosis of HME is based on clinical and/or radiographic findings of multiple exostoses in one or more members of a family. Sequence analysis of the EXT1 and the EXT2 genes is available.

Diagnosis of EDS is made first upon clinical grounds; skin hyper elasticity, easy bruising, dystrophic scarring, and joint hyper motility are the cardinal symptoms, which may be present in different combinations and with variable severity.

Specific diagnosis of all these disorders is made after genetic defect identification.

1.4 Secondary abnormality of glycosylation

The Tf glycoforms pattern characteristic of CDG is not restricted to this condition. Other inborn errors, as galactosaemia and hereditary fructose intolerance give rise to similar glycosylation abnormalities, and need to be excluded. The nature of the metabolic block in protein glycosylation seen in galactosaemia is uncertain; it is tempting to suggest that there is a deficiency of the donor molecule UDP-galactose. In either case the absence of a galactose residue means that there is no substrate for the terminal sialic acid to attach to. An accumulation of fructose-1-P in untreated cases of hereditary fructose intolerance

has been shown to inhibit PMM, causing similar hypoglycosylation as is seen in CDG. Alcohol abuse, hepatic damage and some chronic diseases maybe also induce structure alterations of many plasma glycoproteins (Nihlen U 2001, Keir G 1999).

1.5 Treatment

Unfortunately, an efficient treatment is still not available for the **CDG-Ia** patients. It was reported that mannose supplementation results in an increased incorporation of mannose in patient's fibroblasts (Panneerselvam K 1997), but mannose administration to CDG-Ia patients did not improve the clinical or biochemical features (Mayatepek E 1997, 1998).

Providing PMM-deficient cells with Man-1-P may be a way to increase the GDP-mannose pool, but Man-1-P is not able to penetrate cell membranes due to its high polarity (Rutschow S 2002).

Promising might be results of quite recent experiments (Eklund EA 2005): Two hydrophobic membrane permeable derivatives of Man-1-P were synthesized, and their ability to normalize LLO size and N-glycosylation in CDG-Ia fibroblasts tested, they restored glycosylation to the control cell levels.

The biguanide drug metformin corrected experimentally induced deficiencies in the synthesis of $Glc_3Man_9GlcNAc_2$-P-P-dolichol and N-linked glycosylation. Metformin 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).

Studies with a ketogenic diet in CDG-Ia are ongoing. The rationale for this treatment is the observation, that glucose starvation improves N-glycosylation in fibroblasts from CDG-Ia patients (Körner C 1998).

As to symptomatic treatment, prevention of stroke-like events by using 0.5 mg acetylsalicylic acid / kg per day is recommended. Also, biphosphonates should be considered in patients with recurrent fractures (Grünewald S 2000). Oestradiol therapy has induced growth of breast tissue and pubic hair in two Danish females (Kjaergaard S 2001).

Moreover, any postnatal therapy of CDG-Ia would be difficult: one reason is the prenatal onset of CDG-Ia, demonstrated by the presence of dysmorphic features and neurological dysfunction at birth. On the other hand, the normal foetal growth and the failure to detect hypoglycosylation of Tf in CDG-Ia prenatally suggest that maternal

compensation and/or a developmentally regulated alternate pathway may bypass PMM deficiency "in-utero". Presently, the treatment offered to patients with CDG-Ia remains only supportive.

CDG-Ib 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 supplementation increases the depleted Man- 6-P pool, thereby normalizing the hypoglycosylation of proteins. Mannose also normalizes hypoproteinemia, blood coagulation and effectively treats the symptoms of CDG-Ib like protein-losing enteropathy and hypoglycaemia. An oral dose of 1 g mannose per kg per day divided into five doses is used; higher doses can induce osmotic diarrhoea. Whereas the clinical symptoms disappear rapidly, significant improvement of the Tf IEF pattern during mannose therapy takes several months of treatment to occur (De Lonlay P 1999, Niehues R 1998).

Despite the successful correction of immunodeficiency-related defects in **CDG IIc** (LAD II), correction of the delayed psychomotor development was expected to be more difficult to achieve. However, the patient showed significant psychomotor improvement while on fucose therapy; the sugar crosses the blood-brain barrier, resulting in elevated free fucose levels in the CSF during therapy. Whether or not fucosylation of glycoproteins produced in the CNS and found in the CSF is influenced by fucose therapy is a topic of further investigation. (Marquardt T 1999).

There are no causal therapeutic options for the other CDG types and various O- glycosylation defects; treatment varies widely depending on the exact diagnosis.

Most types of CDG have failure to thrive as one of their major medical problems. These children can be nourished with any type of formula for maximal caloric intake although early in life they seem to do better on elemental formulas. This diagnosis is not associated with any dietary restrictions; they can tolerate carbohydrates, fats and protein.

A developmental delay is typically recognized in CDG patients around four months of age. At this point early intervention with occupational, physical and speech therapy should be instituted.

Many patients with CDG have low levels of factors in the coagulation cascade. The clinical importance of this rarely manifests in every day activities, but must be acknowledged if an individual with CDG undergoes surgery. Consultation with a haematologist to document the coagulation status and factor levels of the patient and to

discuss with situation with the surgeon is important. Infusion of fresh frozen plasma corrects the factor deficiency and clinical bleeding when indicated.

Seizures-Children with CDG-Ia may have seizures in their 2nd or 3rd year of life, which are easily controlled with medication.

Appropriate orthopaedic management for thorax shortening, scoliosis/kyphosis, wheel chairs, appropriate transfer devices for the home, and continued physical therapy to prevent contractures is important.

Prednisone may be useful in patients with MED, WWS and FCMD. Some HEMPAS patients need to withdraw blood in order to prevent hemosiderosis and cirrhosis. Surgical removal of the spleen often alleviates the anaemia. Individuals with HME often need surgery to address angular deformities, pain due to irritation of skin, tendons, or nerves, and leg-length inequalities.

Table 1 Overview of CDG types

Type	Gene	Gene location	Deficient enzyme	Prevailing symptoms
Ia	PMM1, 2	16p13.3-p13.2	Phosphomannomutase	Dysmorphism, hypotonia, cerebellar hypoplasia
Ib	MPI	15q22-qter	Phosphomanno isomerase	Hepatic fibrosis, enteropathy, coagulopathy
Ic	ALG6	1p22.3	Glucosyltransferase I	Moderate form of CDG-Ia
Id	ALG3	3q27	Mannosyltransferase VI	Profound form of CDG-Ia
Ie	DPM1	20q13.13	Dolichol-P-Man synthase I	Similar to CDG-Ia, cortical blindness, microcephaly
If	MPDUI	17p13.1-p12	Dolichol-P-Man I utilization	Typical CDG-Ia symptoms, ichthyosis
Ig	ALG12	Chr.22	Mannosyltransferase VIII	Common CDG-Ia symptoms, low IgG
Ih	ALG8	11pter-p15.5	Glucosyltransferase II	Similar to that of CDG-Ib
Ii	ALG2	9q22	α-1,3 Mannosyltransferase	Typical CDG-Ia symptoms
Ij	DPAGT1	11q23.3	Dolichol-P-N-NAcGlc-1-P-transferase	Similar to that of CDG-Ia
Ik	ALG1	16p13.3	Mannosyltransferase I	Common CDG-Ia symptoms, $\downarrow$ B-cells, IgG
Il	ALG9	11q23	α-1,2 -Mannosyltransferase	Microcephaly, hypotonia, seizures, hepatomegaly
IIa	MGAT2	14q21	N-Acetyl-glucosaminyltransferase II	Developmental delay, dysmorphism, seizures
IIb	GCS1	2p13-p12	Glucosidase I	Dysmorphism, hypotonia, seizures, hepatic fibrosis
IIc	FUCT1	11p11.2	GDP-fucose transporter	Recurrent infections, developmental delay, hypotonia
IId	β4GALT1	9p13	β-1,4-Galactosyltransferase I	Myopathy, Dandy-Walker malformation,coagulopathy
IIe	COG7	16p	COG 7	Dysmorphism, hypotonia, recurrent infections
IIf	CMP		CMP- sialic acid transporter	Thrombocytopenia, no neurological symptoms

Fig. 1 Assembly and processing of linked N-glycans and molecular defects of known CDG types. Illustrations taken from Dupre T 2004
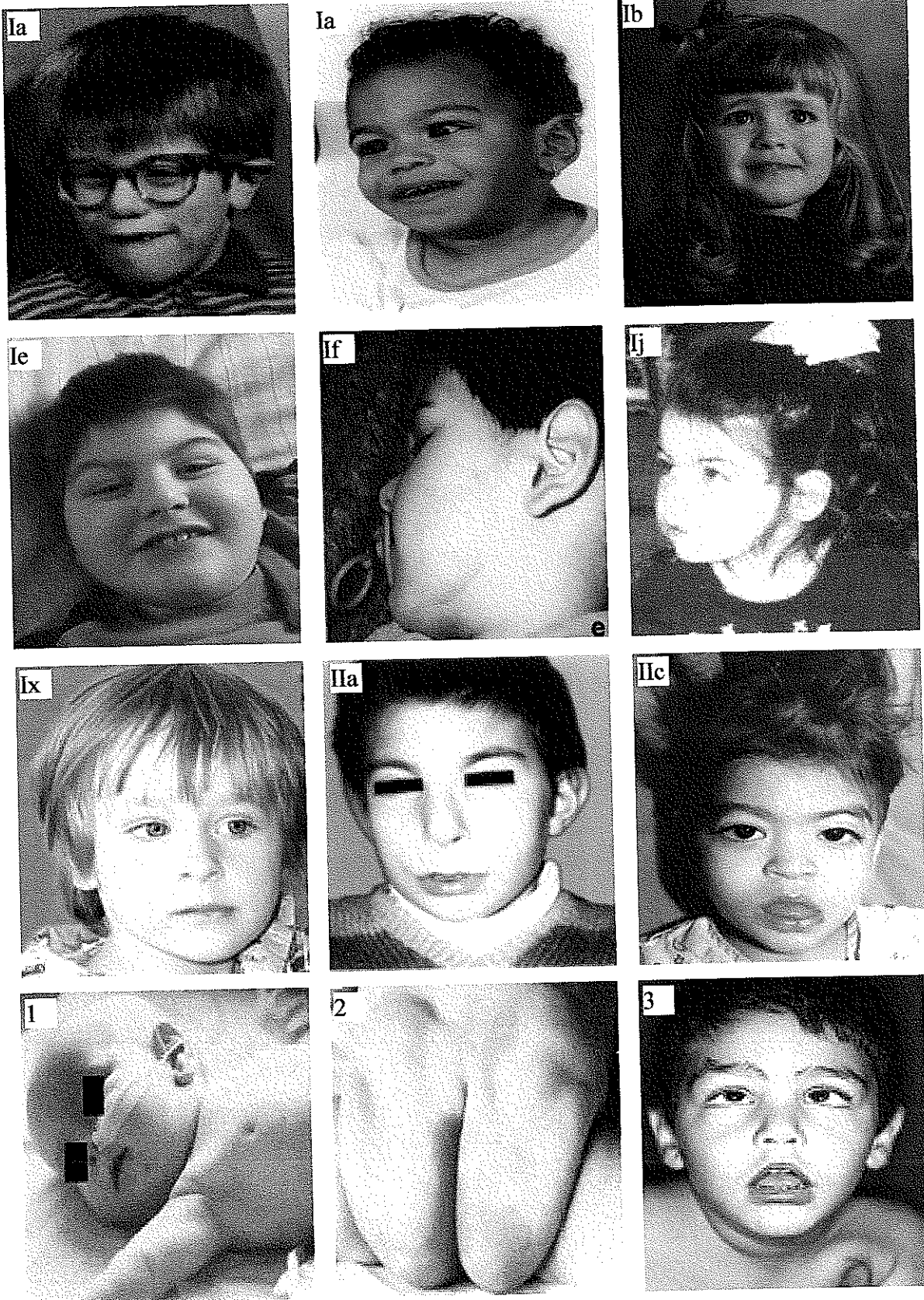
Fig. 2 Some patients of various CDG types (illustrations taken from the "CDG Family Network", http://www.cdgs.com)
Typical CDG symptoms: 1) inverted nipples, 2) fat pads, 3) strabismus

Fig. 1 *Structure of serum iron transport protein human transferrin*

Pentasialo Tf   Tetrasialo Tf   Trisialo Tf

Disialo Tf   Monosialo Tf   Asialo Tf
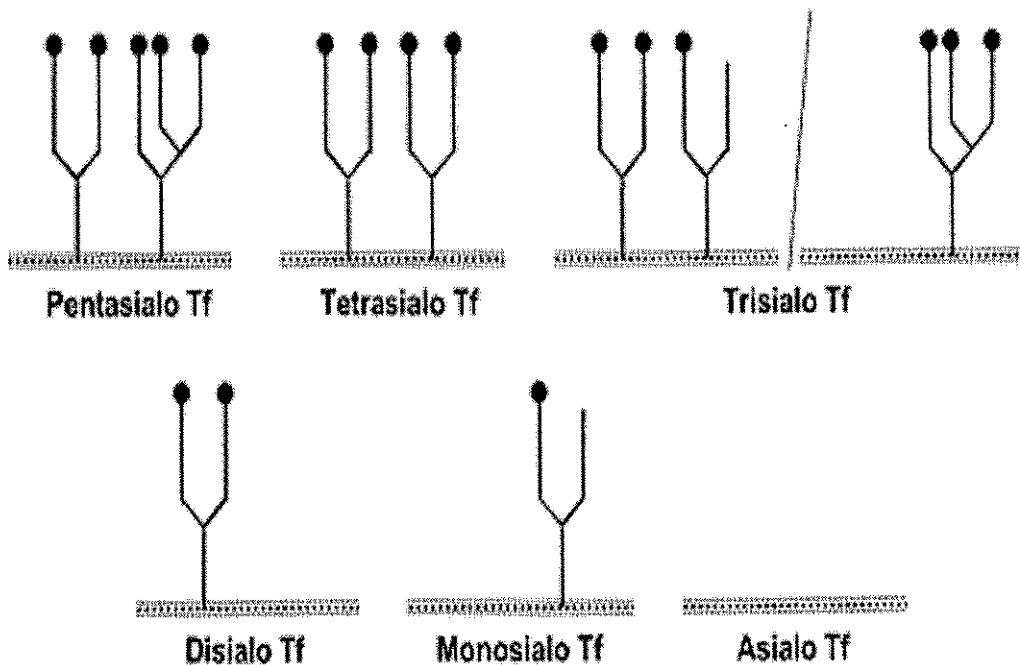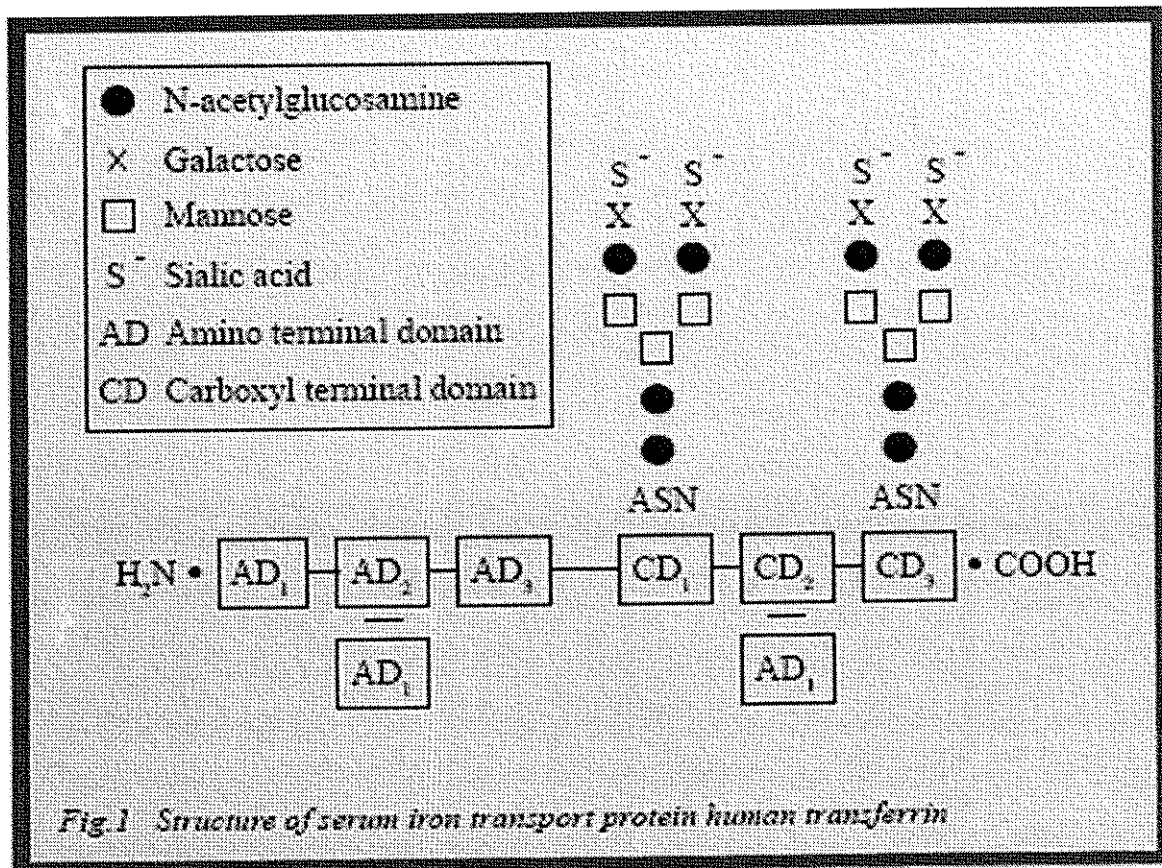
Fig. 3. Composition of Tf and its isoforms

Tf consists of a polypeptide chain with iron-binding sites, and a carbohydrate part. Eight isoforms of Tf can be distinguished: the group of the disialo-, monosialo-, and asialo-Tf is called CDT. Illustrations taken from Wong CK 2001

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 Material and methods

3.1 Chemicals

All chemicals were of analytical grade; use of double-distilled water for all analytical steps is essential.

Chemicals for IEF: Dimethyldichlorosilane, Immobiline DryPlate gels pH 4.0-7.0, 250 x 125 mm, Ampholine pH 3.5 - 5.0, Ampholine pH 6.0 - 8.0, PhastGel Blue R-350, IEF electrode strip, and sample application strips were supplied by Amersham Pharmacia Biotech.

Glycerol, 87 %, trichloroacetic acid (TCA), acetic-acid, 99.5%, methanol, 96%, tris(hydroxymethyl)aminomethane, and $NaHCO_3$ provided Merck, anti-Tf- and anti-α_1-AT- rabbit anti human are products of DAKO, Fe (III)-citrate.$1H_2O$, $FeCl_3$ .6 H_2O, nitrilotriacetic (NTA) acid and neuraminidase type V from Clostridium perfringens produces Sigma. Cysteine.HCL supplied Serva.

Chemicals for HPLC: Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris), and 2-methyl-4-isothiazolin-3-one hydrochloride (MIT) were from Sigma, dextran sulphate, and sodium salt from Fluka, $CaCl_2$ .2 H_2O from Merck, NaCl and pepsin from Lachema.

Chemicals for enzyme essays: Man-1-P- sodium salt (Na), nicotinamide adenine dinucleotide phosphate (NADP), glucose-1,6-bis-phosphate (Glc-1,6-bis-P), Man-6-P-Na, phosphoglucose isomerase (PGI), PMI, glucose-6-phosphate dehydrogenase (Glc-6-P-DH), $MgCl_2.6H_2O$, and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) supplied Sigma.

3.2 Preparation of standard solutions and reagents

3.2.1 IEF

Saturation solution (*Fe-citrate, 10 mmol/L*): Fe-citrate, 245 mg/ 100 mL (warmed to 60 °C); $NaHCO_3$, *0.5 mol/L,* 4.2 g/100 mL water. Both solutions were mixed at ratio 2:1 freshly before use.

Cysteine.HCl *(0.3 mol/L, pH 7.4)*: Cysteine.HCl (94 mg) was dissolved in 2 mL water, adjusted to pH 7.4 with a few drops of of NaOH (2.5 mol/L).

Rehydration solution (*20% glycerol*): 5 mL of glycerol, 87% was mixed with 16.5 mL water.

Acetic acid (*10% and 20% solutions*): 10 mL or 20 mL of acetic acid, 99.5% was mixed with 90, or 80 mL water, respectively.

Ampholines *(2%)*: Anodic and cathodic ampholine solutions, 40%, were diluted with water freshly before use, 1:19, and volumes of 2 mL were used for soaking electrode strips.

Antibody solutions *(anti-Tf 14 g/L; anti-α₁-AT 17 g/L)*: 0.9 mL of each or a mixture of both was used; the stock solution was diluted 1:1 and 1.8 mL volume was applied on gel (250 x 125 mm area).

Fixing solution, *20%*: 20 mg of TCA was dissolved in 100 mL water.

Staining solution *(Coomassie blue, 0.1 in methanol and acetic acid)*: One tablet of Phast Gel Blue R-350 was dissolved in 80 mL water, and warmed at 60 °C for 5 minutes. Then 120 mL of methanol, 96%, was added, well mixed, filtered and stored at 4 °C. Just before use, 50 mL of this stock solution was mixed with 50 mL acetic acid, 20%; after filtration this solution can be use twice, always freshly filtered.

Destaining solution *(methanol, 30%, and acetic acid, 10%)*: 300 mL methanol, 96% was mixed with 100 mL acetic acid, 99.5%, and 600 mL water; after regeneration with carbon filter; the solution can be use twice.

Saline *(0.9% NaCl)*: 9 mg of NaCl was dissolved in 1L water.

Digestion solution *(neuraminidase N-2876, 7.2 mg; 1.4 U/ mg; 2 mg/mL in Tris buffer)*: 1 mg of the enzyme was dissolved in 500 µL Tris buffer, 0.1 mol/L, pH 7.0. Aliquots of 100 µL volumes were stored at -20°C. The solution is stable at -20°C, 200 µL of enzyme solution was used for 50 µL serum sample. *Neuraminidase* (N-2876, 2.1 mg; 4.7 U/ mg, *4mg/mL)* was used for dry spot samples for IEF.

Tris buffer *(0.1 mol/L, pH 7.0)*: 50 mL of Tris solution (1.1 g Tris in 50 mL distilled water) was mixed with 38 mL of 0.2 M HCl (0.3 mL 99% HCL in 50 mL water), and pH of 7 made with cca 5 mL HCl, 0.1 mol/L; the total volume was adjusted to 200 mL by water. This solution was stored at –20 °C.

3.2.2 HPLC

The mobile phases *(Bis-Tris-NaCl)* for gradient elution consisted of the following *solutions*: buffer A was *20 mmol/L* Bis-Tris, adjusted to pH 6.2 with acetic acid, buffer B was buffer A, containing *0.35 mol/L* NaCl, and solution C was 1 mol/L NaCl.

Sample pretreatment and HPLC procedure, originally described by Jeppsson et al. (Jeppsson JO 1993) was used in this study with slight modifications.

Saturation solution was the same as used for IEF; occasionally, two other reagents were tested for 300 µl serum sample: $FeCl_3$ (*10 mmol/L*, 7 µL) mixed with $NaHCO_3$ (*0.5 mol/L*, 10 µL), FeNTA reagent (*10 mmol/L*, 60 µL); FeNTA was prepared by dissolving 275 mg of NTA and 270 mg $FeCl_3$ in 90 mL water, pH adjusted to 7.0 with 1.0 mol/L NaOH, making the final volume of 100 mL by water.

Delipidation solutions A: dextran sulphate in water (*100 g/L*), and B: $CaCl_2$ (*1 mol/L*).

Cleaning solutions: Solution A: NaCl (*2 mol/L*, 2 mL), group of solutions B, applied consequently, consisted from NaCl, NaOH, HCl, NaCl (all *1 mol/L*, 4 mL) with 2-5 ml water in-between, and solution C was pepsin (1 mg), NaCl (1 g), and acetic acid (*0.1 mol/L*, 200 µL) in 34 ml water.

3.2.3 Enzyme assays reagents

Solutions for cells isolation (*0.2, 0.9 and 3.6% NaCl*): These solutions are stable at least for 2 months at 4 °C.

Enzymes solutions: Man-1-P-Na, 0.1 mmol/L, 1.3 mg/mL; Glc-1,6 - bis-P- , 1.0 mg/mL; $MgCl_2$, 5 mmol/L, 20 mg/mL; HEPES, 50 mmol/L, 49.5 mg/mL pH 7.1; NADP, 25 mmol/L, 3.7 mg/mL. Injection water was used for the preparation of all components. The other components were added as an enzyme cocktail, which was prepared as demonstrated in Table 2.

Table 2 Preparation of the enzyme cocktail

Enzyme	µg / 1 essay	µL from original pack
Glc-6-P-DH	10	3.2
PGI	10	1.1
PMI	3.5	0.45

3.3 Instrumentation

Multiphor II apparatus for IEF was supplied by Pharmacia LKB. Milli-Q®, Ultrapure Water Purification System was from Millipore.

Various HPLC systems were used in this study:
ECOM HPLC system (CR), consisting of an LCP 4020 tertiary pump, GP6 gradient programmer, Gilson 234 autosampler, and LCD 2084 UV/Vis a multiple wavelength detector, amended by Chromatographic Station for Windows 1.7 software (DataApex, CR);

HPLC LC-10AD VP equipment from *Shimadzu* (Japan) with SIL-10AD VP autoinjector, SPD-10A VP UV/Vis detector, and LabSolutions software;

Hewlett-Packard 1050 chromatograph with UV/Vis detector and ChemStation software; *Agilent 1100* Series (USA) equipped with a quaternary pump and degasser, autosampler, spectrophotometric multiple wavelength UV/Vis detector and ChemStation software; and *Agilent 1100* system with a diode array detector, and ChemStation software.

Strong anion-exchange columns ResourceQ and MonoQ were from Amersham Bioscience, 0.5 μm inline solvent filters supplied Upchurch Scientific, 0.2 μm filter for mobile phase filtration were provided by Machery-Nagel (Germany), and various types of 0.45 μm disposable sample filters were from Tessek (CR), or Gelman (Canada).

Other devices also used *were the Spectrophotometer UV2* (Spectronic Unicam, United Kingdom), ultrasound bath RK 100 H (Bandelin electronic Ltd, Germany), thermostat BT 120 (Laboratorní přístroje Praha, CR), Cobas Mira (Roche diagnostics, Germany), and centrifuge K70 D (Engelsdorf, Germany).

3.4 Samples

IEF: 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. Beside these, several groups of well defined patients with isolated signs, or clear diagnoses were screened. Serum of premature newborns (12), alcohol abusers (2) and patients with hepatopathy (15) served as pathological reference samples (due to secondary defects of glycosylation as consequence). Also CDG-positive samples (3) obtained from other laboratories have 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 CSF were tested.

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

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

3.5 Sample preparation

Serum and plasma for IEF and HPLC were separated from venous blood by centrifugation (2000 g, 10 min), and stored at -20°C up to the time of analysis. Procedure with other material is described below. For enzyme analysis, EDTA blood was collected.

For Tf / Tf combined with α_1-AT analysis, 50 μL of separated serum sample was mixed with 15 μL saturation solution, and then incubated for 1 hour at room temperature. The sample was 5times diluted with water, and a volume of 1.5 μL was applied on the gel.

When α_1-AT alone was analysed, 90 μL of serum was mixed with 10 μL cysteine.HCL solution, incubated at +4 °C for 1 h, and a volume of 1.5 μL was applied on the gel.

CSF (2 mL) and amniotic fluid (30 mL) were concentrated in vacuum 10times and 30times, respectively, and then saturated as serum; 5 μL of undiluted sample was applied on the gel.

Urine (2.5 mL) was concentrated 5times in vacuum, and saturated as serum; 20 μL of not diluted sample was applied on the gel.

Dry blood / serum sample: A paper disk of 4-mm diameter was cut in pieces, placed in a 1.5-mL plastic tube with 60 μL water, and incubated for 36 hours at 4 °C. Then 40 μL were removed and incubated with 5 μL saturation solution for 1 h, and centrifuged at 2000 g for 10 min; 10 μL (from blood) or 5 μL (form serum) was applied on the IEF gel.

Treatment with neuraminidase: Saturated serum sample (10 μL) was incubated with neuraminidase solution (40 μL) for 24 hours at 37 °C, and diluted 40times with water; 4 μL aliquots were applied on the gel. When using dry blood/serum spot, Tf digestion was combined with sample elution (45 μl water and 15 μl neuraminidase solution was incubated for 36 hours at +37 °C). Tf saturation step (omitted if α_1-AT alone was to be analysed) then followed, the sample was diluted with water 8times, and 5 or 10 μL aliquots of dry serum/blood spot eluate were applied on the gel.

3.6 IEF method

3.6.1 Gel preparation

The gel was removed from the freezer, tempered, and unpacked. The anodic side of the gel was marked by cutting one angle. To prevent the gel from adhering to the

U-framed glass plate, 2 mL of dimethyldichlorosilane was evenly distributed on the glass with a tissue, leaved to dry, and then rinsed with water. Some drops of water were added on the glass plate before placing the gel on it (carefully without air bubbles, rolling the gel flat to ensure of good contact, and blotting away any excess water). The U-framed glass plate was placed on top of the dry gel, and the cassettes were clamped together, standing vertically. The space was then completely filled with about 25 mL bubble free hydration solution using a syringe; the gel re-swelled for 1.5 hour at room temperature (if a minor leakage occurred, the cassette was placed horizontally). The cassette was then opened and gel was quickly blotted (by two wet tissue papers, and two dry ones placed on them), and rolled to remove excess of the solution.

Light paraffin oil (about 1 mL) was pipetted onto the cooling plate of IEF apparatus and the gel was placed in the centre without air bubbles. To maintain a stable gradient, electrode strips soaked in the anode- and cathode- ampholine-solutions, well blotted before use, were applied between the gel and the electrodes.

3.6.2 IEF run

Multiphor II was connected to the power supply, and the gel was allowed to pre-focus for 30 min. Then the samples were loaded into the wells of the sample-strip (placed 1 cm aside from the cathode side) using a micropipette. The apparatus was reassembled and proteins focused. The running conditions with a total 8000 V achieved are summarized in Table 3.

Table 3 IEF conditions

	Time (min)	V	mA	W
Pre-focusing	10	1000	4	4
	20	2000	4	8
Samples application				
Focusing	30	1000	4	4
	240	2000	4	8

3.6.3 Detection

After focusing, the gel (side-up) was placed on a glass supporter, and 0.9 mL of Tf antibodies or 1.8 mL of Tf and - α_1-AT antibodies solution was pipetted at the anodic side, then spread with a glass- stick on the whole gel surface. The gel was then incubated for 45 min at room temperature.

The next steps include an overnight gel washing with saline (1 L), protein fixation with TCA solution (100 mL, 10 min), staining with Coomassie blue (100 mL, 10 min), and final destaining (500 mL, 2 h). The gels were left to dry at room temperature overnight.

Staining was performed at room temperature with continuous shaking; destaining was continued until appearance of clear background of the gel with 4-5 fresh portions (100 mL).

3.6.4 Densitometry

The peak areas or/and heights of the penta-, tetra-, tri-, di-, mono-, and asialo-Tf isoforms were used for calculation (ratio in %). Similarly, the hexa-, penta-, tetra-, tri-, di-, mono- and asialo- α_1-AT were also calculated.

3.7 HPLC method
3.7.1 Preanalysis

Fresh or thawed serum (300 µL aliquot) was saturated with the Fe-citrate solution (90 µL), and incubated for 1 hour at room temperature. Alternatively, two other types of saturation solution were mixed with 300 µL serum for comparison: the $FeCl_3$ (17 µL), or the FeNTA (60 µL), reagents, and then incubated for 1 hour at room temperature.

Serum lipoproteins were precipitated by the delipidation solutions A (3 µL) and B (15 µL), added to the whole volume of the saturated serum, well mixed, and left for 40 min at 4°C; after centrifugation at 2000 g for 10 min, the supernatant (250 µL) was diluted 3-5times with HPLC buffer A. Samples were then filtered through 0.45 µm disposable syringe filters; three different types were compared (to check proteins adsorption) by measuring the protein concentrations in the filtrates (Cobas Mira).

Three paper discs (12 mm-diameters) of dry serum spot sample were cut, placed into a 1.5 ml plastic tube, and eluted with water (450 µL) for 36 hours at +4°C. The eluate (300 µL) was then saturated with a mixture of $FeCl_3$ (7 µL) and $NaHCO_3$ (10 µL), incubated for 1 hour at room temperature, delipidated analogously to serum, cf. at 2000 g for 10 min, and filtered; not diluted.

Digestion with neuraminidase was used for identification of Tf genetic variants. Neuraminidase-treated sample was prepared as follows: Saturated serum (50 µL) was incubated with the digestion solution (200 µL) at 37°C for 24 hours, then the sample was

mixed with the delipidation solutions A (0.5 μL) and B (2.5 μL), and filtered without further dilution.

3.7.2 HPLC analysis

The HPLC procedure is based on anion-exchange chromatographic separation of the individual Tf glycoforms using linear salt gradient elution at a flow rate (FR) of 1.0 mL/min at room temperature, followed by photometric detection of the Fe-Tf complex at 460 nm.

Saturated, delipidated, diluted and filtered samples, either freshly prepared, frozen, or stored for 12 hours at +4°C were analysed; volume of 100 μL was injected.

For elution we used a linear Bis-Tris-NaCl gradient: The mobile phases were freshly prepared (possibly stored overnight at +4 °C, and well tempered the next day), filtered through 0.2 μm filter, and continuously degassed by sonication. The FR of 1 mL/min, and alternatively 0.7 mL/min were used. Detection was done at 460 nm, and additionally the wavelength of 470 nm was tested for comparison.

Apart from that one described by Jeppsson et al. (1993), other programs of linear salt gradient were also tested (Renner F 1997, Turpeinen U 2001). Finally we adopted that one used by Turpeinen (personal communication), with a total run time 49 min. HPLC conditions on various systems are illustrated by Table 4.

Table 4 Comparison of gradient profiles used for HPLC analysis of Tf isoforms; FR = 1 ml/min. 1) Jeppsson JO 1993, 2) Renner F 1997, 3) Turpeinen U 2001, 4) Turpeinen U 2004; personal communication.

I. Jasco, MonoQ (1)				II. Merck L4250, MonoQ (2)				III. Spectraflow 783, MonoQ, ResourceQ (3)				IV. Agilent 1100, ResourceQ (4)			
Time (min)	A %	B %	C %	Time (min)	A %	B %	C %	Time (min)	A %	B %	C %	Time (min)	A %	B %	C %
0.0	100	0	0	0.0	90	10	0	0	100	0	0	0	100	0	0
3.0	100	0	0	3.0	90	10	0	1	100	0	0	1	100	0	0
5.0	90	10	0	3.1	85	15	0	2	90	10	0	28	60	40	0
18.0	66	34	0	7.0	85	15	0	24	60	40	0	29	0	0	100
18.1	0	0	100	18.0	70	30	0	25	0	0	100	33	0	0	100
22.0	0	0	100	18.1	0	0	100	28	0	0	100	34	100	0	0
22.1	100	0	0	22.0	0	0	100	29	100	0	0	49	100	0	0
32.0	100	0	0	22.1	100	0	0	39	100	0	0				
				32.0	100	0	0								

Column cleaning was done according to the manufacturer's instruction: the regular cleaning of MonoQ column was performed by solution A, and more rigorous cleaning was done by solution B. Radical cleaning was done after 50 serum samples with solution of pepsin (1 mg), NaCl (1 g) and acetic acid (0.1 mol/L, 200 µL) in 34 ml water. This solution was pumped through the column with a retrograde FR of 1 ml/min for 10 min. The column ends were sealed, and the whole column was stored at 37 °C overnight.

The ResourceQ column was cleaned after each series of analysis by reverse flow with 0.5 mol/L NaCl (30 min), followed by water (30 min). Then the column was returned to its original position and washed with water for 30 min. More rigorous cleaning was the same as that described for MonoQ; no pepsin solution was used for this column.

The columns were stored in 20% ethanol; re-equilibration was done with water, buffer A, buffer B, and buffer A (volumes 5 mL) at a FR 1 mL/min.

3.8 Enzyme essay

Screening of Tf isoforms in the most common CDG type Ia is completed by measurement of cellular PMM activity.

PMM activity was assayed according to the procedure of van Schaftingen (van Schaftingen 1995). A two-step procedure has been adopted for isolation of peripheral blood leucocytes, which were freshly prepared. The cell disintegration was achieved mechanically by several passings of leucocyte pellets through a 25 G needle (Barnier A 2002).

The assay was modified by using Glc-1,6-bis-P instead of Man-1,6-bis-P, as a cofactor (Chabas A, personal communication, Niehues R 1998).

3.8.1 Cells isolation

EDTA blood (10 mL) was incubated in thermostat at 37 °C for exactly 30 min. Of the two layers (bottom– erythrocyte, supernatant– plasma with proteins and leucocytes), the upper layer was transferred to 10 mL tube, centrifuged for 10 min at 700 g and the supernatant removed. The leucocyte pellet on the bottom was suspended in 5 mL NaCl (0.2%), the tube was covered with parafilm, and mixed several times by gentle inversion of the tube. After 2 min, 1.5 mL NaCl (3.6 %) was added, mixed again by gentle inversion, and centrifuged for 10 min at 2500 g; the supernatant was removed out. If the pellet was contaminated by erythrocytes, 100 µL cold water (5 min at −20 °C) was added,

and after 50 sec, the tube was briefly vortexed to allow complete lysis of erythrocytes. Then 1 mL saline was added, mixed several times by gentle inversion, and centrifuged for 10 min at 1000 g. The supernatant was removed, and the leucocyte pellet used for enzyme essay (it can be stored in 150 mL/L glycerol at −80 °C for one month).

3.8.2 Cells lysis

The leucocyte pellet was resuspended in 50 μL of injection water, and 3 μL aliquot was inspected microscopically. After that, cells were disrupted by repeated (30x) passage through a syringe needle (25 gauge- 5/8-inch). In order to prevent the leucocytes waste in the needle and syringe, they were flushed by 250 μL water. The pellet was frozen for 10-15 min, thawed, and sonicated for 10 min. Disruption of leucocytes was checked inspected microscopically (3 μL), and the sample was used for enzyme essay; protein concentration (preferable range is 2-10 mg/L) was determined in a 65 μL aliquot.

3.8.3 Enzyme activity

The total volume of incubation solution was 1 mL, containing cell extract, Glc-1,6-bis-P, Man-1-P-Na, enzymes cocktail (Glc-6-P-DH, PGI and PMI), $NADP^+$ and $MgCl_2$ in HEPES. The volume of cell extract was calculated according to the protein concentration in the pellet (an equivalent of 100 μg was used).

The enzyme essay was processed in two steps (Table 5): in the first, the reaction mixture without the enzyme cocktail was incubated for 90 min at 37 °C. The reaction was stopped by heating for 5 min at 80 °C, and then shortly cooled on ice, centrifuged for 3 min at 1000 g, and the supernatants (sample and blank) were separated. The second step was incubated with the enzyme cocktail in the presence of NADP and the supernatant from the first step in thermostat-water bath for 1 hour at 37 °C. The absorbance was read 5times in 15 min intervals at 340 nm; the first time (zero) before the NADP addition, and the last time after the final heating.

The blank was prepared as the sample by two steps, but without Man-1-P-Na in the first step.

Table 5 Measurement of PMM activity in leucocytes

Reaction step 1		
Component	Sample (µL)	Blank (µL)
Man-1-P-Na	50	-
Glc-1,6-bis-P	80	80
MgCl$_2$	100	100
Cells extract	120	120
HEPES	650	700
Reaction step 2		
Supernatant	400	400
Enzyme cocktail	30	30
HEPES	520	520
Read the absorbance, and then add to each tube:		
NADP	50	50
Read again in 15 min intervals!		

4 Results

4.1 IEF

4.1.1 Preanalysis

Serum samples are preferred for analysis of Tf isoforms. Even if plasma is usually not recommended for analysis, a slight increase of CDT was documented in only 3 of 60 plasma samples analysed. In control CSF, considerably higher mono-, and asialo-Tf was found, while pentasialo-, tetrasialo-, trisialo- and disialo-Tf were decreased, when compared to serum (Fig. 4). Analysis of amniotic fluid resulted in an IEF pattern similar to that obtained with serum (Fig. 4). No useful results were obtained from urine analysis (Fig. 4).

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 was analysed for comparison: sharp bands of Tf isoforms could be detected in the properly saturated sample, while tailing is noted in the native serum (Fig. 5, Lane S1). Various Fe-solutions (citrate, chloride, and FeNTA) were tested, but no significant difference was noted (Fig. 5, Lanes C5-C7). When samples intended for HPLC (prepared by removal of serum albumin and lipoproteins) were analysed, tailing occurred (Fig. 5, Lane S3).

Various volumes and concentrations of samples were tested; best results were obtained when 1.5 µL volume of the saturated serum sample, diluted 5times, was applied on the gel (Fig. 5, 6 and 9A). Optimal conditions for dry spot samples were achieved when 10 µL (eluate of blood dry spot), or 5 µL aliquots (elute of serum dry spot) were applied on the gel; no distinct difference was noted, when compared to serum (Fig. 6A). Using this type of material, both glycoproteins (Tf and α_1-AT) could be analysed with nearly the same results, as obtained with serum (Fig. 6).

Separated and combined analysis of Tf and α_1-AT on one gel gave comparable results. Slight increase of serum CDT detected by HPLC (Agilent 1100) in one alcohol abuser was reflected by the similar ratios of Tf isoforms found by IEF of serum dry spot (Fig. 6B, Lane A8).

For complete Tf desialisation, 150 µg of neuraminidase is sufficient for 50 µL serum (Fig. 7, Lane N4). Similar conditions are used for samples eluted from dry serum/blood spots. A possible contamination of the adjacent samples with the enzyme in the course of IEF is illustrated in Fig. 7, Lanes C2 and C3.

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4.1.2 Stability of samples and reagents

Whole blood transported at room temperature is usable for 6 hours, and the separated serum for 24 hours, at maximum, as compared to the 7-day interval, which is considered safe for the refrigerated serum, and 6 months for serum frozen at -20 °C. Iron-saturated samples, stored at $+4$ °C and -20 °C are applicable for 72 hours and 3 month, respectively. The number of times, when a sample can be frozen and thawed safely is 4-6.

The Immobiline DryPlate gels should be stored at -20 °C, the shelf life of gel is about 18 months. After gel rehydration it can be stored at 4-8 °C for one week before the analysis.

The Tf and α_1-AT antibodies are stable for at least one year at 4-8 °C. To check the stability of the antibodies, the relevant controls should be run simultaneously with patient specimens.

The buffers for gradient HPLC elution should be freshly prepared and stored at room temperature during analysis for the shortest period necessary, usually up to 3 days with good results (Turpeinen U 2001); bacteriostatic agent MIT (0.66 mmol/L), added to buffers prolongs their availability time up to one month, when refrigerated. The stability of the other reagents is listed in Table 6.

Table 6 Stability of reagents

Solutions for	Storage / Stability
rehydration	should be freshly prepared before use
saturation - Fe citrate	at least 1-2 months at $+4$ °C
saturation - FeNTA	at least 1 year at 4°
delipidation	at least 1-2 months at $+4$ °C
fixation	at least 1-2 months at $+4$ °C
staining	at least 1-2 months at $+4$ °C
destaining	at least 1-2 months at $+22$ °C
Cysteine.HCL	should be freshly prepared before use
Neuraminidase	stable at -20°C; 4 freeze-thaw cycles
Ampholines	should be freshly diluted before use
Tris buffer	solution stored at -20 °C

4.1.3 Technical problems

A leakage of hydrating solution along the cassette (when only part of gel was used) could be prevented by placing the cassette horizontally.

The effects of the prolonged gel blotting after rehydration (fuzzy bands) are demonstrated in Fig. 8C.

Freshly filtered staining solution should be used; otherwise precipitation of the dye occurs (Fig. 8A). Prolonged time of destaining and frequent solution renewal improved the results (Fig. 8A).

In spite of involuntary interruption of the power supply (up to 2 hours), no negative effect was noted when time-delayed IEF continues later on.

4.1.4 Analysis of Tf

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 6times, 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 hours (8000 Vh), and detected by the direct immunofixation (RAHU anti-Tf, Coomassie blue), followed by densitometry.

A maximum of 52 samples could be applied on the gel. Under certain circumstances, e.g. when the analysis has to be repeated for confirmation of suspicious results, when analysing a dry spot-material, or when the neuraminidase-digested samples are spotted, it was necessary (safer) to leave more space between the samples. Later on, by use of smaller pieces of gel (cut before rehydration), the method was adapted for lower series, which allows obtainment of results more quickly.

In healthy controls, 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.

For comparison, the control and CDG specimens are always simultaneously run with unknown samples.

The relative concentration of particular Tf isoforms was expressed as a percentage of the total Tf (sum of six Tf isoforms, from asialo- to pentasialo-Tf)

Distribution of Tf isoforms in our set of controls and some CDG patients has been established (Tables 7-9). No gender differences from the viewpoint of both aspects, hypoglycosylation and distribution of genetic variants, have been recognized. The reference values of CDT were higher in healthy children (no signs of CDG, or presenting of secondary causes which increased CDT) than in adult control.

Table 7 Reference values of serum Tf isoforms in children (n= 35)

Tf isoforms	Mean (%)	SD	x – 2 SD	x + 2 SD
0	0.8	0.7	-0.5	2.1
1	1.3	0.9	-0.9	2.6
2	7.0	2.4	2.2	11.9
3	10.7	3.1	4.5	17.0
4	63.2	6.9	49.5	77.1
5	17.0	6.3	4.3	29.6

Table 8 Reference values of serum Tf isoforms in healthy adults (n= 100)

Tf isoforms	Mean (%)	SD	x – 2 SD	x + 2 SD
0	0.6	0.6	-0.6	1.7
1	1.0	0.9	-0.8	2.7
2	6.4	1.5	3.8	9.7
3	11.8	4.0	3.7	19.8
4	60.2	7.1	46.2	74.6
5	20.0	5.2	9.6	30.4

Table 9 Range of serum Tf isoforms (%) in controls (children) and CDG patients; comparison of results

Tf (%)	Wevers R 2000 Control (n=30)	CDG Ia (n=12)	Our results Control (n=35)	CDG Ia (n=3)
0	0.0 – 2.6	5.4 – 29.5	0.0 – 1.9	7.9 – 19.8
1	0.0 – 2.6	0.1 – 7.1	0.0 – 3.3	3.4 – 5.4
2	1.6 – 2.6	17.0 – 37.7	3.3 – 13.5	28.5 – 30.6
3	2.5 – 15.6	5.0 – 12.5	4.4 – 17.5	8.3 – 12.4
4	51.2 – 72.2	20.6 – 49.5	51.0 – 78.3	29.7 – 34.9
5	12.1 – 30.8	3.9 – 19.3	7.3 – 29.6	8.2 – 11.1
6	0.0 – 9.0	0.0 – 5.9	-	-

The within run (n=1, x=4) and between run (n=6, x=6) reproducibility of Tf fractions was tested. The coefficient of variation (CV) of intrassay and interassay of the peaks-area ratios were 10% and 15% for disialo-, 14% and 16% for trisialo-, 2% and 3% for tetrasialo-, 11% and 13% for pentasialo- Tf, respectively.

4.1.5 Analysis of α_1-AT

Since abnormal IEF pattern of other glycoproteins, apart from Tf might confirm a generalized defect of glycosylation, a method for separation of α_1-AT was introduced; similar analytical conditions can be used (Fig. 9A).

Further optimalization of the IEF method was introduced: it consists in simultaneous separation of both serum glycoproteins, initially analysed in two separated gels and placed apart. Since the position of individual isoforms doesn't overlap, both antibody solutions can be applied on the different parts of gel surface for selective detection. Simultaneous analysis of Tf and α_1-AT in the same gel was tested with good results (Fig. 9A).

Finally, having been inspired by Artuch et al. (2003), an antibody cocktail for detection of both glycoproteins in one gel was tested, and successfully used since then. The samples of CDG Ia and our CDG patient showed clear abnormalities.

The reference values of α_1-AT are demonstrated in the Table 10.

Table 10 Reference values of serum α_1-AT isoforms in children (n= 13)

α_1-AT isoforms	Mean (%)	SD	x - 2 SD	x + 2 SD
0	0.1	0.3	-0.5	0.7
1	0.1	0.3	-0.5	0.7
2	9.5	4.9	-0.3	19.3
3 + 4	36.6	1.4	33.2	38.8
6	34.5	5.0	24.5	44.5
7	19.2	2.6	13.8	24.2

4.1.6 Abnormalities of glycosylation

Serum or plasma samples of patients with any of the CDG- typical symptoms, or suspicious biochemical abnormalities were analysed.

In the group of children with signs of an inherited metabolic disease (IMD) (n= 1100), namely the following symptoms have been be recorded: brain atrophy, epilepsy, psychomotor or mental retardation, failure to thrive, hydrocephalus, neurological and nephrologic abnormalities, pigmentary retinopathy, retinal dystrophy, blindness, hepatitis, hepatic cirrhosis, fibrosis, and other non-specific symptoms.

4.1.6.1 Primary abnormality

A patient with typical clinical symptoms and abnormal IEF pattern was identified as the CDG type IIx (see Chapter 4.4). The frequency of CDG in our set of patients has been calculated to be approximately 1: 1100.

Serum samples of three CDG Ia patients kindly provided by other laboratory served as pathological reference samples in this study; they showed a typical CDG I- IEF

pattern with lower tetrasialo-, and a marked increase of disialo-, and asialo-Tf; see Fig. 10 and 23. Our CDG patient showed higher disialo-, but only slight increase in asialo-Tf, beside the lower tetrasialo-Tf; see Table 11 and Fig. 23.

The IEF pattern of α_1-AT in the three CDG Ia patients was characterized by increased asialo- and monosialo- isoforms, together with lower tetrasialo-α_1-AT, when compared to controls. Our patient, however, showed only increase in monosialo- α_1-AT (Table 12 and Fig. 22).

Table 11 Distribution of Tf isoforms (%) in controls and CDG patients

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

Table 12 Distribution of α_1-AT isoforms (%) in controls and CDG patients

Samples	α_1-AT isoforms					
	0	1	2	3 + 4	6	7
CDG Ia 1	5.6	7.8	12.4	31	29.4	13.8
CDG Ia 2	6.2	6.1	11.9	30.5	32.0	13.3
CDG Ia 3	6.0	6.3	10.9	32.7	31.0	13.1
Our patient	0.1	4.8	9.6	32.3	39.2	14.0
Controls (mean)	0.1	0.1	9.5	36.6	34.5	19.2

4.1.6.2 Secondary abnormalities, non CDG

Certain glycosylation abnormalities were detected in about 7.2 % (n= 85) of the total group of patients, mostly associated with final 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), epilepsy (14/40), bronchial asthma (1/11), nephrotic syndrome (1/12), obesity (1/5), speech disorders (1/4), acute tubulo-interstitial nephritis (1/1), Wilson disease (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 hepatopathy, hypotonia, psychomotor retardation, convulsions, failure to thrive, anaemia, and psychic abnormalities. Some of these conditions are illustrated in Fig. 6C.

Apart from that, a group of adults with severe hepatic disease (including hepatitis C, cirrhosis, fibrosis, and liver failure, n=15) 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.

Alcohol load (60 g per day for 7 days) in one adult volunteer (occasional drinker) did not result in any noticeable signs of hypoglycosylation, in contrast to the slight increase of CDT found in the blood samples (dry spots) from a chronic alcohol abuser, provided by another laboratory.

Due to reported influence of some common chronic diseases on protein glycosylation, a group of children with cystic fibrosis (CF) (n=40) and Crohn's disease (n=24) were tested; an increase of CDT was detected in three patients with CF and one patient with Crohn's disease.

Serum Tf isoforms were also analysed in healthy newborns with a gestational age between 36-38 weeks; among the 12 of the newborns tested, 7 of them show higher CDT.

Effect of long term treatment with cytostatics (methotrexate, n=15), antibiotics (n=10), corticosteroids (n=10), antiepileptics (n=13), antirheumatics (salazopyrin, n=11; chlorochin, n=5) has been followed; only slight signs of hypoglycosylation were found in three children treated by methotrexate or phenobarbitals (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.

4.1.7 Tf and α_1-AT genetic variants

In this study, seven different phenotypes of Tf have been recognized (Fig. 10), and their frequencies calculated. Only the variants C_1C_1 (in 86 %), and C_1C_2 (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 C_2C_2 (0.6 %), and C_1C_3 (0.3 %), as well as heterozygous CB (0.2 % for Tf C_1B_{1-2}) and CD (0.1 % for both Tf C_1D_2 and Tf C_1D_{4-5}) phenotypes were found (Fig. 10).

Apparently higher incidence of the Tf C_1C_2 subtype, noted in the group of patients suspected of an IMD (20 %; n=1100) and especially in two smaller groups of children with Crohn's disease (29.2 %; n=24) and CF (27.5 %; n=40), when compared to healthy controls (16 %; n=100) was not in fact significant (Chi square and Fisher's exact tests), see Table 13. No differences could be found in other subgroups of patients tested.

An abnormal IEF pattern was found in a 14-years old boy with the diagnosis of ankylosing spondylitis (coming under a group of patients with chronic diseases). IEF revealed a considerable, but isolated increase in the asialo-Tf band (Fig. 10). This finding, not typical for the most common CDG types, together with a lack of CDG symptoms prompted us to rule out a slow (cathodal) Tf CD variant. However, just one band, reflecting asialo-Tf C_1, appeared after Tf desialisation by neuraminidase (Fig. 10), 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 C_1D_{4-5} variant, was demonstrated (Fig. 10). A finding of the same Tf phenotype in the patient's father (Fig. 11), and Tf C_1 with normal isoforms in the mother, together with a normal result of α_1-AT analysis in the boy and his parents supported the presence of the rare Tf variant.

To demonstrate a risky similarity of Tf variants and higher CDT, IEF patterns of Tf isoforms in typically symptomatic CDG Ia patients are shown for comparison (Fig. 10).

Table 13 Frequencies of two common Tf phenotypes in controls and in various patient groups

Groups	Number	Tf C_1		Tf C_1C_2	
		N	%	N	%
Controls	100	84	84.0	16	16.0
Bronchial asthma	11	11	100	-	-
Crohn's disease	24	17	70.8	7	29.2
Cystic fibrosis	40	27	72.5	11	27.5
Epilepsy	40	34	85.0	6	15.0
Nephrotic syndrome	12	10	83.0	2	17.0

α_1-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 procedure described by Jeppsson et al. (Jeppsson JO 1993), using the ECOM system and MonoQ column, but our results were not satisfactory: Only three peaks of the main Tf isoforms (tetra-, penta-, and trisialo-Tf) could be detected on chromatograms in controls, whereas the important disialo-Tf, the main discriminator between controls and CDG patients was hardly visible, let alone the other two minor, CDG-pathognomic peaks, corresponding to asialo- and monosialo-Tf.

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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 FR, and then comparing various HPLC systems, namely detectors, weighing up the optimal wavelength setting, and the choice of chromatographic column.

4.2.1 Preanalysis

First of all we focused our attention on sample pretreatment.

Since the saturation step is evidently a critical point of the Tf analysis (based on the Fe^{3+}-Tf complex detection at 460 nm), 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 (see Fig. 12), due to the different charges of the incompletely saturated Tf molecule.

Of the three different iron solutions tested, the serum sample saturated with $FeCl_3$ (see Chapter 3.2.2, and Fig. 12) showed the highest peaks of the Tf isoforms detectable, namely the tetrasialo-Tf, in comparison with the slightly lower, otherwise similar results with the Fe-citrate and FeNTA solution. When the standard FeNTA reagent was tested, a peak with a retention time (RT) close to that of tetrasialo-Tf was noted; its use for Tf saturation may possibly result in false interpretation (Fig. 12).

Saturation of samples with the Fe-citrate solution at 22°C showed higher peaks when compared to those incubated at 37°C in the same 60 min interval.

In contrast to serum, the EDTA plasma showed a peak eluting at the position of trisialo-Tf (compared to EDTA blank sample) (Fig. 12).

As there had been a few problems with frequent increases of pressure in the HPLC system reaching the allotted upper limit of the column (1.5 mega Pascal (MPa) only for the ResourceQ), the mobile phases degassing, and delipidation steps were thoroughly checked up, namely the chemicals used and the order of their addition to serum, as well as the final centrifugation; however, we were not able to find any connection. No different results were noted when a lipid-free and non-delipidated sample was analysed, as demonstrated in Fig. 12.

To exclude substantial loss of protein, due to its possible sorption on the disposable syringe filter before sample application on the column, three types of filters were compared (Tessek and Gelman). No difference in total protein concentration, measured in the non- filtered sample and the three different filtrates, was found.

Sample storage and stability of all solutions used in the procedure were also carefully controlled (see chapter 4.1.2). Apart from serum and plasma, eluates of dry spot serum were also tested on the Agilent 1100 system: the CDT isoforms were below the detection limit of the HPLC method, as illustrated by Fig. 13.

4.2.2 HPLC columns

Two anion-exchange chromatographic columns were used and compared; the MonoQ is longer and narrower in comparison to the ResouceQ type, so that better separation of Tf isoforms could be expected. Finally we chose the more economic ResourceQ giving similar results (Fig. 14) (confirmed also by Turpeinen U 2001).

4.2.3 HPLC systems

The preparation, degassing and storage of mobile phases were thoroughly checked, but without any positive effect of eventual improvement on the (permanently low) sensitivity of analysis. Slower FR did not result in a better separation, as illustrated in Fig. 15 (compare flow-rate 0.7 and 1.0 ml/min).

An improvement of Tf detection when reading absorbance at 470 nm instead of the original wavelength set at 460 nm was reported (Helander A 2003); however, we could not see any significant difference (see Fig. 16).

We had been forewarned of the insufficiency of some systems with UV-Vis detectors (e.g. Hewlett-Packard 1090, Turpeinen U 2001 and personal communication) for this application. So, we took the opportunity to test other HPLC systems available, such as Hewlett-Packard 1050, Shimadzu LC10AD VP, and Agilent 1100.

The analysis was performed on Hewlett-Packard 1050 chromatograph, but results were again not satisfactory. Better separation was achieved with the Shimadzu LC10AD VP equipment on the same ResourceQ column. In healthy individuals, in addition to pentasialo-, tetrasialo- and trisialo-Tf, also the physiological disialo-Tf was detected by the Shimadzu and Agilent 1100, but not with the ECOM system.

The sample of our CDG patient was analysed by both ECOM and Shimadzu chromatographs: An increase of disialo-Tf, more prominent using the Shimadzu system,

was noted, while the slight increase of asialo-Tf, found by IEF, was under the detection limit of both HPLC systems. Certain increase of asialo- and disialo- was detected in an alcoholic patient by the Agilent 1100 system.

In our experiments, the within day and between day imprecision (coefficient of variation, CV) for the RT of tetrasialo-Tf values were 1.8 % (n=7) and 5.7 % (n=20), respectively.

4.2.4 Review of results

Various systems showed different detection sensitivity for Tf isoforms. Analysis of control serum by ECOM system resulted in separation of the main three peaks (tetra-, penta-, and trisialo-Tf), but the disialo- and lower-sialo-Tfs was under the detection limit. In our CDG patient, an increase in the disialo-Tf, but not the other CDT peaks (asialo-Tf), was found (Fig. 17, A).

Analogous separation, but with higher yield in controls as well as in our CDG patient could be obtained on the Shimadzu system (Fig. 17, B). When Agilent 1100 multiple wave lengths UV-Vis detectors 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); see Fig. 17, C.

Various samples (of newborns, patients with CF, or those treated with antiepileptics) showing slight abnormalities by IEF method did not present any CDT-typical peaks with the ECOM HPLC system (Fig. 18).

With the ECOM and Shimadzu systems we have been successful in differentiating between the common Tf C_1 and some other genetic variants of Tf. A serum sample with rare Tf-C_1D_{4-5} variant analysed on the Shimadzu system showed two main peaks reflecting the tetrasialo-Tf C_1 and the tetrasialo-Tf D_{4-5} (eluted comparatively earlier), and three minor peaks matching with the penta- and trisialo-isoforms of Tf-C_1 allele, and pentasialo-glycoform of Tf D_{4-5} allele. The same sample analysed with the ECOM system under the same conditions showed only two main peaks (tetrasialo-Tf C_1 and the sum of trisialo-TfC_1 and pentasialo-Tf D_{4-5}), in addition to two smaller peaks (pentasialo-Tf C_1 and tetraisialo-Tf D) (Fig. 19).

Another rare heterozygous Tf C_1B variant was identified, showing two main peaks of tetrasialo-Tf C_1 and tetrasialo-Tf B (delayed elution) with a minor peak matching pentasialo-Tf B, see Fig. 19.

In addition to heterozygous Tf variants, a rare homozygous Tf C_2C_2 was also distinguished (by a shifted RT of the main Tf isoforms, see (Fig. 19). Similar to IEF, the HPLC conditions do not allow identification of the common Tf C_1C_2, and other heterozygous subtypes of Tf C_1 (Fig. 19).

The Tf C_1C_1 glycoforms patterns after neuraminidase treatment shows one rather broad peak at the position of the asialo-Tf C_1, while the completely desialisated Tf C_1D is characterized by two main peaks (asialo-Tf C_1 and asialo-Tf D allele), see Fig. 20.

Different RTs of Tf isoforms under the same working conditions (the same column, FR and mobile phases) were obtained with various HPLC systems (Shimadzu, ECOM, and Agilent 1100), due to different length of capillaries and the overall arrangement.

4.3 Enzyme essays

After the leucocytes were isolated and disrupted, the protein concentration was measured by turbidimetry.

Parameters of enzyme essay and PMM activity results in our CDG patient and the other family members (controls) are presented in Table 14; volumes of the EDTA blood used for leucocytes isolation, and the supernatant after blood warming, protein concentration in the cells lysate, and enzyme activities are given.

Table 14 Results of enzyme activity in controls, our CDG patient and his family

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

PMM activity in controls is 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 NADPH.

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, followed prenatally for intrauterine hypotrophy and a finding of intestinal obstruction (double bubble sign). Born full term as

the first child of healthy non-related Czech parents without remarkable family history, his Apgar score was 6, 6, and 7, birth weight 2310 g, body length 45 cm, and head perimeter 32.5 cm. He presented with dysmorphic features (Fig. 21), including dolichocephaly, low hairline, high nasal bridge, thin lips, microgenia, gothic palate, blepharophimosis and epicanthus, in addition to other malformations, such as arachnodactyly, unusual palmar creases, sinus sacralis, pes equinovarus, hypospadias, inguinal hernia, cryptorchism, vesicourethral reflux, peripheral pulmonary stenosis, and patent foramen ovale. Shortly after birth a surgery was undertaken for the correction of intestinal malrotation and duodenal stenosis (Ladd syndrome). Neonatal brain US, ophthalmic fundus, radiographic and electrocardiogram evaluation were normal, as well as biotinidase activity, lactate and ammonia levels, chromosome analysis, and immunological investigations. Plasma and urinary amino acids, organic acids, purines and pyrimidines, sugars, oligo- and mucopolysaccharides did not reveal any pathological changes.

At age 4 months, the brain showed partial agenesis of the corpus callosum, and abnormal EEG delta waves above its posterior quadrant. Prolonged activated partial thromboplastin time (APTT) and thromboplastin time (PT) were found in addition to anaemia and elevated transaminases; AT III was within the physiological range.

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 disialo-Tf and slightly increased asialo-Tf (Table 11). Abnormalities of α_1-AT and TBG proved a generalized defect of N-glycosylation (Fig. 22). 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 leucocytes 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 (Fig. 23) and normal expression of sialyl Lewis X antigen in neutrophils (Fig. 24) excluded CDG IIb, and both types IIc and IIf, respectively. The patient was classified as having an unidentified form of CDG type II.

The finding of a hyposialylation IEF pattern of apo C-III with increased apo C-III$_0$, normal apo C-III$_1$ and decreased apo C-III$_2$ 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.
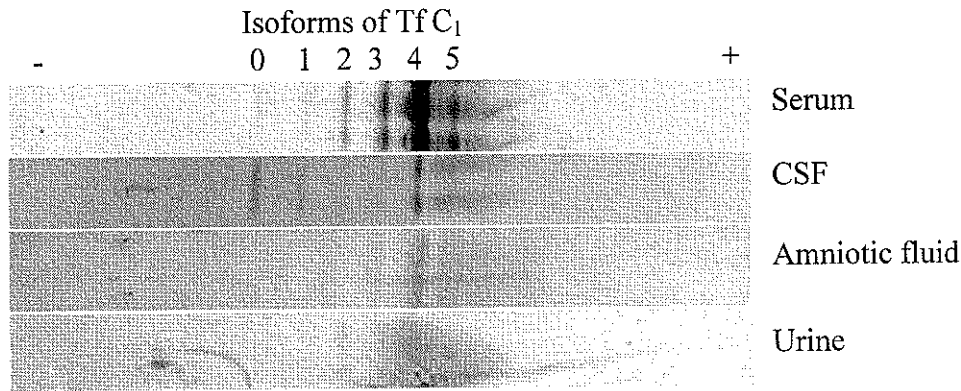
Fig. 4. IEF analysis of Tf isoforms in different types of materials of healthy individuals

Serum: 5times diluted, 1.5 μL applied. CSF:10times concentrated, 3 μL; note the lower tetrasialo- and higher asialo- and monosialo-Tf, compared to serum. Amniotic fluid: 30times concentrated, 3 μL. Urine: 5times concentrated, 20 μL.

0=asialo-, 1=monosialo-, 2=disialo-, 3=trisialo-, 4=tetrasialo-, 5=pentasialo-, 6=hexasialo-, 7=septasialo-, 8=octasialo-Tf, or α_1-AT.
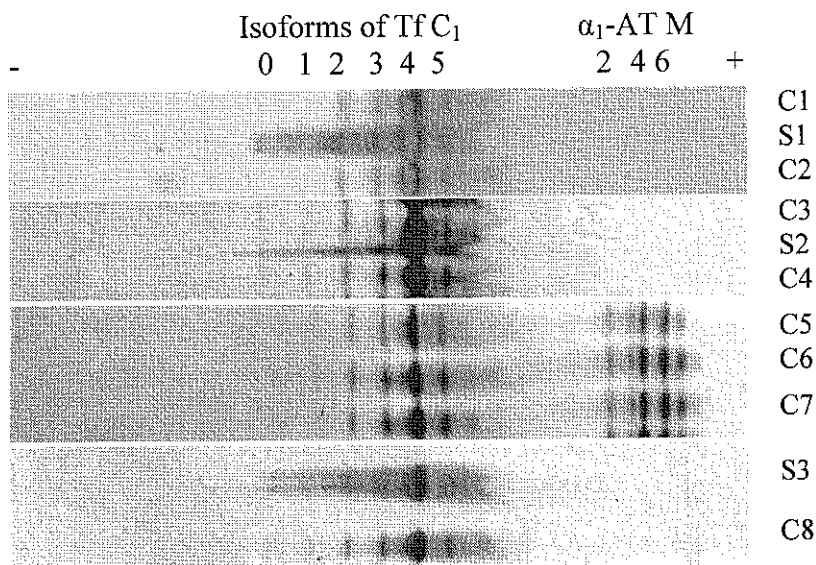

Fig. 5. Effect of preanalytical conditions of Tf-isoforms IEF analysis on Immobiline. DryPlate gel pH 4-7; serum samples saturated, 5times diluted, and 1.5 μL applied on the gel

Lanes C1 — C4, C6, C8 and C11: serum saturated with Fe-citrate. Lanes C5-C7: serum saturated with various Fe^{3+}-reagents; FeNTA, Fe-chloride, and Fe-citrate, respectively. Lane S1: native serum without saturation (note tailing of the sample with appearance of other cathodal bands on the dark background). Lane S2: haemolytic serum sample. Lane S3: saturated and delipidated serum, prepared for HPLC analysis; see the tailing.
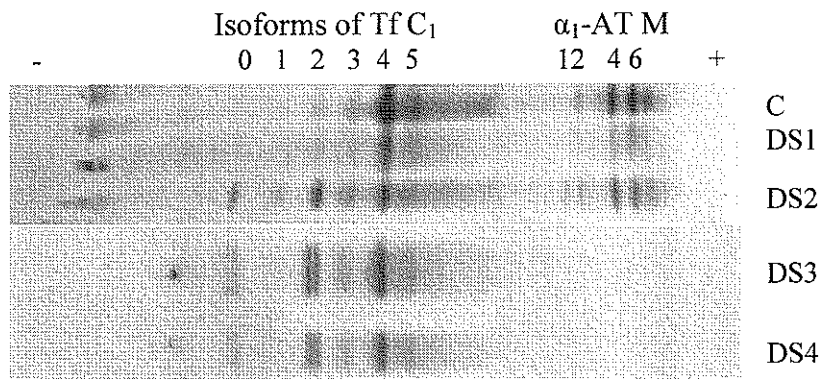
Fig. 6A. IEF of Tf and α_1-AT isoforms from dry spot (DS) samples

Lane C: serum sample (1.5 µL), healthy control. Lane DS1: serum dry spot (5 µL), healthy control. Lanes DS2 and DS4: serum dry spot (5 µL), CDG Ia patients; see the prominent increase of disialo- and asialo- Tf, note also increased monosialo-α_1-AT compared with the control. Lane DS3: serum dry spot (10 µL), CDG Ia patient.
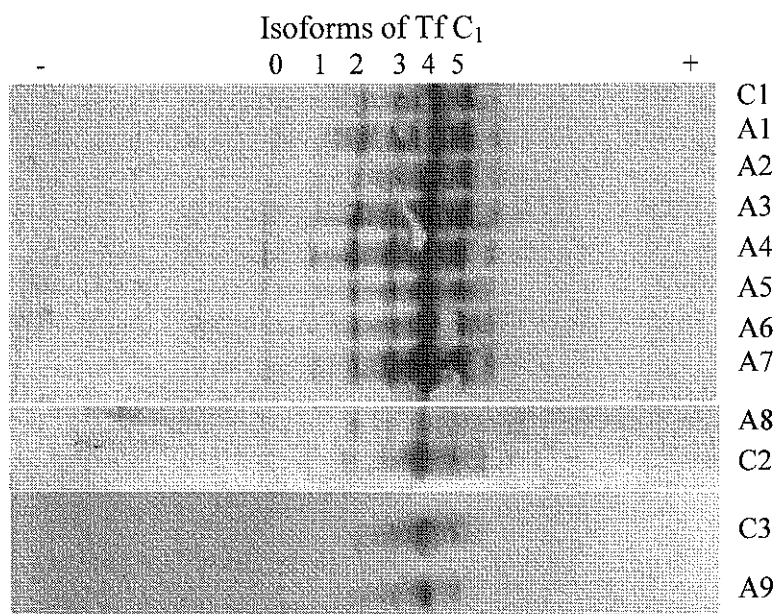
Fig. 6B. Effect of alcohol abusing (A) on Tf isoforms analysis

Lanes C1 – C3: controls. Lanes A1 – A7: patiens with acute alcohol intoxication; see the increase of asialo- and monosialo-Tf in comparison with controls. Lane A8: chronic alcoholic patient, dry serum spot; see the decrease of tetrasialo-Tf and increase of disialo- and asialo-Tf in comparison with control. Lanes C3 and A9: a volunteer before and after alcohol intake (70 mg per day for one week); no increase in CDT was noted.
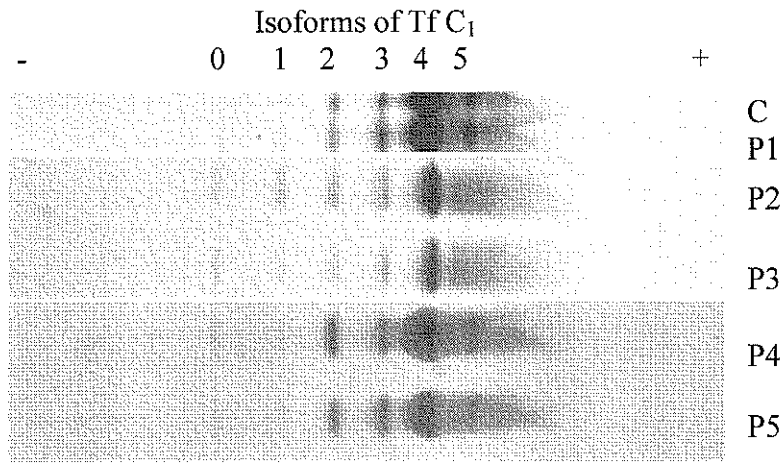
Fig. 6C. Secondary increase of CDT under various conditions

Lane C: control. Lane P1: Hashimoto disease. Lanes P2 and P3: newborns. Lane P4: hepatitis C. Lane P5: cystic fibrosis. Note the increase of monosialo- and asialo-Tf in samples P1 – P5 compared to control.
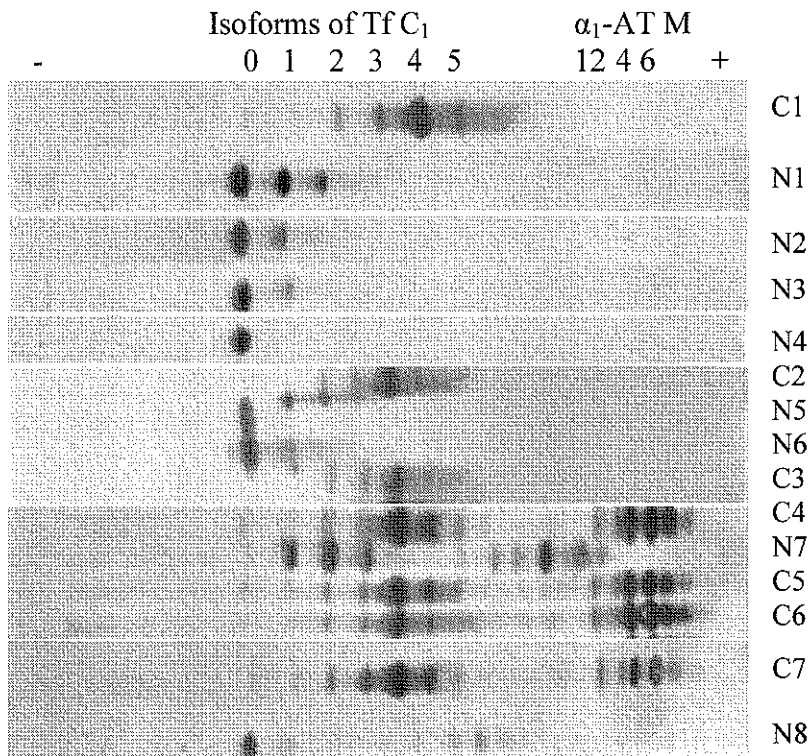
Fig. 7. Effect of serum Tf and α_1-AT digestion with neuraminidase (N)

Lanes C1 – C7: samples without neuraminidase. Lanes N1 – N4: control serum sample incubated at 37 °C for 24 hours with various amounts of neuraminidase (10, 15, 20, and 40 μL, respectively). Lanes N5 and N6: effect of contamination with neuraminidase during IEF run; note a monosialo-Tf in native samples, adjacent to samples pre-treated with neuraminidase. Lanes C4 – C6, N7 and N8: isoforms of Tf and α_1-AT. Lane N7: effect of contamination with neuraminidase during sample preparation. Lane N8: optimal concentration of neuraminidase (40 μL) for combined Tf and α_1-AT analysis.

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Isoforms of Tf C_1
0 1 2 3 4 5

Isoforms of Tf C_1
0 1 2 3 4 5

Isoforms of Tf C_1
0 1 2 3 4 5
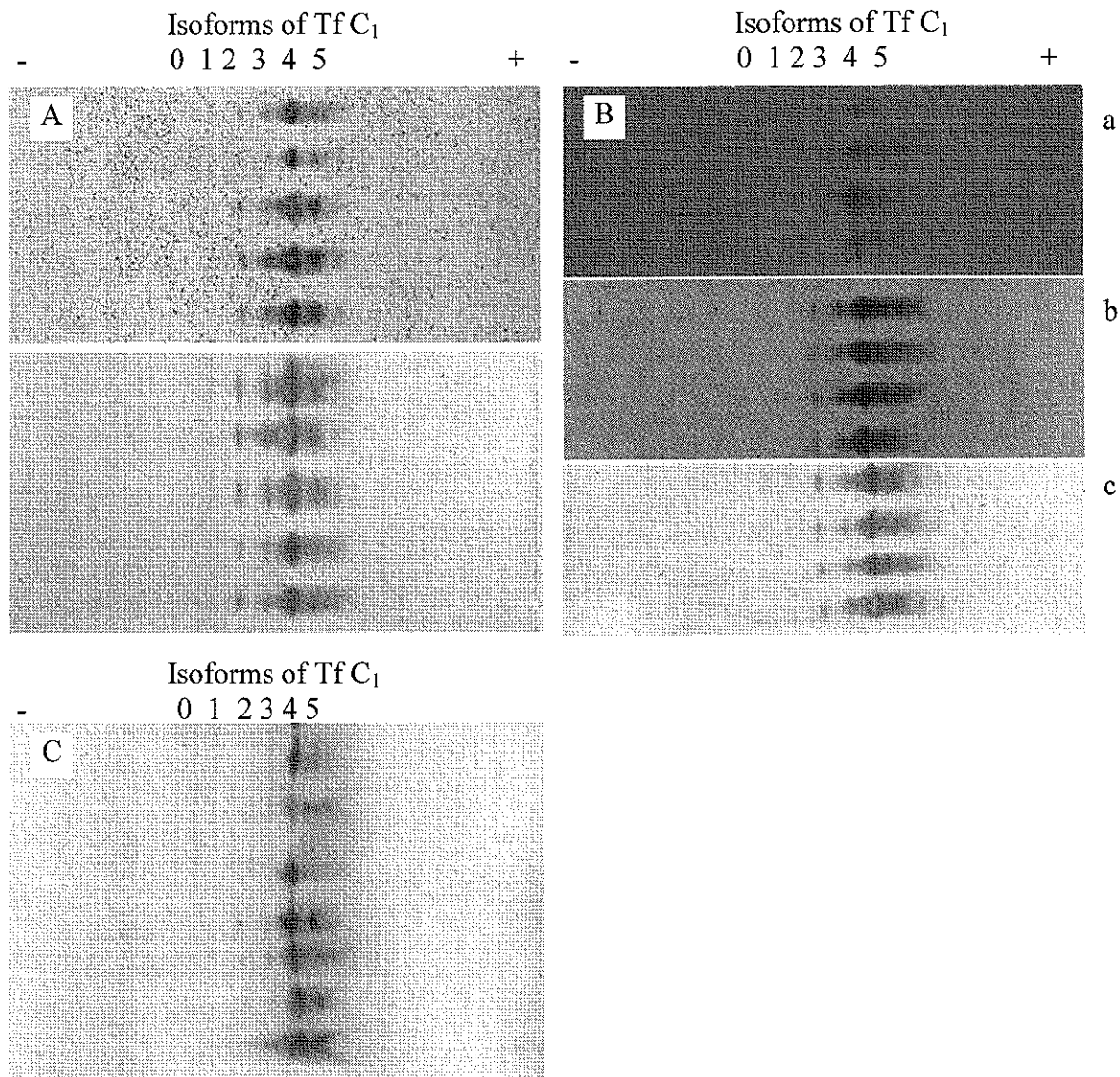
Fig. 8. Some technical problems of analysis of Tf isoforms by IEF

A) Effect of the staining-solution filtration on the gel background: a) before, and b) after filtration.

B) Time-effect of the gel destaining; a) 30 min, b) 1 hour, c) 2 hours.

C) Effect of gel overdrying; note the scattered and blurred pattern of Tf isoforms in control serum.
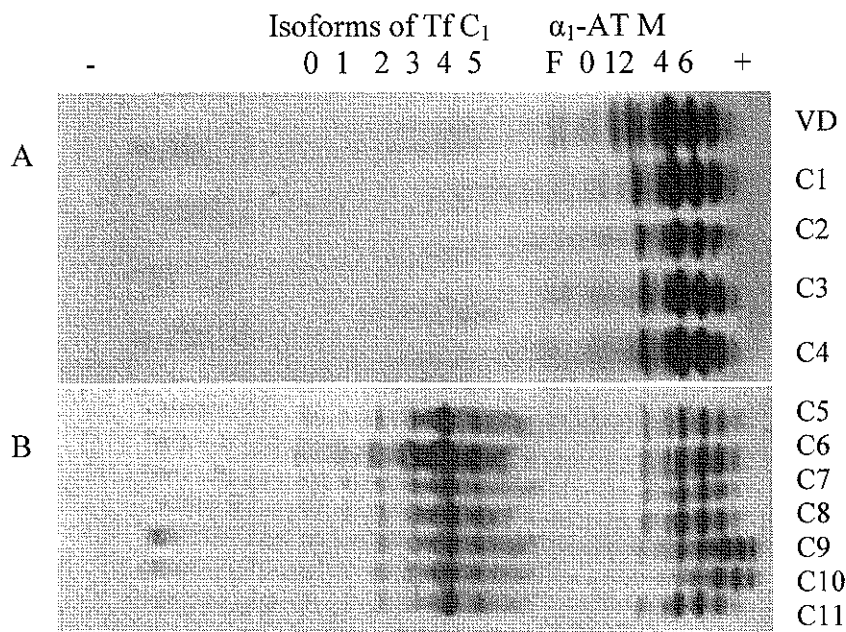
Fig. 9. IEF of α_1-AT and Tf in CDG screening

A) Analysis of α_1-AT in native serum (non-saturated, non-diluted), 1.5 µL applied.
B) Combined analysis of α_1-AT and Tf in one run; 1.5 µL of 5times diluted serum.
Lane VD: our CDG patient (increased monosialo- α_1-AT, F: fucosylated form of α_1-AT.
Lanes C1 - C11: controls.
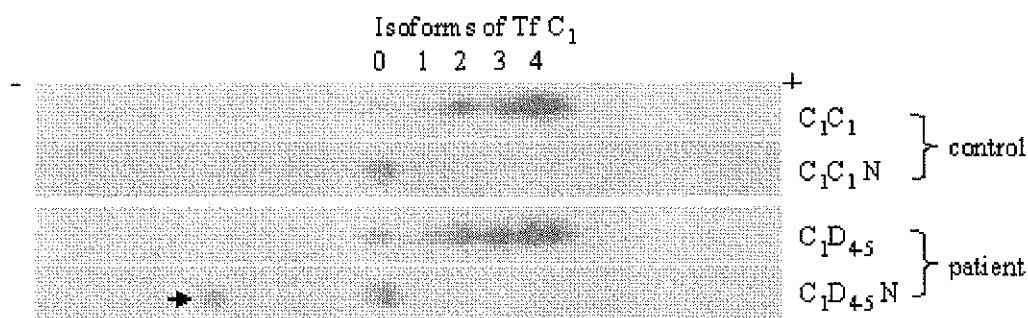
Fig. 11. Serum Tf variants by IEF on the Ampholine PAGplate gel of pH range 3.5 – 9.5
in the patient (C_1D_{4-5}) and control (C_1C_1) before and after digestion by neuraminidase (N);
two asialo-Tf bands reflect Tf C and Tf D alleles; arrow indicates asialo-Tf D_{4-5}.

Isoforms of Tf C_1
0 1 2 3 4 5

C_1C_1
C_1C_2

C_1C_1 ⎱ CDG
C_1C_2 ⎰

C_1C_1N ⎱ both controls
C_1C_2N ⎰ and CDG

C_1C_1 ⎱
C_2C_2 ⎬ newborns
C_1C_1 ⎰

C_1C_1N ⎱
C_2C_2N ⎬ newborns
C_1C_1N ⎰

C_1C_3

C_1C_3N

C_1B_{1-2}

$C_1B_{1-2}N$

C_1D_2

C_1D_2N

C_1D_{4-5} boy

$C_1D_{4-5}N$

C_1C_1 mother

C_1C_1N

C_1D_{4-5} father

$C_1D_{4-5}N$
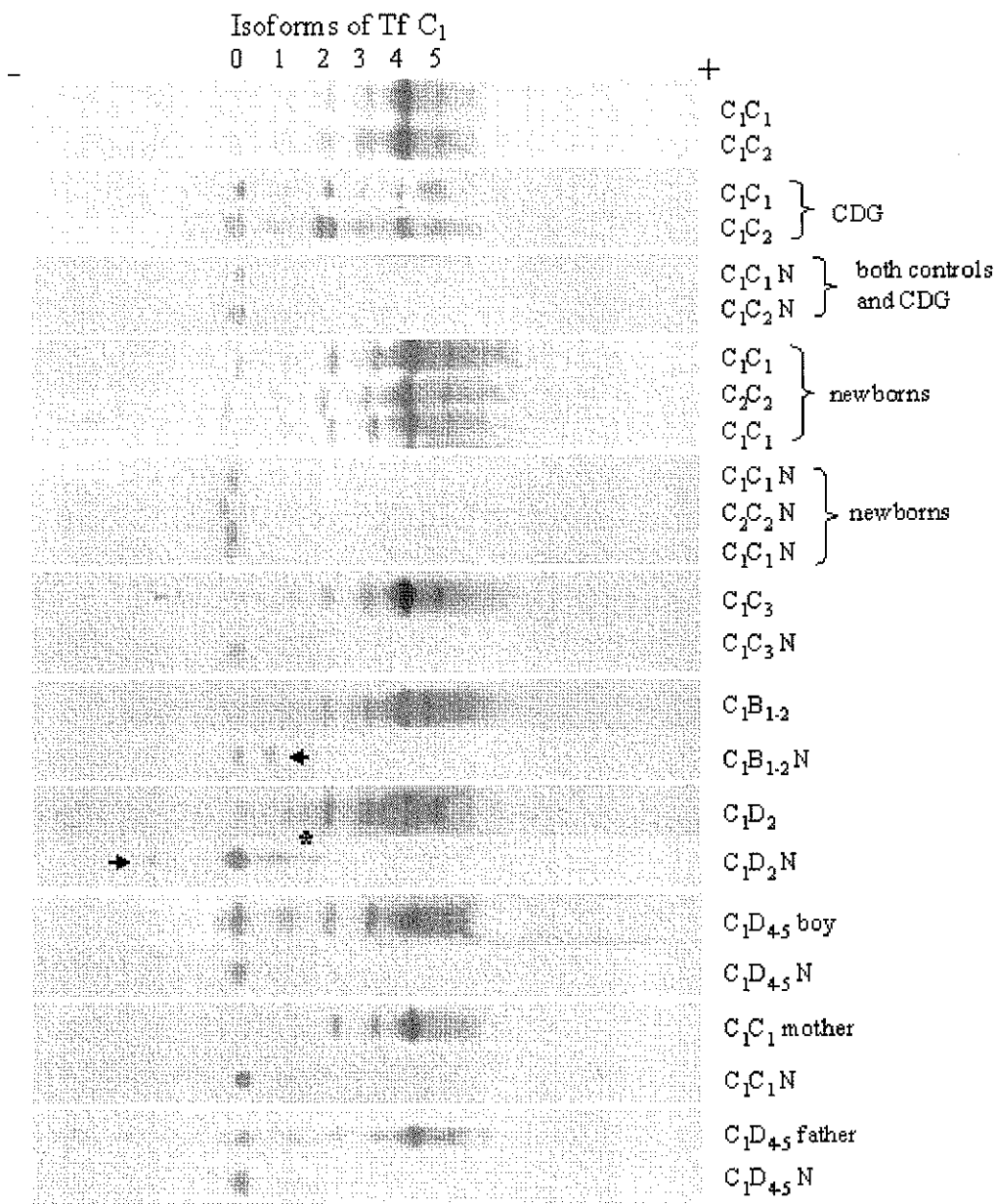
Fig. 10. Tf variants by IEF on the Immobiline DryPlate gel of pH range 4 – 7 in controls (non-CDG), newborns and CDG patients (higher CDT); effect of treatment with neuraminidase (N); all digested samples were diluted 10times. Asterisk marks tetrasialo-Tf D_2, arrows indicate Tf B_{1-2} and Tf-D_2 asialo-isoforms.

60
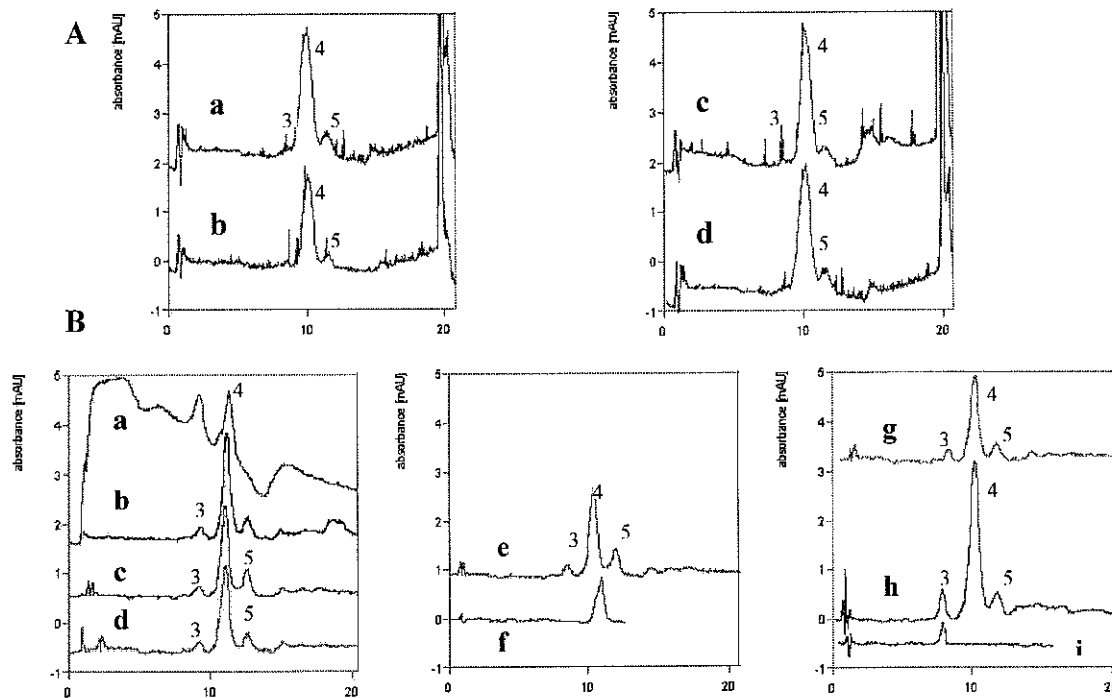
Fig. 12. HPLC separation of Tf isoforms on the ECOM system in controls under various conditions of preanalysis

A) Results on MonoQ column with elution gradient profile I (Jeppsson JO 1993): The same serum sample Fe_3-citrate saturated (a) at $22^{\circ}C$, (b) at $37^{\circ}C$, both delipidated. The same serum sample, Fe-citrate saturated at room temperature (c) nondelipidated, and (d) delipidated. B) Results on ResourceQ column with gradient profile IV (Turpeinen U, 2004; personal communication): (a) Non-saturated serum compared to serum saturated with (b) Fe-chloride, (c) Fe-citrate, and (d) FeNTA; (e) serum saturated with FeNTA solution, compared to (f) FeNTA standard, showing a peak closed to tetrasialo-Tf; (g) Fe-citrate saturated serum sample; (h) Fe-citrate saturated EDTA-plasma, and (i) EDTA standard, interfering with trisialo-Tf; 3 = trisialo-, 4 = tetrasialo-, 5 = pentasialo-Tf.

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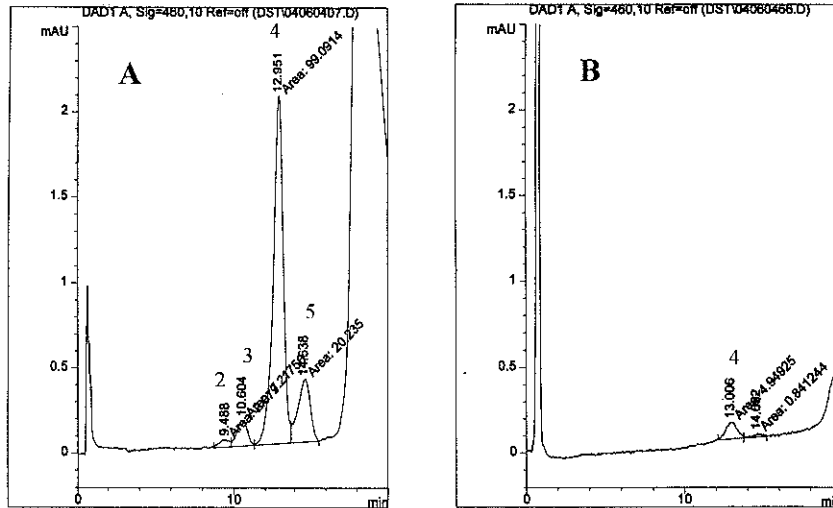
Fig.13. HPLC profiles of Tf isoforms eluted from dry spot (control serum) on Agilent 1100 system and ResourceQ column with gradient profile IV (Turpeinen U, 2004; personal communication), (A) serum, (B) dry spot
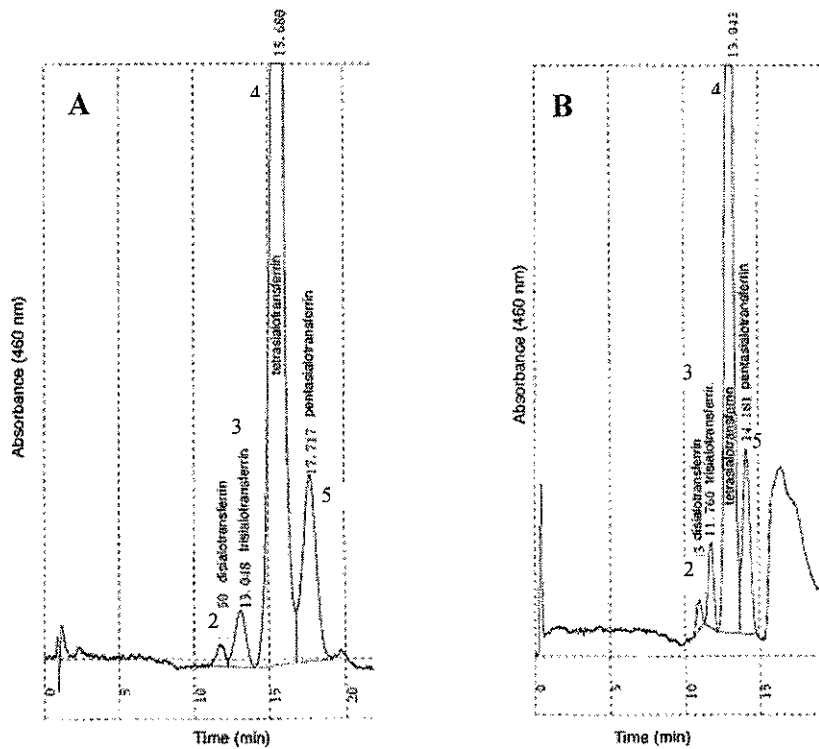
Fig. 14. Comparison of MonoQ and ResourceQ columns

Analysis of serum Tf on Agilent 1100 chromatograph with gradient profile III (Turpeinen U, 2004) on the (A) MonoQ, and (B) ResourceQ columns.
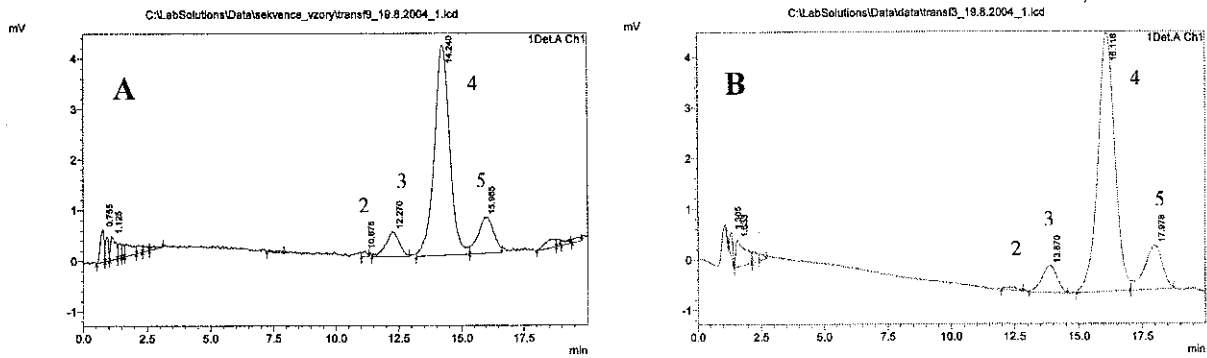
Fig. 15. Effect of the flow rate on Tf isoforms separation on Shimadzu system with ResourceQ column, gradient profile IV (Turpeinen U, 2004; personal communication): (A) 1 ml/min, (B) 0.7 ml/min; prolonged time of the analysis, but without any effect on separation.
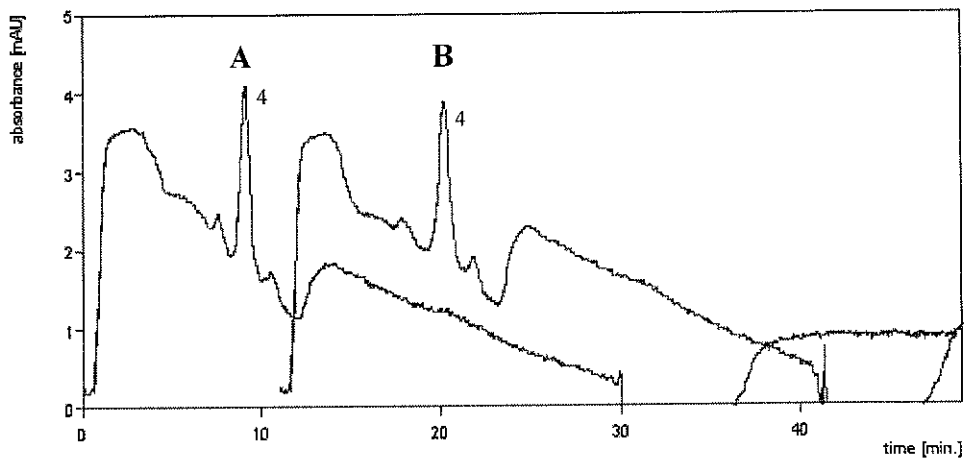
Fig.16. Comparison of two wavelengths used for the iron-Tf complex detection (on the ECOM system with multiple wavelength detector, baseline integration, on ResourceQ column and gradient profile IV); A) 460 nm, (B) 470 nm
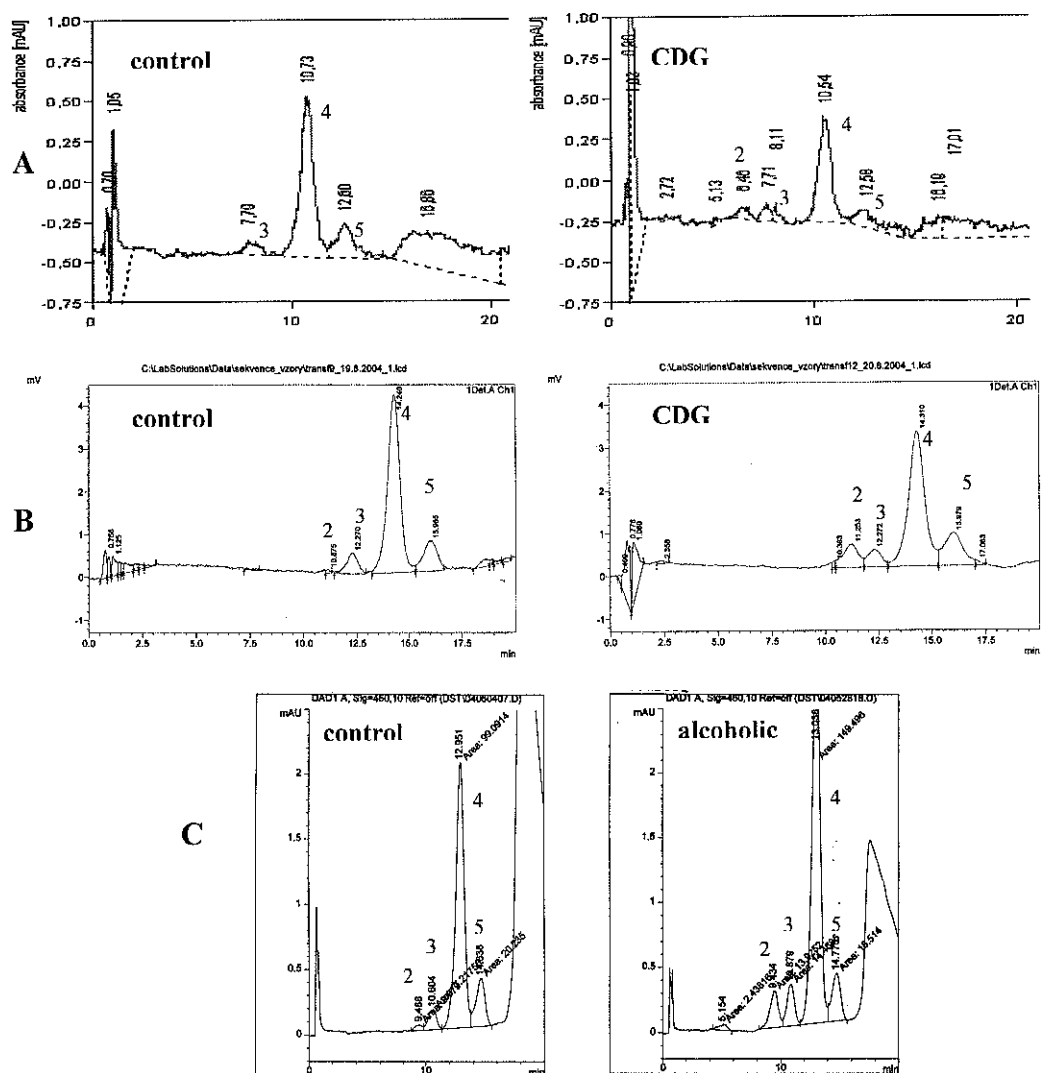
Fig. 17. Distribution of serum Tf glycoforms analysed by various HPLC systems on ResourceQ column with gradient program IV with the (A) ECOM, (B) Shimadzu, and c) Agilent 1100 chromatographs (Turpeinen U, 2004; personal communication). Three peaks corresponding to tetra-, penta-, and trisialo-Tf in controls with additional peak of disialo-Tf in the alcoholic and the CDG patient can be found by all systems; the disialo-isoform can also be detected in controls, but not when using the ECOM chromatograph; (mV corresponds to mAU).
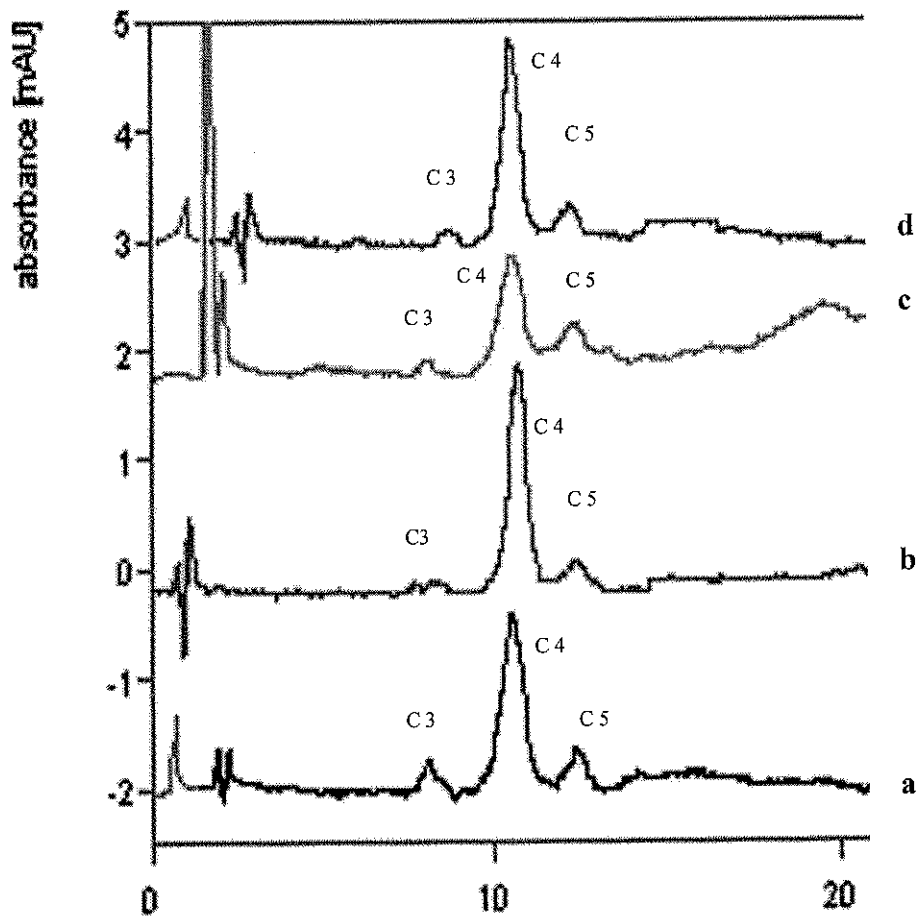
Fig. 18. HPLC analysis of Tf in serum samples with increased CDT (found by IEF) with the ECOM system on ResourceQ column and gradient profile IV; (a) control, (b) newborn, (c) patient treated with antiepileptics, and (d) child with cystic fibrosis.
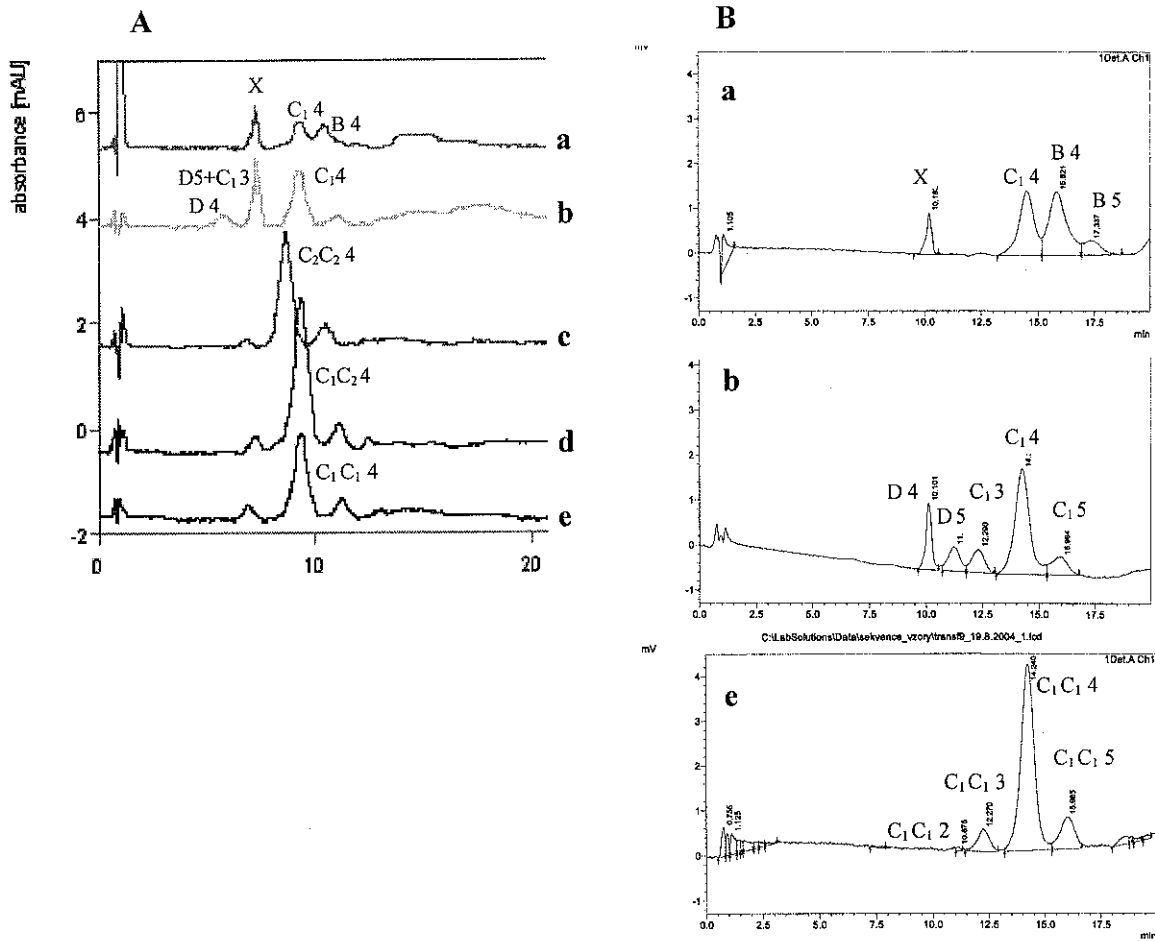
Fig. 19. Genetic variants of Tf on the ResourceQ column and gradient profile IV with the A) ECOM, and B) Shimadzu system; (a) Tf C_1B (plasma, X - unknown), (b) Tf C_1D; in addition to main peak of tetrasialo-Tf C_1C_1 note another peak, reflecting the second Tf D allele, (c) Tf C_2C_2, (d) Tf C_1C_2, (e) Tf C_1C_1; note shifted retention times of all isoforms in Tf C_2C_2, while heterozygous Tf C_1C_2 cannot be differentiated.
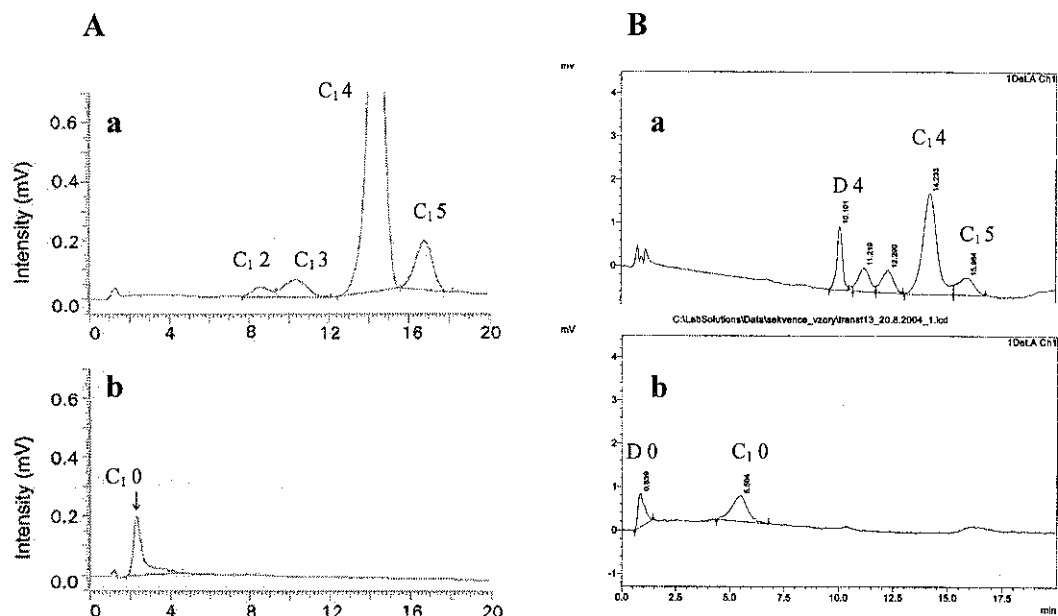
Fig. 20. Effect of sample treatment with neuraminidase on Tf isoforms

(A) Merck L4250 system, MonoQ column with gradient profile II, Tf C_1 genetic variant reported by Renner [17], and (B) Shimadzu system on ResourceQ column with gradient profile IV, Tf C_1D genetic variant; (a) before, and (b) after digestion with neuraminidase.
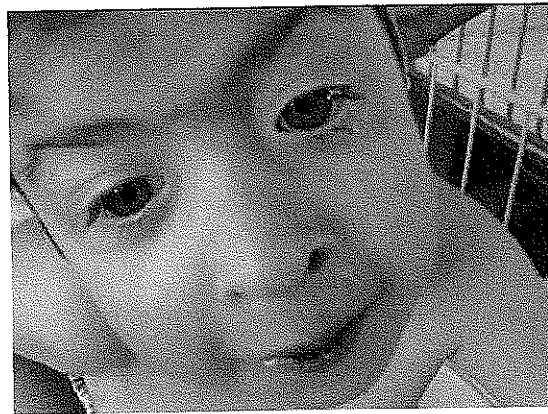
Fig. 21. Clinical features of CDG patient (facial dysmorphy, dolichocephaly. low hairline, high nasal bridge, blepharophimosis, kyphosis and scoliosis)
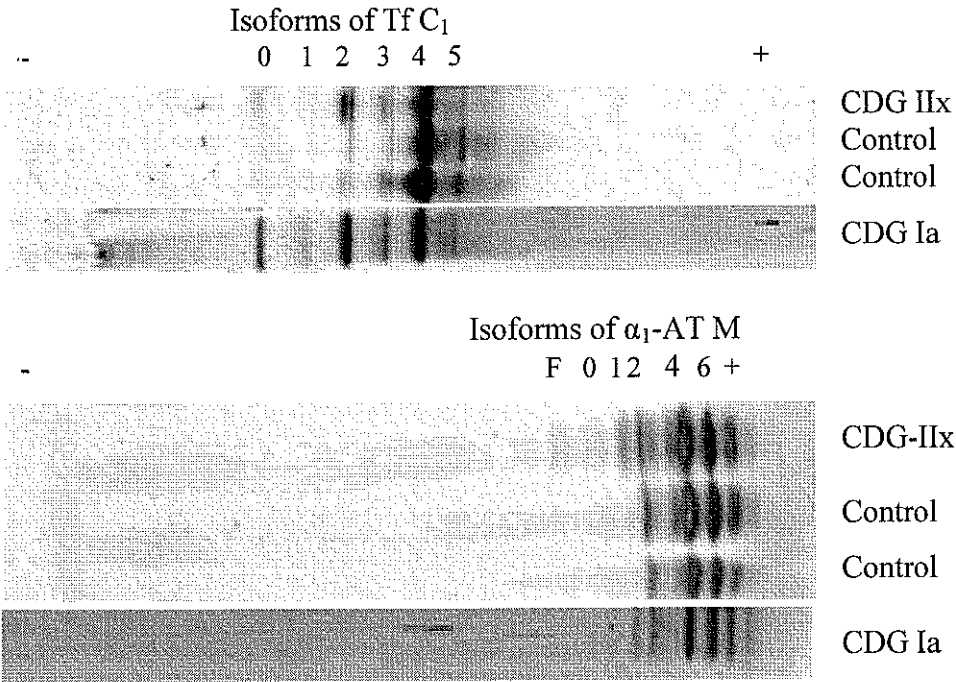
Fig. 22. IEF of serum Tf and α_1-AT on Immuobiline DryPlate gel with pH range of 4-7 See an increase of monosialo-α_1-AT, and disialo-Tf with asialo-Tf in our CDG patient (CDG-IIx), compared to controls. F= fucosylated form of α_1-AT, 0=asialo-, 1=monosialo-, 2=disialo-, 3=trisialo-, 4=tetrasialo-, 5=pentasialo-, 6=hexasialo-, 7=septasialo-, 8=octasialo-Tf, or α_1-AT.
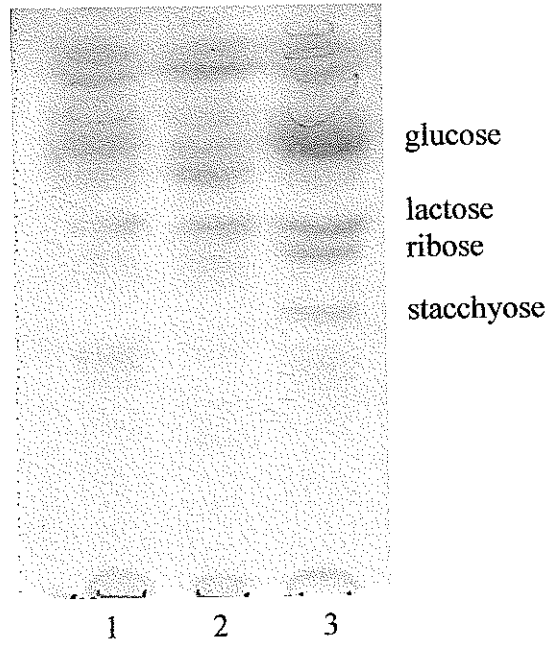
Fig. 23. TLC of oligosaccharides in urine on Kieselgel 60 F_{254} plate (0.2 mm; Merck), developed by n-butanol - acetic acid – water, 2:1:1. Bands were visualized by spraying with a solution of orcinol/sulfuric acid and heating for 5 min at 130°C (De Praeter DM 2002). Lane 1: control. Lane 2: our CDG patient. Lane 3: standards (mono, di-, tri-, and tetrasaccharide).
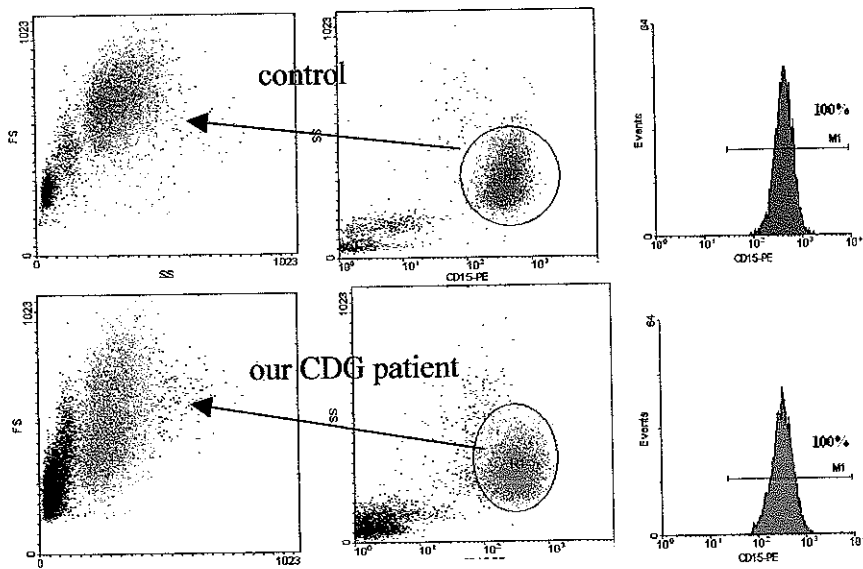
Fig. 24. Sialyl-Lewis X antigen on granulocytes followed by flow cytometry: expression of the antigen in a control and the patient was similar.

5 Discussion

5.1 Preanalysis

5.1.1 Material

Tf is present in different biological fluids with various concentrations.

Amniotic fluid can be source of Tf and may be useful in the prenatal diagnosis. Using a sample of 30times concentrated amniotic fluid; well-separated Tf isoforms could be detected by IEF in controls. It was thus supposed that the prenatal diagnosis of CDG would be possible by the analysis of foetal blood or amniotic fluid (Stibler H 1991). However, Clayton et al (Clayton P 1993) was not able to find any abnormalities of Tf or α_1-AT before the 2-3 weeks after birth. Stibler et al. (1994) examined a twin pregnancy in a family with a previously affected girl. On the 17 week of gestation, Tf and α_1-AT were found to be normal compared with age-matched controls. However, at birth the twins showed signs of the disease, further confirmed by the relevant tests on the serum. The prenatal diagnostic failure may be due to maternal metabolism, compensating for the defect in the foetus. The total Tf/ CDT ratio in amniotic fluid might possibly have a diagnostic potential. The problem of maternal contribution may be elucidated by further studies of foetal secretory glycoproteins (Stibler H 1991).

In addition to serum, also CSF is a suitable material for this method since it contains all Tf glycoforms. In this study, well-separated Tf isoforms and physiologically higher CDT (compared to serum) could be detected in controls. Increased CDT levels in CSF were found in CDG Ia patients in comparison to controls (Rauch SD 2000), unfortunately, CSF was not available in our CDG patient; moreover, there were no reports on validity of this material in other CDG types.

Desialylated Tf (ß -2Tf or Tau protein) is an isoform (asialo-Tf), physiologically not present in blood, nasal mucus, tears or mucosal discharge. It is produced by neuraminidase activity in the brain and is usually detectable only in CSF, perilymph, and aqueous humour. Thus, analysis of Tf isoforms may be used also in the diagnostics of CSF leakage due to unhealed fistula (Blennow K 1995, Roelandse FW1998).

ß-Trace glycoprotein is another N- glycosylated protein, synthesized mainly in the epithelial cells of the choroid plexus, and is found in CSF in concentrations ~35times higher than in plasma; it thus represents a potentially useful marker for N- glycosylation defects in the nervous system (Pohl S 1997, Grünewald S 1999).

The urine is an easily obtained material, widely used in the screening of IMD; only small volume is necessary for detection of Tf genetic polymorphism by IEF (Kishi

K 1990). Sample desalting, or more sensitive detection could possibly avoid our failure in analysis of this material; however, it makes the procedure rather complicated.

Abnormal Tf also accumulates in other tissues, mainly the liver: delipidated homogenate of liver biopsy has been successfully analysed in the CDG diagnostics (Stibler H 1991).

Dry spots are used in the investigation of some common metabolic disease. This 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 newborn samples. IEF thus provide a means for early diagnosis of the CDG in microliter volumes of capillary blood (Stibler H 1991).

Also the use of dry spot samples was adopted in our laboratory, and various conditions were tested: samples of three CDG patients showed distinct abnormalities of both Tf and α_1-AT. The results of analysis in our CDG patient were similar to those obtained from serum samples.

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.

5.1.2 Saturation

Tf can bind a variety of metal ions (e.g. Fe, Al, and Cu) with variable affinity in the presence of bicarbonate, the naturally occurring synergistic anion, and as it is the one that forms by far the strongest metal complexes with Tf, it was used in saturation. Iron binding to Tf is very sensitive to pH changes (release of iron from Tf starts at pH<6.3), thus acidic pH should be avoided. (Van Campenhout A 2004). The pI of the Tf molecule decreases by ~0.2 pH units with each Fe^{3+} ion bound. Native serum Tf is only partly saturated (30%); in fact, not even the N- and C-terminal binding sites are equivalent in terms of iron uptake and release. Therefore a thorough „in vitro" saturation is essential for reproducibility of results (Arndt T 2001). Since the iron-saturation step is evidently a critical point (namely of the HPLC analysis, based on the Fe^{3+}-Tf complex specific detection at 460 nm) it was thoroughly studied.

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 the citrate plasma presents an additional peak on HPLC, partially

co-eluting with the tetrasialo-Tf (Helander A 2003). We noted higher CDT values in a small number of plasma-heparin samples, analysed by IEF.

When we tested native samples, small and not well-separated peaks appeared on HPLC, and similarly a tailing of Tf isoforms bands was noted on IEF.

We were further looking for optimal working conditions including various iron-reagents, temperature and time of saturation: Of the three different iron solutions tested, no significant differences by IEF were found in our experiments. In HPLC analysis, the $FeCl_3$ resulted in the highest peaks of Tf-isoforms, when compared with the Fe-citrate and the FeNTA reagent, 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).

5.1.3 Delipidation

Since the lipoproteins and the other serum proteins may precipitate at pH 6.2 during the HPLC analysis, thus causing a gradual rise of pressure in the system, serum delipidation prevents this problem and prolongs column lifetime. However, delipidation reduces the serum CDT concentration by 22% (Arndt T 2001). We compared results of parallel HPLC analysis of one serum sample with and without delipidation; no prominent differences were observed.

Delipidated samples were also analysed by IEF with the view of testing it's possible suitability for both methods; tailing of the Tf bands was observed, possibly due to the dextran sulphate additive, which can bind to proteins thus changing both their pI and molecular weight.

5.1.4 Neuraminidase

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 (Helander A 2001, Henry H 1999); after digestion of sialic acid residues, all homozygote Tf variants present with just one peak/band at the site of 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.

Increasing the neuraminidase concentration and prolonging the incubation time leads to a more efficient removal of sialic acid (Fermo I 2004, Van Campenhout A 2004, Rauch SD 2000). In our hands, incomplete digestion of Tf by neuraminidase showed itself by an increased number of Tf isoforms on IEF gel, while complete desialization of homozygous Tf was presented with only one band (asialo-Tf). It has been achieved with an enzyme concentration 8 μg / 1 μL serum Tf, incubated at 37 °C for 24 hours.

Improperly digested Tf may be attributable to other reasons: 1) the pH optimum for the enzyme is 5.8–6.0, while the loading of Fe_3 is performed at physiologic conditions (pH 7.4). 2) Fe_3, as well as other heavy metals (and oxygen), inhibit the enzymatic activity of neuraminidase.

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. This system has a multi-step programming capability allows gels to be prerun, loaded, and run; controlled parameters include temperature, voltage, current, and power. It allows efficient and uniform cooling of ultra thin gels, and thus it improves the resolution at high voltages. The PhastSystem automates both the running and staining steps on mini-gels.

Both polyacrylamide and agarose gels can be used for IEF (Hackler R 2000, Petrén S 1989). The cross-linked polymer of acrylamide is significantly more annoying to prepare than agarose gels, since it is a potent neurotoxin and should be handled with care. In comparison to agarose, polyacrylamide gels have a rather small range of separation, but very high resolving power. In the present study, as in most of the reported references on IEF of Tf, polyacrylamide gels were used.

Apart from hydrated gels ready to use, Immobiline Dry gels are available; they require rehydration with 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. Farther, blank gels can be used and adapted to any pH-range. One of

their advantages is the choice of different additives in the reswelling solution, and rapid equilibration in the ampholyte mixture. These gels should be used after rehydration.

The samples are conveniently applied by use of a silicon applicator strip, which is available for 52 samples. When a confirmation of results was necessary, or when analysing digested sample, a reduced number of samples (26) is preferred to avoid contamination. When larger volumes are applied, direct pipetting on the gel surface or on pieces of cellulose paper is possible. When just a part of gel was used, the applicator was laid out edges of the gel, or shorted to required length. Thorough cleaning of used application strip by 0.1 M NaOH is highly recommended after each analysis for prevention of streaking in the next run. The origin of the occasionally occurring obscure defects (white spots) disturbing the evaluation of the stained gel fails to be explained.

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) and thus a blotting technique is preferred. Immunoblotting is also useful when the antigen of interest is insoluble or readily degraded and cannot be easily immunoprecipitated. 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).

Purified rabbit anti human Tf and α_1-AT antibodies provided in liquid form are used in concentration 0.3 μL of each per cm^2 of gel area of each Tf and α_1-AT were used, when a cocktail of antibodies was used, no cross reaction between anti-Tf and anti α_1-AT was noted.

We have found that recommended overnight gel washing by shaking was not necessary, and was thus omitted. The gel was left standing for 12 hours in saline solution without shaking; no substantial difference in the background quality of the gel was noted.

Coomassie blue staining is quantitative, relatively simple and convenient procedure. It is sensitive for a range of 0.5 to 20 μg of protein; thus, 1.5 μl of sample diluted 5times containing about 0.6 μg of Tf and α_1-AT was applied on the gel. A 50-fold more sensitive silver staining is essential for detection of low levels of glycoproteins (Hackler R 2000, Roelandse FW 1998) and may be used in the case of CSF, or urine, in place of sample concentration.

The length of time for staining ranged from 8-12 minutes. Irregularities may be prevented by agitation when staining. The destaining step is essentially limited by the rate of diffusion of the coomassie molecules from the gel matrix into the surrounding solution; it was monitored visually.

Evaluation of gels was made after densitometry and data digitalisation. In general, for bands identification it is recommended to use marker proteins of known isoelectric points, which can focus independently on the point of application. Isoforms of Tf and α_1-AT in our methods were identified on the basis of their position, derived from the pH range of gel, and by comparison of the obtained results with data from literatures (Gorge A 1983, Kuhle P 1979, Weidinger S 1984).

5.3 HPLC

In our laboratory we introduced 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, Source15Q (Amersham Bioscience), and columns high-flow-packed with Poros HQ10 (Perseptive BioSystems) (Bean P 1997).

The MonoQ HR 5/5 is a high-resolution pre-packed column. The bed volume is 1 ml of resin and it can hold up to 20-50 mg of protein, although for optimal resolution only 5 mg should be loaded. It works at pressures less than 4 MPa; such a requirement might present a great challenge. The most serious limitation for MonoQ column is that to obtain its potential high resolving power, the usage conditions must be carefully worked out; that usually means a lot of empirical work to determine the "ideal" running conditions with each system. MonoQ column gets clogged surprisingly easily and requires constant cleaning and maintenance for optimal performance. As it gets dirtier (and this is almost unavoidable over time), the column will retain a larger proportion of protein due to non-specific adsorption (up to 50% of protein at the end of its life). The accumulation of strongly retained material on an HPLC column can reduce its lifetime. By modifying the packing surface, these retained materials can cause shifts in peak retention, loss of resolution and efficiency, as well as degradation of peak shape. Inline filters, changed after series of about 70 analyses protect and elongate the life of column. However, Jeppsson reported that over 1000 analyses were performed on MonoQ with no serious problems

(Jeppsson JO 1993; similarly Turpeinen (Turpeinen U 2001) used one ResourceQ column for about 1000 runs. Farther problem with the MonoQ column is its high price. Therefore, for routine analysis the ResourceQ column was used as more economic, giving similar results to those observed on MonoQ (Turpeinen U 2001).

Various types of the Bis-Tris-NaCl buffer gradient were compared (Tables 4). To conclude, for each HPLC system the appropriate gradient profile should be adapted to achieve an optimal separation of Tf isoforms. Demand for perfect purity of mobile phases presents a challenge in this application. Both buffers A and B are stable at least for three days at room temperature (Turpeinen U 2001), and for one month at 4°C with the bacteriostatic MIT additive (Renner F 1997). The pH range of 6.2 - 7.0 was used for buffers in Tf analysis, as the acidic pH leads to release of iron from Tf molecule; therefore the pH control of samples and mobile phases is thus advisable.

High detector sensitivity is essential for analysis of Tf isoforms, because of low concentration of CDT glycoforms in serum and low molar absorbency, despite the high specificity of the iron-Tf complex at 460 nm. For illustration, the tetrasialo-Tf peak height on Agilent 1100 in controls is about 2-3 mV, and those of disialo-, mono- and asialo-Tf reach on the maximum 0.05 mV only.

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 (Jeppsson O 1993), LKB 2141 (Bean P 1997), Merck L4250 (Renner F 1997), Agilent 1100 (Helander A 2003, Turpeinen U, personal communication), as well as some other Agilent types (D'Ottavi A 1997, Simonsson P 1996), 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.

The working pressure with both the ECOM and the Shimadzu systems was about 1.4 MPa due to contribution of the individual parts: pre-column capillary 0.3 MPa, pre-column filter 0.1-0.2 MPa, column 0.6 MPa, and post column capillary 0.3 MPa. The low upper limit recommended for the columns (4 MPa for MonoQ and only 1.5 MPa for ResourceQ) led to occasional pump stops thus greatly complicating analyses on the ECOM HPLC system.

It is generally assumed that the HPLC analysis of Tf provides reproducible separation and relative quantification (Helander A 2003). Only a few samples were tested on Shimadzu system. The physiological disialo- Tf was not detected by ECOM system in control samples; therefore, the peak areas of tetra-Tf alone were calculated with a result of 20.6, and 20.4 respectively. The within-day and between-day CV values for peak areas of disialo- Tf were reported to be < 5% (Helander A 2003).

Some drawbacks connected with supposedly simple HPLC procedure of Tf glycoforms are commented. In general, they are based on the 1) low molar absorbency despite high specificity of the iron-Tf complex at 460 nm, 2) low concentration of CDT glycoforms in serum, 3) claims of good performance of spectrophotometric detector at 460 nm, and 4) low pressure limit for ion-exchange HPLC columns. The other inconveniences, such as 5) long time of analysis with gradient elution, and 6) high expanses of HPLC column may be compensated by the automation possibility, and by a choice of comparatively good columns: the more economic ResourceQ, and the other for twice the price, MonoQ. Lack of 7) a clearly defined CDT analyte group thus complicating analysis as well as the pre- and post-analytical steps was another drawback; now at last standard Tf-glycoforms are commercially available (IBR, Matzingen, Germany).

5.4 Enzyme essay

CDG type Ia forms about 85% of the all CDG subtypes so far known. PMM and PMI 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. In some patients, high PMM activities had been reported in fibroblasts, in contrast to a decrease found in leucocytes. This difference corresponds to the fact that the enzyme residual activity is higher in rapidly dividing fibroblasts with active protein synthesis, than in leucocytes with little or no protein synthesis (Grünewald S 2001).

Various experiments were performed to achieve the highest yield of the leucocytes; the best results were obtained by combination of two methods described by Beutler (Beutler E 1970).

Leucocytes were isolated from blood samples, incubated in an opened tube for exactly 30 min (otherwise not good separation or haemolysis). 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. A common alternative to erythrocyte lysis is Ficoll density-gradient centrifugation. In contrast to erythrocyte-lysis procedures, Ficoll method recovers only mononuclear cells (lymphocytes and monocytes) and removes granulocytes.

The leucocyte pellet should be white with possible traces of erythrocytes. After effecting erythrocyte lysis, all steps should be performed as quickly as possible. All sugar substances, the enzyme cocktail and NADP should be freshly prepared.

The original PMM enzyme assay, based on the measurement of the produced NADPH (van Schaftingen E 1995) was adopted with slight modifications: enzyme substrate glucose-1,6 biphosphate, instead of the originally recommended mannose-1,6-bi-P was used. EDTA - plasma is preferred, giving statistically higher results, when compared to the samples with heparin (Barnier A 2002).

The reference values of PMM activity in our group of controls are similar to those reported in the literature (van Schaftingen E 1995, Grünewald S 2001, Barnier A 2002); enzyme activity in our CDG patient and his family was normal, and thus the most common CDG type Ia can be excluded. Heterozygotes had intermediate PMM activity values with a large range of variation (Matthijs G 1998), and thus PMM measurements may be not useful for the identification of carriers.

PMM values in the children younger than 2 years are higher, when compared with older individuals; this could be caused by the high metabolic activity related to growth in the first months of life. PMM mean value is lower in women compared to men; however, no such a difference was reported in individuals younger than 2 years (Barnier A 2002).

PMM deficiency results in a multisystem disease with dysmorphy and severe neurological involvement, and usually only mild liver, intestinal, and other organ disease (Jaeken J 1993, 1997). PMI deficiency causes the same kind of sialo-Tf IEF pattern as PMM deficiency, but the clinical manifestations are different (mainly hepatic and intestinal symptoms); various explanations were speculated for the fact, e. g. PMI

deficiency can be by-passed by the hexokinases, in contrast to PMM. (Niehues R 1998) see Fig. 25.

Fig. 25 Enzymatic reactions involved in the assays for determination of PMM activity
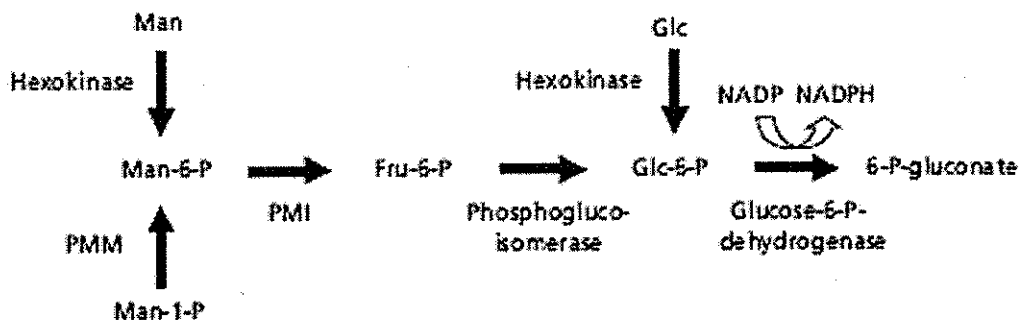
Man: Mannose; Glc: Glucose; Fru: Fructose

5.5 Our CDG patient

At age 5.5 years, the clinical pictures of our patient is characterized mainly by microcephaly, oligophrenia, dyslalia, 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, proteins C, S, D-dimer and AT III are basically normal. IEF of Tf and α_1-AT permanently shows hyposialylation patterns; EMG is normal, EEG is only slightly abnormal.

Apart from common mental retardation, muscular hypotonia, and facial dysmorphy, the patient also presented with skin, skeletal, and urological malformations, partial agenesis of corpus callosum, and Ladd syndrome (to our knowledge not reported in CDG before). Neither signs of cerebral hypoplasia, one of the most constant CDG symptoms (Jaeken J 2003), nor lipodystrophy and inverted nipples, also frequently present in CDG, have been detected in our patient.

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).

A recent analysis of CDG-II cases identified mutations in different subunits of the COG complex. This complex consists of 8 subunits (COG1-8) that forms a ~800kDa bilobed network that is found both in the cytoplasm and on Golgi membranes, where it is thought to play a role in the targeting of e.g. glycosyltransferases to their appropriate resident cisternae (Annaert W 2003, VIB Dept. of Human Genetics, KULeuven).

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 cutis laxa were the most prominent directly after birth and improved thereafter, while our patient had a belated appearance of mild skin anomalies.

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 has been monitored only by ultrasonography. A third boy was born in 39th week of gestation, fortunately without any apparent abnormalities (now at the age of 10 months). IEF of Tf was performed the first day of life from umbilical-blood spot, showing a slight increase of disialo-Tf (physiological in newborns); when repeated at the end of the first month of life the tests for Tf and α_1-AT were normal.

The CDG disease was found around the world (Jaeken J 2003); the highest frequency (1 : 40.000) was reported in Scandinavians countries (Kjærgaard S 2004).

The incidence of CDG may be even higher than presently recognized; genes involved in glycosylation have been estimated to account for as much as 1% of all human genes, thus it may be predicted that the current list of glycosylation disorders is far from being complete (Freeze H 1999). Since 2001, 11 CDG Ia patients have been diagnosed in Czech Republic (Honzík T 2003); our patient is the first one with a combined N- and O-glycosylation defect.

5.6 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% respectively), 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, CDG positive serum samples have already been referred at the first week of life (Stibler H 1991). False results were obtained from the brother of our CDG patient when the test was performed directly after the birth. Repeated analyses in the 1st, and the 6th months of age for both Tf and α_1-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 enzyme important for the correct processing of glycans is attributable to galactose-1-P, which is increased in galactosemia patients; a similar pathogenesis is assumed in the congenital fructosuria (Keir G 1999, Bean P 1996).

Changes in the amino acid sequence of the Tf polypeptide chain (namely the D genetic variants) should also always be 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, Dupré T 2000), after two years of follow-up of IEF Tf pattern in our CDG patient, no distinct changes were 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 G1990).

Higher CDT was also reported in rheumatoid arthritis, hemochromatosis (Arndt T 2001), CF (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 CF; 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-oligosacharide 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).

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

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

5.7 Tf and α_1-AT genetic variants

Tf shows great genetic (protein) polymorphism, which occurs at various frequencies among different populations. At least 36 Tf protein variants are known (Arndt T 2001); most of them represent subtypes of the major Tf variants C with minor changes in pIs of the isoforms. The most common is Tf C (>99 %; subtypes C_1 - C_{16}), whereas Tf B (B_0 – B_2) and D (D_1 - $D_{4.5}$) variants are rare (Kamboch KM 1987).

The frequencies of Tf variants in Europe are as follows: homozygous Tf C_1 >85%, Tf C_2 1-3%, heterozygous Tf C_1C_2 about 10%, Tf C_1B 0.7% and Tf C_1D 0.2% (Beckman LE 1998).

Tf variants have been characterized by amino acid sequencing. The amino acid substitutions of Pro70Ser for Tf C_2, Asp277Gly for Tf D_1, Gly652Glu for Tf B, Gly394Arg for Tf Devans, Ile378 or 381 Arg for Tf Dshaw and His300Arg for Tf Dchig have been reported (Tsughida S 2000).

Since the Tf CB and Tf CD interfere with the usual IEF and also HPLC chromatography pattern, it is necessary to confirm results. For the differentiation of higher CDT and 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.

Especially, the D_{4-5} Tf allele may simulate a CDG-positive result, as the prominent tetrasialo-Tf D_{4-5} band shifts cathodally to the position of the asialo-isoform C Tf (which is physiologically only faint, otherwise CDG-pathognomic). In the serum of our patient with a rare C_1 D_{4-5} variant, only one band related to the asialo-Tf of the C_1 subtype appeared after digestion with neuraminidase; repeatedly we could not detect any asialo-Tf D, neither when analysing a new sample of the patient, nor in the serum of his father (a carrier of the same allele). Since the asialo-Tf D_{4-5} focuses close to the position of the application strip, it may be unclearly visible. In such a case, gels of larger pH range are needed to demonstrate high cathodic shift of the asialo-Tf D_{4-5}.

An asymmetrical ratio of the Tf C_1- and D_2 variants (only faint tetrasialo-isoform D in contrast to the intensive tetrasialo-Tf C) was noted in both native sample, and that digested by neuraminidase (weak asialo-isoform D band) in one patient; the change of the amino acid sequence in the protein of the rare Tf D_2 variant may possibly result in changes of immunodetection specificity. Similar asymmetry in serum α_1-AT MZ and MS variants is well known (Jeppsson JO 1982); unequal expression of some Tf alleles in horse has also been reported (Niini T 1997).

An interesting case of misinterpretation of a rare Tf variant for higher CDT was reported by Welker (Welker MW 2004): a young man was refused reapplication of his driving license because of a supposedly elevated alcohol-positive CDT test, but finally IEF of Tf revealed a Tf D genetic type.

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 (Petrén S 1989), Alzheimer's disease (Namekata K 1997, and lower Fe-binding capacity (Wong CT 1986). Tf C_3 is suggested to show certain protection against some types of smoking-derived lung-carcinoma (Beckman LE 1999). On the other hand, comparably lower occurrence of Tf C_1C_2 is reported in patients with CF (Pascali VL 1984).

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. Suggested higher frequency of C_1C_2 Tf in a few rather small groups of children was not significant; studies on an extended number of patients are necessary.

Tf variants are used as markers of population's admixture. A highly significant heterogeneity was found in Scandinavian countries with respect to Tf C_3, B_2, B_1 and Tf DCHI variants (Beckman L 1998).

The diagnosis of CDG can be made by examination of the complex N-glycans on various glycoproteins. Owing to the possible false negativity in some CDG patients, in dubious cases, analysis of more than one plasma glycoprotein is recommended. α_1-AT is one of those, which is often examined in CDG patients (Stibler H 1998, Mills K 2003).

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.

IEF of plasma α_1-AT leads to the detection of eight bands. Physiologically, 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, whereas pentasialo- and trisialo-α_1-AT are present in fewer amounts. Decreased tetrasialo- and increased disialo-, monosialo- and asialo- α_1-AT are pathognomic for CDG.

The pH optimum for the most common α_1-AT variant M lies between pH 4 -5, nevertheless for practical reasons we tested the conditions similar to those used for Tf (gels of pH range 4-7). Different antibody application, skipping the iron-saturation step and sample pre-treatment with cysteine (to prevent an anomalous patterns resulting from the aged sera) are the minor variations of the method used for the α_1-AT analysis.

The rare S, Z, and F genetic variants should be properly recognised for a reliable interpretation of results. Thus, samples pretreatment with neuraminidase, and the parents

testing for identification of their α_1-AT phenotypes, in addition to Tf analysis, may help in the results interpretation.

By IEF, in addition to confirmation of CDG diagnosis, also primary deficiency of α_1-AT can be detected, further confirmed on the basis of genetic analyses.

α_1-AT phenotype might have an impact on disease outcome as well as on the risk of secondary IgA nephropathy (Szonyi L 2004). A significant association between HIV-1 infection and the presence of allelic variant M_2 was observed (Hayes VM 2003). In addition to that, also an increased frequency of heterozygosity for the Z α_1-AT among patients with ulcerative colititis was noted (Elzouki AN 1999), and significantly higher frequency of MS variants (Lolin YI 1995). The MZ phenotype was statistically more common in patients with hemochromatosis than in the general population (Rabinovitz M 1992).

In this study, we have analysed α-AT in a relative small group of patients, and thus eventual correlation of α_1-AT variants and diseases could not be followed.

Apart from Tf and α_1.AT, also TBG, AT III, ferritin, haptoglobin, orosomucoid, ß-hexoaminidase, vitamin D-, retinal-binding proteins, α_2-HS-glycoprotein, plasminogen, and Zn-α_2-glycoprotein showed abnormal results in CDG patients (Stibler H 1998, Yuasa I 1995, Macchia PE 1995, Wuyts B 2001).

5.8 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. They are based on fractionation of CDT and non-CDT isoforms on anion exchange microcolumns, before quantification of CDT isoforms. They include CDTect-RIA, CDT-EIA (Pharmacia & Upjohn, Uppsala, Sweden), %CDT-TIA (Bio-Rad, Oslo, Norway) and Tinaquant-%CDT/Tf (Roche Diagnostic GmbH, Germany).

The CDT assays can be used as screening tools, since they are commonly available in clinical laboratories. However, the fraction referred to as CDT is not precisely defined and contains, in addition to asialo-, monosialo-, and disialo-isoforms, also variable amounts of trisialo-Tf, whereas part of disialo-Tf retained on the column. These essays often produce low results for CB Tf and high results for CD Tf- variants, and may thus cause incorrect interpretation of results, since 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; moreover, the anti-human Tf antibodies show no cross reactivity to other proteins, which reflects the specificity of this method. 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.

The IEF patterns are pathognomic of hypoglycosylation, but they do not indicate the defect and do not distinguish between the different subtypes of CDG; but still, two main types of IEF pattern can be recognized: type I consists of higher asialo-, and disialo-Tf with mainly decrease of tetrasialo-Tf, while higher asialo-, monosialo-, disialo-, and trisialo- Tf with decrease tetrasialo-Tf is characteristic for CDG type II.

Apart from analyses of Tf isoforms, investigation of other glycoproteins, e.g. thyroxin binding globulin, AT III, and α_1-AT may be also used to confirm the general defect of glycosylation in CDG. The IEF pattern of α_1-antichymotrypsin seems to allow a differentiation between CDG-Ia and CDG-Ic (Fang J 2004). Differentiation of CDG type Ia from the other CDG I subtypes, using IEF of β-trace protein isoforms in CSF has also referred (Grünewald S 1999).

IEF of serum Tf remains the most powerful test for first step CDG screening. However, not all rare forms (such as CDG type IIb, IIc, IIf 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 and E, characteristic markers of some O-glycosylation defects (e.g. MEB), may be determined (Hackler R 1994, Assmann B 2000).

Some membrane bound glycoproteins (e.g. red cell-membrane band 3 and glycophorin) might serve as additional indicators of glycosylation defects, namely in CDG type Ig (Zdebska E 2003), or HEMPAS (Marquardt T 2003), respectively.

Some of the rare Tf variants that cause problems in CDTect minicolumn chromatography, and they cannot be differentiated by this method. Most of Tf variants can be detected by HPLC procedure. In contrast to IEF, the common Tf C genetic subtypes show only minor changes in charge, and therefore they cannot be distinguished by HPLC (Helander A 2001). 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, disadvantage of HPLC method is a need of 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 the rapidity, sensitivity and good precision, even 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 15.

Table 15 Comparison of commercial kits for common screening methods of Tf analysis

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

Analysis of glycan by sensitive matrix-assisted laser desorption ionization - time of flight - mass spectroscopy (MALDI-TOF-MS) is complementary to IEF for 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. Consequent structural analysis of glycans, achieved by sequential digestion by specific exoglycosidases follows, so that the defective steps in the processing pathway could be identified. 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 MS is the best technique. Currently, a general strategy combining HPLC, CE, MS and exoglycosidase sequencing represents the most effective way to approach the analysis of glycans.

5.9 Indications for CDG screening

The association of neurological signs, ocular abnormalities, cutaneous findings and hepatodigestive signs (cytolysis, fibrosis) is highly suggestive of a CDG. The disease probably remains largely under diagnosed.

Following frequent reports on the diversity in CDG patients, the screening criteria should be very wide. Thus, the CDG diagnosis is to be considered in all cases with: Body dysmorphia (especially inverted nipple and lipodystrophiahypotonia), failure to thrive, psychomotor retardation, neurological findings (central hypotonia, ataxia, cerebellar hypoplasia, olivopontocerebellar atrophy, seizures unresponsive to treatment, stroke-like episodes), ophthalmologic abnormalities (nystagmus, strabismus, pigmentary retinopathy, cataract), hepatopathy, hepatic fibrosis, chronic diarrhoea, clotting abnormalities, and multiorgan failure. At a later age, in adolescence or adulthood, the presentation may include in addition to a suggestive history: peripheral neuropathy with or without muscle wasting, progressive scoliosis with truncal shortening, joint contractures, hypothyroidism, absent puberty in females and small testes in males.

Laboratory findings, leading to suspicion of CDG include: High activities of aminotransferases, arylsulphatase A, increasing TSH, prolonged time of APTT, recurrent leucocytosis, lower serum levels of β_2-mikroglobulin, ceruloplasmin, α_1-AT, transferrin, plasminogen, orosomucoid, protein binding vitamin D and protein binding retinol, coagulation factors II, V, VII, IX, X, XI, AT III, protein S and C, TBG, T3, T4, rT3, unexplained hypoglycaemia, decreased serum IgG, decreased cholinesterase activity, thrombocytopenia, hypoalbuminemia, hypogammaglobulinemia, hypocholesterolemia, and proteinuria

Imaging studies, which help in CDG diagnostics: Skeletal deformations, namely kyphosis and scoliosis can be found by RTG. In some cases, signs of dysostosis are detected. Large kidneys with cysts and diffuse hyperechogenity may be demonstrated by US. The liver is usually normal or modestly enlarged, but effusion of the pericardium and hypertrophic obstructive cardiomyopathy are not unusual findings. MRI reveals small brain stem, dilated ventricles, enlarged sulci of cerebral hemispheres and vermis, cerebellar atrophy, atrophy of the pons and olives. Ophthalmologic methods, electromyography, electroencephalography may show abnormal results. Nerve conduction velocities are decreased especially in the motor nerves.

5.10 Diagnostic algorithm

The algorithm of CDG investigation begins with physician's suspicion, based on clinical symptoms and/or biochemical findings (low level of various serum glycoproteins). CDG screening usually starts with IEF of serum Tf isoforms, but other methods, and also other glycoproteins are convenient for CDG screening.

When excluding all possible secondary causes, positive IEF results usually reliably lead to diagnosis of CDG. Decrease of serum coagulation factors should be followed, and of other glycoproteins, e.g. α_1-AT and TBG. Investigation of Tf isoform by other methods (HPLC or CE) may help in confirmation of results, which is further proved by enzyme assays, analysis of glycan structures and mutation analysis.

However, negative IEF result doesn't exclude rare CDG type IIb, IIc, and IIf, so that the detection of oligosaccharides in urine or structure of membrane antigens should be follwoed.

The tetrasacharide Glc3Man found by TLC of urine is typical for CDG type IIb. Bombay phenotype is an O blood group, which lacks antibodies anti-B and anti-A; the absence of Bombay blood phenotype excludes CDG type IIc. Normal expression of sialyl-Lewis X antigen on the surface of leucocytes excludes CDG types IIc, and IIf. Glycophorin A and the band III in erythrocyte membrane may be pathognomic for HEMPAS and CDG type Ig, resp.

Moreover, electrophoretic analysis of serum apo C-III and apo E, as well as α-DG in skeletal muscle may be helpful for detection of O-glycosylation defects, which possibly constitute a new CDG subgroup.

IEF of Tf will detect all known CDG types I, CDG-IIa, CDG-IId, CDG-IIe and the others, so far designed CDG-x.

False positive results can be found in newborns, in patients under medication, with galactosemia and fructosuria, anaemia, chronic disease, alcoholics, samples contaminated by neuraminidase (sepsis, uraemia), and Tf genetic variants.

For excluding the secondary causes of CDT elevation it is recommended to rerun the analysis from a new collection, possibly to repeat the test after a suitable period of time of recovery from illness.

However, for excluding Tf genetic variants, the sample should be treated with neuraminidase, and analysis of samples from patient's parents may be considered; one of the parents should have the same variant as the patient (however, the combination of the genetic variant and the abnormal glycosylation of the same glycoprotein is possible).

Analysis of blood and urine sugars help to exclude galactosemia and fructosuria (symptoms improve by a diet, in contrast to CDG).

Confirmation of results may vary according to the IEF pattern: If IEF of Tf has the type I pattern, then PMM, and PMI assays should follow. If these enzymes are normal, other CDG types I are investigated by analysis of LLO in fibroblasts, followed by the analysis of suspected enzyme and identification of the gene mutation.

When IEF of Tf has the CDG type II pattern, then glycan analysis, e.g. by sensitive MALDI-TOF-MS is indicated. This method that identifies the compromised enzymatic step, points to the cell type-specific glycan processing pathways. The diagnostic procedure is accomplished by testing enzyme activity, allows links to genetic mutations, and indicates proteins and pathways affected in the disease process.

Diagnostic algorithm of CDG

Suspicion of CDG (clinical symptoms, biochemistry)

IEF of serum Tf (+ α_1-AT, TBG, ...)

Secondary causes

Positive

Negative

Other diagnoses

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

O-GLYCOSYLATION defects
IEF of α-dystroglycan, Apo C-3
and Apo E, mutation analysis

CDG type I
(different for Id, Ie)

CDG type II
IIa, IId, IIe

S-α_1-antichymotrypsin + CSF.
β-trace protein in dif dg. Ia/Ic

↓ IgG; Ery band III +
glycophorine A (Ig)

CDG type II
IIb, IIc, IIf

Enzymes of CDG
subtypes Ia / Ib / Ic

Urine oligosaccharides (IIb)
Bombay blood phenotype (IIc)
Sialyl Lewis X (IIc, IIf)

Decreased

Normal

Abnormal

Analysis of LLO
(Lipid-linked oligosaccharide)

Abnormal

Normal

Structure of glycan
(MALDI-TOF MS)

CDG Ix

Abnormal

Normal

Other diagnoses

Enzymes of subtypes
CDG Id/Ie/If/Ig/Ih/..../Il

Enzymes of subtypes
CDG IIa/IIb/ - IIf

Abnormal

Normal

Normal

Abnormal

CDG Ix

CDG IIx

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

*) The most common diagnostic procedure for subtype CDG Ia - in red

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 α_1-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 obtainment 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 an 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_1D_{4-5} protein variant, as proved by neuraminidase treatment, analysis of serum α_1-AT, and by investigation in the family of the affected adolescent boy.

The most prominent IEF abnormality found in a 4-year old child might correspond to CDG type IIx; also α_1-AT and TBG analysis revealed abnormal IEF-profile. The PMM activity in leucocytes shows normal results. The finding of a hyposialylation IEF pattern of apolipoprotein 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 α_1-AT.

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