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Invited critical review

Screening and diagnosis of congenital disorders of glycosylation

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Abstract

The aim of this paper is to review the diagnostics of congenital disorders of glycosylation (CDG), an ever expanding group of diseases. Development delay, neurological, and other clinical abnormalities as well as various non-specific laboratory changes can lead to the first suspicion of the disease. Still common screening test for most CDG types, including CDG Ia, is isoelectric focusing/polyacrylamide gel electrophoresis (IEF). IEF demonstrates the hypoglycosylation of various glycoproteins, usually serum transferrin. Other methods, such as agarose electrophoresis, capillary electrophoresis, high-performance liquid chromatography, micro-column separation combined with turbidimetry, enzyme-(EIA) and radio-immunoassay (RIA) have also been used for screening. However, these methods do not recognize all CDG defects, so other approaches including analysis of membrane-linked markers and urine oligosaccharides should be taken. Confirmation of diagnosis and detailed CDG subtyping starts with thorough structure analysis of the affected lipid-linked oligosaccharide or protein-(peptide)-linked-glycan using metabolic labeling and various (possibly mass-spectrometry combined) techniques. Decreased enzyme activity in peripheral leukocytes/cultured fibroblasts or analysis of affected transporters and other functional proteins combined with identification of specific gene mutations confirm the diagnosis. Prenatal diagnosis, based on enzyme assay or mutation analysis, is also available. Peri-/post-mortem investigations of fatal cases are important for genetic counseling. Evaluation of various analytical approaches and proposed algorithms for investigation complete the review.

Keywords: CDG; Hypoglycosylation; Investigations; Methods; Inborn errors

Contents

Abbreviations: 2-D, two-dimensional; aAT, α_1-antitrypsin; ACE, affinity capture and elution; AEC, anion exchange chromatography; ALT, alanine aminotransferase; apoC-III, apolipoprotein C-III; Asn, asparagine; AST, aspartate aminotransferase; AT III, antithrombin III; CDG, congenital disorders of glycosylation; CDT, carbohydrate deficient transferrin; CE, capillary electrophoresis; CMP, cytidine monophosphate; COG, conserved oligomeric (hetero-octameric) Golgi complex; CRP, C-reactive peptide; HPLC, high performance liquid chromatography; EIA, enzyme immunoassay; ER, endoplasmatic reticulum; ESI, electrospray ionisation; IEF, isoelectric focusing; LAD II, leukocyte adhesion deficiency type II; LC, liquid chromatography; LLO, lipid-linked oligosaccharide; MALDI, matrix-assisted laser desorption/ionization; Man, mannose; GDP, guanosine-diphosphate; MS, mass spectrometry; NLG, peptide-/protein-*N*-linked oligosaccharide; P, phosphate; PAD, pulsed amperometric detection; PMI, phosphomannose isomerase; PMM, phosphomannomutase; PNGase F, peptide *N*-glycosidase F; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SELDI, surface-enhanced laser desorption/ionization; TBG, thyroxin-binding globulin; Tf, transferrin; TIA, turbidimetric immunoassay; TLC, thin layer chromatography; TOF, time of flight method of detection.

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1. Introduction

In 1978, an unusual combination of abnormalities in several circulating glycoproteins was found in twin infants with psychomotor retardation [1]. These findings finally led to the diagnosis of the Carbohydrate-Deficient Glycoprotein Syndrome, later renamed to Congenital Disorders of Glycosylation (CDG) [2]. CDG represents a large family of autosomal recessive, mostly multi-systemic disorders. They are caused by various defects in 1) the biosynthesis or transfer of the lipid (dolichol-phosphate (P))-linked oligosaccharide (LLO) precursor in *N*-glycosylation, localized in the cytosol and the endoplasmic reticulum (ER) (CDG type I, subtypes a-m), or 2) defective processing of protein *N*-linked glycan (NLG) in the ER and the Golgi, namely trimming and elongation of glycan chain or intracellular trafficking (CDG type II, eight subtypes) [3–5]. Impaired glycoprotein maturation and expression, or structural changes thus lead to production of abnormally glycosylated secretory glycoproteins, lysosomal enzymes, and membrane proteins. As some of the enzymes involved in the glycosylation process (glycosyltransferases) need to be *N*-glycosylated themselves, defects in early glycosylation steps (CDG type I) may also result in defective processing (CDG type II), and thus conceal the original fault [6,7]. For review of 21 different CDG types identified so far see Table 1.

New unclear subtypes with unknown genetic basis (named CDG-Ix or CDG-IIx) [8] await precise characterization, i.e., similar to disorders selectively affecting specific pathways (mucolipidosis II and III, called I-cell disease and pseudo-Hurler polydystrophy, respectively) [9], or specific cell types (HEMPAS, type II congenital dyserythropoietic anaemia) [3,10]. On the other hand, CDG group has now expanded to *O*-glycosylation abnormalities, namely in the cases when combined *N*- and *O*-glycosylation defects have been found (including the CDG types IIc, IIe/Cog7, IIf, IIg/Cog1, and IIh/Cog8) [5,11–14]. In fact, about 24 different genetic defects involving isolated *O*-glycosylation disorders are formally not included in the CDG group. These will marginally be mentioned here since their biochemical characteristics have recently been reviewed [4,8,15–17].

Hundreds of genes are involved in several classes of glycoconjugation. This diversity could potentially lead to a large number of defects in biosynthetic pathways resulting in developmental alterations, when not lethal. Despite the ever-increasing number of CDG subtypes, the CDG Ia is still the most common form diagnosed thus far.

2. Review of investigations

2.1. Clinical symptoms

The defective glycosylation of proteins throughout the body explains the broad, diverse and severe, mostly multi-systemic nature of this autosomal recessive disease.

Symptoms in CDG type Ia are often age-dependent. In the neonatal or early infantile period the defect may present itself by atypical morphology (e.g. facial dysmorphism, microcephaly, inverted nipples and fat pads), nonimmune hydrops fetalis, failure to thrive, defects of immune system, strabismus, nystagmus, developmental delay, seizures, axial hypotonia and hyporeflexia, cerebral hypoplasia with loss of both grey and white matter, especially affecting the pons and cerebellum. Although the cerebellum atrophy may progress through early childhood, it is stable in adolescence. Intracranial hemorrhage in a term neonate, or transient stroke-like episodes derived from blood coagulation defects might occur, and, later on, retinopathy and peripheral neuropathy gradually develops. Skeletal deformations, namely

Table 1
Currently identified CDG types

CDG type	Gene/location/*MIM*	Affected protein/EC/*MIM*	Prevailing symptoms	References
Ia	PMM2/16p13.3-p13.2/*601785*	Phosphomannomutase/EC 5.4.2.8/*212065*	Facial dysmorphism, hypotonia, seizures, psychomotor retardation (PMR), lipodystrophy, stroke-like episodes, cerebellar hypoplasia, coagulo-, hepato-, entero-, cardio (hypertrophic non-obstructive)-myopathy, peripheral neuropathy.	[30,31]
Ib	MPI/15q22/*154550*	Man-P isomerase/EC 5.3.1.8/*602579*	Liver fibrosis, vomiting and/or diarrhea with protein-losing enteropathy, coagulopathy, oedema, ascites, hyperinsulinemic hypoglycemia, no PMR (oral mannose therapy).	[32,33]
Ic	ALG6/Ip22.3/*604560*	Dol-P-Glc:Man9 GlcNAc2-PP-Dol-α-1,3-glucosyltransferase EC 2.4.1.-/*603147*	Similar to CDG-Ia, moderate form; 2nd the most common after CDG Ia.	[34,35]
Id	ALG3/3q27.1/*608750*	Dol-P-Man:Man5GlcN Ac2-PP-Dol-α-1,3-mannosyltransferase(mannosyltransferase IV)/ EC 2.4.1.-/*601110*	Similar to CDG-Ia, but more severe; optic nerve atrophy.	[36]
Ie	DPM1/20q13.13 /*603503*	GDP-Man:Dol-P-mannosyltransferase(Dol-P-Man synthase I, Dol-P mannosyltransferase I)/ EC 2.4.1.83/608799	Similar to CDG-Id; cortical blindness, coagulopathy, hypertrophic obstructive cardiomyopathy.	[35,37]
If	MPDUI/17p13.1-p12/*604041*	Dol-P-Man utilization factor 1/*609180*	Severe PMR, short stature, ichthyosis, erythema, retinopathy.	[38]
Ig	ALG12/22q13.33/*607144*	Dol-P-Man:Man7Glc NAc2PP-Dolα1, 6-mannosyltransferase/EC 2.4.1.-/*607143*	Similar to CDG-Ia; low IgG, male genital hypoplasia, frequent infections; abnormal band III and glycophorin A in red cells.	[39,40]
Ih	ALG8/11pter-p15.5/*608103*	Dol-P-Glc:Glc1Man9 GlcNAc2PP-Dol-α-1, 3-glucosyltransferase (α-1,3-glucosyltransferase II)/ EC 2.4.1.119/*608104*	Similar to CDG-Ib; dysmorphism, renal failure, no encephalopathy.	[41]
Ii	ALG2/9q22/*607905*	GDP-Man:Man1Glc NAc2-PP-Dol-α-1, 3-mannosyltransferase (mannosyltransferase II)/ EC 2.4.1.-/*607906*	Similar to CDG-Ia; normal at birth, intractable seizures, hepatomegaly, hypomyelination, coloboma iridis.	[42]
Ij	ALG7 (DPAGT1)/11q23.3/*191350*	UDP-GlcNAc:Dol-P-GlcNAc-phosphotransferase/ EC 3. 5.3.1.8/*608093*	Similar to CDG-Ia, severe PMR.	[43]

Ik	ALG1 16p13.3/*605907*	GDP-Man:GlcNAc2PP-Dolβ-1,4-mannosyltransferase (mannosyltransferase I)/EC 2.4.1.83/*608540*	Similar to CDG-Ia, but more severe; intractable seizures, fever, nephrotic syndrome, early death (hydrops fetalis), low B-cells, low IgG.	[44–46]
Il	ALG9/11q23/*606941*	Dol-P-Man:Man(6)(8) GlcNAc2PP-Dolα1,2-mannosyltransferase/EC 2.4.1.-/*608776*	Severe microcephaly, hypotonia, seizures, hepatomegaly.	[47]
Im	hDK1/TMEM15/9q34.11/*610746*	Dol-kinase/EC 2.7.1.108/*610768*	Microcephaly, hypotonia, hair loss, ichthyosis, hyperkeratosis, dilative cardiomyopathy, tetraplegia, early death.	[48]
IIa	MGAT2/14q21/*602616*	UDP-GlcNAc:α-6-d-mannoside-β-1,2-Glc NAc-transferase II/EC 2.4.1.143/*212066*	Developmental delay, dysmorphism, seizures, small stature, relative macrocephaly, no cerebellar atrophy, no peripheral neuropathy; abnormal Tf.	[49]
IIb	GLS1 (CWH41)/2p13-p12/*601336*	α-1,2-Glucosidase I/EC 3.2.1.106/*606056*	Dysmorphism, hypotonia, seizures, hepatomegaly, hepatic fibrosis, early death; normal Tf.	[50]
IIc/LAD II	FUCT1/SLC35C1/11p11.2/*605881*	GDP-fucose transporter I/EC 2.7.1.38/*266265*	PMR, hypotonia, microcephaly, infections, neutrophilia, no sialyl-Lewis^X- and H-antigens; Bombay blood phenotype, normal Tf; combined *N*- and *O*-defect (partially effective oral fucose therapy).	[11]
IId	β4GALT1/9p13/*137060*	UDP-Gal:β-GlcNAc-β-1,4-galactosyltransferase (β-1,4-galactosyl-transferase I)/EC 2.4.1.38/*607091*	Moderate PMR, macrocephaly, hypotonia, myopathy, Dandy-Walker malformation, hemorrhage, abnormal Tf.	[51]
IIe or II/COG7	COG7/16p12.1/*606978*	COG, subunit 7/*608779*	Dysmorphism, hypotonia, infections intractable seizures, hepatomegaly, progressive jaundice, cardiac failure, diarrhea, early death, abnormal Tf and apoC-III; combined *N*- and *O*-defect.	[12,52,53]
IIf	SLC35Al/6q15/*605634*	CMP-sialic acid transporter/*269920*	Thrombocytopenia, hemorrhages, no neurological symptoms, no Lewis^X antigen, abnormal platelet glycoprotein; Bombay blood phenotype, normal Tf, abnormal apoC-III; combined *N*- and *O*-defect.	[13,54]
IIg or II/COG1	COG1/17q25.1/*606973*	COG, subunit 1/*606978*	Dysmorphism, macrocephaly, mild cerebellar atrophy, hypotonia; abnormal Tf and apoC-III; combined *N*- and *O*-defect.	[14]
IIh or II/Cog8	COG8/16q22.1/*606979*	Disruption of the COG1-COG8 interaction (+ß1, 4-galactosytransferase)	PMR, microcephaly, abnormal Tf and apoC-III; combined *N*- and *O*-glycosylation defect.	[5]

CMP, cytidine monophosphate; COG, conserved oligomeric Golgi complex; Dol, dolichol; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; EC, enzyme commision number; GDP, guanosine-diphosphate; LAD II, leukocyte adhesion deficiency type II; Man, mannose; MIM, Mendelian Inheritance in Man; P, phosphate; PP, pyrophosphate; PMR, psychomotor retardation; UDP, uridine-diphosphate.

kyphosis and scoliosis can be found. There is substantial childhood mortality due to severe infections or organ failure. In adolescence the acute events become less frequent, and adult stage defines namely a permanent, nonprogressive ataxia, stable mental retardation and peripheral neuropathy.

Apart from these signs, tetraspastic paresis, hydrocephalus, macrocephaly, cardiopathy, renal failure, leukocytosis with severe episodes of pneumonia, extreme hyperthermia, ichtiosis, and wrinkled skin may appear in patients with various CDG subtypes. Hepato-intestinal symptoms are typical for CDG type Ib (clinically distinct from the type Ia and most other CDGs; without neurological symptoms) and CDG Ih. For details see Table 1 and Refs. [3,4,18]. In some rare CDG subgroups, only one or few patients have been identified, and thus the relevance of clinical description and screening results is limited. The clinical diversity of CDG is remarkable even within each group, namely the type Ia, so phenotype may range from severe multivisceral disease to only very mild symptoms [18–20]. Clinically, nearly asymptomatic or atypical forms (with presentations not seen before in CDG) have been repeatedly identified [7,21,22]. These findings should alert to the possibility of CDG misdiagnosis. It is thus recommended that CDG be considered in any case of an unexplained clinical syndrome.

Various other studies such as ophthalmic and electromyographic examination, X-ray, ultrasound, magnetic resonance imaging (MRI), and computer tomography (CT) may facilitate disease diagnosis. Histological findings in brain, eye [23], liver [24–26], intestine [25,27], and peripheral nerve [28] tissue have been useful in CDG Ia patients.

The *O*-glycosylation defects, characterized mainly by muscle dystrophy, structural eye abnormalities and brain malformation [29], have been reviewed elsewhere [4,17]. As can be appreciated, clinical diagnosis alone is exceedingly difficult due to the non-specific features of the disease combined with great variability in disease severity.

2.2. Biochemical findings

The majority of plasma proteins are glycoconjugates, where one or more carbohydrate chains (glycans) are covalently linked by *N*-glycosidic links to amino acid (asparagine, Asn) side chains of the polypeptide. Disturbances at any step in the metabolic pathway lead to hypo- or misglycosylation. Primary defects may involve monosaccharide synthesis in the cytoplasm, one of the glycan-specific transferases, glycosidases, nucleotide sugar transporters, and structural proteins in the Golgi and ER. As consequence, carbohydrate side chains of glycoproteins can be partly or totally missing from the protein (CDG I), or structurally altered (CDG II). However, in CDG diagnosis, secondary causes of hypoglycosylation (mainly due to functional enzymes inhibition) should be excluded, e.g. untreated galactosemia, hereditary fructose intolerance, and other liver pathology (including chronic alcohol abuse) [55], inflammatory diseases (rheumatoid arthritis) and cancer. Other conditions, such as temporary abnormalities in hemolytic uremic syndrome, and bacterial infections, due to massive neuraminidase production [56–57] should be considered.

Basically, the biochemical screening and diagnostics of CDG involve detection of hypoglycosylated serum or membrane bound glycoproteins, low intracellular enzyme activity and identification of structurally defective lipid-linked or protein (peptide)-linked glycans and their metabolic intermediates.

2.2.1. Common laboratory investigations

In CDG patients, pathological results of common biochemical tests may be found. These include abnormal liver function tests, low plasma cholesterol and cholinesterase activity with proteinuria (CDG type Ia). Hypoalbuminemia, hypoglycemia with inadequately increased insulin production, and high activities of aminotransferases are typical of CDG Ib [3,58]. Disproportionate aminotransferase activity, i.e., increased aspartate aminotransferase (AST) and normal alanine aminotransferase (ALT), appeared [59] characteristic for CDG subtypes II. This finding was not observed in CDG type I. High plasma and cerebrospinal fluid levels of glycine [60], as well as thrombocytopenia and high plasma ferritin [15] have been occasionally noted. Elevated plasma activity of lysosomal hydrolases, namely aspartylglucosaminidase [61] has been frequently found in CDG type I patients. Increased β-hexosaminidase activity in amniotic fluid has been used as a marker of CDG Ia [62].

2.2.2. Total serum glycoproteins

The concentration of plasma glycoproteins in CDG type I are frequently altered including α_1-antitrypsin (aAT), thyroxin-binding globulin (TBG), and transferrin (Tf). The concentration of these proteins is usually low early in life. This phenomenon is similar to numerous clotting factors and their inhibitors including factors V, XI, II, X, antithrombin III (AT III), proteins C, S, and heparin cofactor II. Thyroid hormones including triiodothyronine (T3), thyroxine (T4), and reverse T3 (rT3) are mostly subnormal. The levels of all these factors increase with age and later stabilize [3,18,63,64].

2.2.3. Hypoglycosylated serum glycoproteins

Altered glycosylation in CDG patients can be detected by analysis of many serum glycoproteins [65,66] (with some exceptions, e.g. factors II, X and fibrinogen) [67,68], and by β-trace protein in the cerebrospinal fluid [69,70]. The most convenient and commonly used is Tf (iron-binding protein, synthesized and metabolized mainly in the liver), because of the relatively simple mechanism of glycosylation (Tf has two disialo-biantennary *N*-glycans as the major oligosaccharide at Asn-432 and Asn-630) [68]. Serum Tf separates into several isoforms owing to different degrees of glycosylation, and thus different amounts of terminal sialic (*N*-acetylneuraminic) acid residues, physiologically the most abundant being the tetrasialo-Tf (four sialic acid residues attached to two biantennary complex glycans on each of the two *N*-glycan sites, Asn413 and Asn611) [71]. Under the CDG conditions, hyposialylated Tfs are relatively increased, especially the disialo-, asialo-, monosialo-, and trisialo-Tfs, due to the complete missing of carbohydrate chains in the type I, or structural abnormalities (truncation, giving rise to a predominant disialo-band) in some CDG subtypes II. These sialic acid-deficient isoforms, resulting in a loss of negative charges,

often called carbohydrate deficient transferrin (CDT) are generally regarded as a marker for most of primary and secondary defects of glycosylation, and their measurement has been widely used as a screening tool.

Originally, Tf isoforms were analyzed by various electrophoretic techniques (namely to identify increased CDT in alcohol abuse). CDT may also be detected by high performance liquid chromatography (HPLC) and commercially available assays. Recently, mass spectrometry (MS) procedures have also been used for to screen for CDT.

Isoelectric focusing (IEF) is an electrophoretic method that separates proteins according to their isoelectric points (pI). Following IEF, immunofixation, or immunoblotting is performed using a specific antiserum to identify the separated bands. Bands of interest may be then quantitated by densitometric scanning. IEF has been instrumental in separation and identification of isoforms of Tf, ferritin, TBG, AT III, aAT, hexosaminidase, orosomucoid, haptoglobin, α_1-antichymotrypsin, α_1-acid glycoprotein, α_2-antiplasmin, vitamin D-binding protein, retinal-binding protein, α_2-HS-glycoprotein (fetuin), plasminogen, and Zn-α_2-glycoprotein [67,68,72]. The IEF patterns of some glycoproteins have been reported to be characteristic of several CDG subtypes [73]. Changes of apolipoprotein C-III (apoC-III), a characteristic marker of some (core 1) *O*-glycosylation disorders can also be monitored by IEF [13].

Analysis of Tf is used as a standard screening method. Serum, cerebrospinal fluid and whole blood/serum-dry spots on Guthrie-type filter paper are suitable specimens for CDG screening [60,74–76]. Other suitable specimens include plasma [55], urine [77], or delipidated liver biopsies homogenates [60]. In some laboratories, Tf isoforms are first purified from serum by immunoaffinity chromatography (see later). Total iron saturation (Fe_2-Tf) is necessary prior to IEF because the co-focusing effect of incompletely saturated iso-Tf (Fe_0-Tf and Fe_1-Tf) could impede interpretation [78]. Both polyacrylamide and agarose gels can be used in the analysis [75,79]. A Tf IEF kit is also commercially available (Servalyt Precotes ™, Heidelberg, Germany). Numerous types of electrophoretic equipment including the special PhastSystem (Amersham Biosciences) and various IEF conditions have been described [76,80,81]. Detection by immunofixation is performed immediately after IEF by exposure to anti-Tf antibody at room temperature and subsequent Coomassie Brilliant Blue staining [80]. Altenatively, silver staining is a higly sensitive technique that can be used to detect low levels of glycoproteins in cerebrospinal fluid [19,82]. In some cases, immunoblotting with an appropriate primary and secondary enzyme-linked (horseradish peroxides) antibody may be preferred. [19,78,83]. Sample storage and other pre-analytical conditions have been tested and described in detail by Arndt [55]. At least two different Tf IEF patterns, roughly corresponding to the two main types CDG I (entire sugar chains of mostly normal structure are absent) and CDG II (glycan structure is altered) can be distinguished [3,63]. These include increased disialo- and asialo-Tf at the expense of tetra-Tf and other highly sialylated isoforms (type I pattern), and additional trisialo- and monosialo-Tf involvement (type II pattern).

The large genetic polymorphism of glycoproteins has to be taken into consideration. For example, at least 38 protein variants of Tf can be distinguished in humans [55] with the most frequent type being C_1 [84]. IEF banding patterns of other Tf variants can shift thus impeding interpretation of test results. Results should be compared before and after digestion of sialic residues by neuraminidase to reduce all glycoforms to asialo-Tf. This process ensures that any heterogeneity is, thus, unique to genetic Tf variants [85,86]. Screening of parental Tf patterns may also be useful in patient evaluation. For a description of the IEF procedure and our experience with interpretation refer to [87,88].

In addition to IEF, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining or Western-blotting has been used to detect serum Tf and other glycoproteins in screening for *N*-glycosylation defects (including apoC-III for *O*-glycosylation/combined abnormalities) [17,83,89]. Modifications can include the use of an antibody cocktail to simultaneously identify different glycoproteins (e.g. Tf, aAT, and haptoglobin) within the gel [90]. Alternatively, SDS-PAGE with lectin blotting may be used for detecting CDT alterations [51].

Two-dimensional (2-D) electrophoresis combines protein separation via charge (IEF) and molecular weight (SDS-PAGE). This technique has been useful in the study of various glycoproteins including Tf and aAT in CDG diagnostics [85,91]. These proteins are directly silver stained or immunodetected.

Agarose gel electrophoresis is a method of choice used for CDG screening. Medium electroendoosmotic agarose gel, veronal buffer (pH 8.6, $I=0.085$) and direct passive immunoblotting have been used [19] to separate and detect Tf isoforms.

Capillary electrophoresis (CE) is another technique successfully applied in the determination of serum Tf isoforms [92–94]. A simple and reliable assay has been developed that uses uncoated fused-silica capillary (Beckman Coulter P/ACE 5000 system) with UV detection (214 nm) and a special buffer system (supplied by Analis Namur, Belgium; CEofix™-CDT kit) [95,96]. A fully automated method for precise detection of CDT isoforms is now commercially available on Capillarys (Sebia, Evry, France).

Compared to electrophoresis, chromatographic methods are less sensitive and specific, but in contrast to IEF, they can provide precise and accurate quantitation of Tf isoforms.

In commercial CDT tests, the sum of asialo, mono-, di-, and a portion of trisialo-Tf CDT is determined relative to total Tf after *in vitro* iron saturation of Tf. The procedure is based on a (micro-column anion-exchange) chromatographic separation of the hyposialylated fraction from the normal Tf, followed by radioimmunoassay, immunoturbidimetry, or enzyme immunoassay (e.g. CDTect and CDTect EIA assay kits, Pharmacia & Upjohn, Uppsala, Sweden; RIA %CDT and TIA %CDT, Axis-Shield, Oslo, Norway; results are expressed as %CDT values relative to total Tf) [55,81,86,97].

Liquid chromatography (LC) is routinely used to determine serum Tf isoforms for the alcohol-abusers detection [55]. Separation by anion-exchange HPLC was first described by Jeppsson [98]. In this technique, iron-saturated and delipidated serum was analyzed on a Pharmacia MonoQ™ HR column using gradient elution with BisTris–NaCl buffer pH 6.2. Eltuion

of the iron-Tf complex was monitored at 460 nm. Further modifications and use of alternative columns led to the subsequent development of a semi-automated procedure for CDT quantitation [99–101]. Commercial HPLC kits ClinRep® CDT for Tf analysis are available (Recipe, München, Germany), C-Fer (Metzingen, Germany), and %CDT HPLC (Axis-Shield, Oslo, Norway).

Affinity chromatography on an immobilized lectin column in combination with other techniques is often employed in the field of glycoprotein isolation and fractionation [51,102,103].

Methods, based on MS analysis of the charged (native or immunoaffinity-isolated) serum Tf isoforms (using ion-trap or TOF, time of flight detection), have recently been introduced. In electrospray ionization-mass spectrometry (ESI-MS), a purified sample is exposed to a strong electric field and a counter flow of nitrogen gas. This process produces an electrospray that causes evaporation of the solvent and desorption of charged ions into the mass spectrometer. The assay reliably identifies underglycosylation of Tf and allows discrimination between CDG-I and CDG-II defects (the loss of entire glycans versus the loss of simple sugars differentiation) [103]. In matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS), the purified sample is added to a matrix (ultraviolet-absorbing crystals) applied to a stationary probe and then exposed to high energy for analytes desorption and ionization. Surface-enhanced laser desorption/ionization-mass spectrometry (SELDI-MS) is another versatile and convenient screening tool for altered glycoprotein (serum Tf) identification in CDG type-I. Immobilization of anti-Tf antibodies on reactive-surface protein chip arrays (RS100, Ciphergen, Surrey, UK) selectively enriched Tf by at least 100-fold, a process that facilitated detection of various patterns of glycoforms in patients with the known or yet unidentified CDG I types. [104].

It must be realized, however, that various screening approaches based on detection of hypoglycosylated glycoproteins cannot recognize all CDG defects. As such, additional and alternative studies such as analyses of membrane antigens or thin layer chromatographic (TLC) separation of urine oligosaccharides should be considered.

2.2.4. Membrane-linked markers

In addition to secretory glycoproteins of hepatic origin, some membrane-bound glycoproteins might serve as alternative indicators of a glycosylation defect. In CDG type Ia and Ig, a single *N*-glycan of the red cell membrane band 3 (AE1) can be separated into two hypoglycosylated fractions by SDS-PAGE. Glycophorin A (the major sialoglycoprotein of the red cell membrane) is insufficiently glycosylated with respect to *O*-linked glycans [105]. This markear appears pathognomic for other subtypes that have not been clearly classified thus far (CDG-*x*) [3].

Loss of lectin binding ability to specific glycoconjugates is another indication of hypoglycosylation. For example, binding of fluorescently labeled lectins to washed platelets has been investigated by flow cytometry. This approach has been successfully used in the analysis of membrane-bound glycoproteins defective in CDG type Ia and IIa [106]. Differences in platelet adhesion (increased in the first and decreased in the second CDG type) may serve as a rough discriminator between both main CDG types.

For some rare CDG types with fucose defects (IIc and IIf, not detectable by the sialyl-based Tf-IEF), the membrane abnormality is the only CDG screening marker [3,4]. Lost expression of some glycoproteins, such as blood-group antigens (Bombay phenotype, assayed by serological testing) on erythrocytes, or sialyl Lewis^X (CD15s) antigen on neutrophils (e.g. by flow cytometry using monoclonal antibodies) should be assessed [11,107]. Abnormal apoC-III profile in CDG IIc and IIf is indicative of combined *O*-glycosylation defect.

2.2.5. Urine oligosaccharides

Thin layer chromatography is commonly used for the analysis of urine oligosaccharides in the screening of inherited metabolic diseases [108]. A prominent abnormal tetrasaccharide band (Glc_3Man) is a characteristic finding in CDG type IIb. In these patients, no abnormalities in the IEF patterns of serum Tf and CSF β-trace protein have been detected [50,109].

Clinical symptoms and abnormal screening results (IEF, CE, HPLC) that demonstrate the Tf-isoforms pattern 1 justify the use of a simple enzyme assay and mutation analysis for detection of the most common CDG subtype Ia. In the case of negative results, an appropriate enzyme assay option (the CDG subtype-specific) should be performed only after comprehensive analysis of the affected LLO (or the NLG, if the Tf-based CDG screening pattern 2 was found).

2.2.6. LLO, NLG and glycopeptides

CDG differentiation, namely the subtypes II identification requires detailed analysis of glycan structure. However, neither ESI-MS nor MALDI-MS can reliably identify the defect in the sugar chain of intact glycoprotein, e.g. Tf. Under these circumstances, released glycans must be analyzed.

In order to obtain structural data, *N*-linked glycans may be enzymatically (peptide *N*-glycosidase F, PNGase F) or chemically cleaved from gel-separated or serum proteins. Various profiling and characterization procedures include SDS-PAGE [110], CE [111], or LC [40], usually combined with MS techniques preferably TOF and also possibly in tandem MS/MS arrangement [112–114].

A standard method of oligosaccharide quantitation is HPLC following fluorescence-labeling via reductive amination (2-aminobenzamide) [112]. Released glycans can also be purified with porous graphite carbon as a separation medium for oligosaccharide alditols and analysed by ESI-MS [110]. Alternatively, oligosaccharides may be directly identified by MALDI-MS [113].

Many studies have relied upon metabolic labeling of oligosaccharides with sugar precursors. Using this technique, glycosylation efficiency, levels of various metabolic intermediates (e.g. Man-1-P or guanosine-di-phosphate (GDP)-Man) and structural changes of the LLO and NLG can be monitored. This approach is especially important for LLO analysis given its relatively low abundance and inherent limitations of available methods. As such, variable increase in the amount of truncated LLO, i.e., lacking sialic and sugar residues, can be detected. Using *in vitro* studies, cultured fibroblasts (controls and patients)

are biosynthetically labeled with radioactive sugar precursors such as 2-[^{3}H]-mannose or [^{14}C]-glucosamine. These are then harvested by trypsination, counted, sonicated, and solubilized. LLO are subsequently released from their lipid carrier by mild acid hydrolysis of the pyrophosphate bond (the NLG cleaved enzymatically) and characterized by HPLC [34,39,40,115]. Anion-exchange chromatography (AEC) with pulsed amperometric detection (PAD) has often been used for selective analysis of saccharides [116]. Recently, a facile, accurate, and sensitive non-radioactive method for LLO and NLG characterization based on fluorophore-assisted carbohydrate electrophoresis (FACE) has been described [117]. Alternatively, released glycans are fluorescence-labeled and examined by LC/MS [112].

Analysis of glycopeptides isolated from the enzymatic digests of glycoproteins in combination with MALDI-MS or LC/MS (or LC/MS/MS) is used for characterization of site-specific or subclass-specific glycan profiles in CDG II diagnosis [113].

2.2.7. Enzyme assays

Decreased enzymes activities are usually evaluated in peripheral blood leukocytes or cultured fibroblasts. Phosphomannose isomerase (PMI) catalyzes conversion of fructose-6-phosphate to mannose-6-phosphate which is then isomerized by phosphomannomutase (PMM) to mannose-1-phosphate required for synthesis of the substrate for LLO. The activity of PMM and PMI was markedly deficient in leukocytes, fibroblasts, and liver cells of CDG patients' type Ia and Ib, respectively. Alternatively, amniocytes and chorionic villi can also be used for prenatal detection [118]. The assay is based on a coupled NADP(+)/NADPH enzyme system. The reaction product is measured photometrically (340 nm) or fluorimetrically (excitation/emission, 340/460 nm) [30,32,118].

Ohkura [119] developed an assay using [^{3}H]-mannose-6-phosphate as a substrate. Intermediate product formation was monitored by paper electrophoresis. More sensitive procedures are, however, based on direct detection of impaired substrate-product conversion (mannose-1-phosphate to mannose-6-phosphate in CDG type Ia) by AEC-PAD. Using this assay, a markedly decreased activity of PMM was found also in obligate heterozygotes [120].

A complex procedure called affinity capture and elution (ACE) combined with ESI-MS assay was used for the determination of the affected enzymes in the multiplex analysis of CDG types Ia and Ib. Enzymatic products that are isomeric with their substrates (in cultured cell homogenates) are subjected to another enzymatic reaction and the resulting products are quantitated by MS [121].

Although the enzyme is known in most CDG types (Table 1), its assay has not always been developed (types Ih, Il). For review of proteins involved in *O*-glycosylation disorders see Ref. [15,17].

2.2.8. Transporters and COG subunits

Defect of GDP-fucose transporter leading to impaired import of cytosolic GDP-fucose into the Golgi is a cause of CDG type IIc. Radiolabeled fucose bound to nucleotide sugars or glycoproteins has been tested on patient-derived fibroblasts using a fucose-specific, lectin-staining procedure [11].

CDG type IIf relates to structural changes of cytidine monophosphate-(CMP)-sialic acid transporter (a CMP antiporter between cytosol and the Golgi complex). Biochemical and genetic analyses including immunofluorescence and deconvolution microscopy for localization have been reported [122].

Intracellular membrane trafficking pathways have also been studied. Various procedures have been described for COG analysis and diagnosis of CDG types IIg (COG1) [14], IIe (COG7) [52], and IIh (COG8) [5]. These procedures include Western-blot and MS analysis of NLG, immunofluorescence labeling of intermediates in cell cultures, transfection experiments, and mutation analysis.

It is important to note that results attained by glycans analysis and specific enzyme (or functional protein) assays should be confirmed by genetic analysis.

2.3. Genetic analysis

More than 50 genes contribute to the synthesis, orientation, shuttle and transfer of the LLO to proteins and to recycling by-products, compared to some 500 genes estimated to participate in oligosaccharide synthesis and function [3,15].

The molecular basis of at least 21 different types of CDG has been identified so far (Table 1). The substitution R141H (which is never observed in the homozygous state) in PMM2 gene (CDG Ia) is the most frequent mutation among Caucasians. More than 90 other, mostly missense mutations in this gene have been discovered [123,124]. A survey of all published mutations in CDG and updates can be viewed on the OMIM web address.

Use of RNA may also complement testing. Total RNA can be extracted from a variety of sources including fresh EDTA-anticoagulated whole blood, cultured skin fibroblasts or amniotic and chorionic villi cells. Mutations can be identified by a combination of simple CE system for the single-strand conformational polymorphism analysis, (real-time) polymerase chain reaction, and DNA sequencing [35,125,126]. Screening strategies for gene mutation analysis (including denaturing HPLC platform or *in vitro* expression testing in either yeast or mammalian mutant cells strains after transfection) has been detailed [127,128]. Homozygous or compound heterozygous mutations, namely in the coding sequence (but also in promoters, introns, and splice junctions) are then confirmed at the genomic level in the patient and their relatives [31,63,129].

Due to the severity of the disease, prenatal diagnostic services (provided that both disease-causing alleles are identified) based on combined mutation and linkage analysis with polymorphic markers are often required [118,130].

3. Discussion

There are a growing number of diseases characterized by changes in glycosylation status, the largest of which involve *N*-glycosylation defects [124]. Over a thousand CDG patients, belonging to one of the 21 known subtypes has been identified

thus far. However, there are very few (less than 10) identified cases in each subgroup (except for CDG-Ia-c). It is assumed that disorders of glycosylation are much more common with an estimated cumulative disease incidence of 1:20,000. CDG occurs worldwide. Affected individuals can present with a range of clinical symptoms, the most frequent being a developmental delay. Some CDG subtypes have nothing more in common. Nevertheless, the overlap of phenotypes and variation of symptom severity (both within and between the different subtypes) precludes reliable specific clinical delineation. Therefore, the biochemical analysis of circulating/membrane bound glycoproteins is the only practical screening method presently available for detection of a major number of CDG subtypes. Comprehensive analysis for all known CDG defects via gene chip detection might, however, present a viable option in the future. Present day choice for the first-level CDG diagnostic is, unfortunately, limited by method availability.

3.1. Screening methods

Although many serum glycoproteins are affected in CDG disorders, Tf is the most sensitive marker. Of potential screening methods, IEF of serum Tf is the most widely used due to its low sample volume requirement (1 μL), low cost, and ability to be performed in most clinical laboratories. IEF, however, tends to be labor intensive due to its complex nature and is not amendable to high throughput. Although densitometry may be performed on IEF gels (stained or overlaid with antibody), this approach is, at best, only semi-quantitative.

In contrast, HPLC analysis of Tf isoforms (AEC using absorbance at 460–470 nm) provides reproducible results and can be automated for large sample numbers. In addition, no antibody is needed. Disadvantages of HPLC include column cost, large sample volume (200 μL), use of gradient elution and frequent and complex column regeneration.

Determination of Tf by CE with UV detection is reliable, rapid (within 15 min) and provides absolute concentrations of the sialo-Tf fractions. Commercial kits are available. Although some reports [101] have indicated that serum compounds may interfere with measurement at 214 nm (e.g. CRP by coeluting with the monosialo-Tf peak), the optimized CE assay represents a reliable alternative to HPLC due to its increased resolution, short analytical time and ease of system reconditioning.

CDG screening can also be based on the analysis of serum CDT by commercial CDTect assays available in a varitye of formats (TIA, EIA, or RIA). All of these are useful for screening, but rare Tf protein variants may cause erroneous results. For example, the B and D phenotypes are more readily identified by HPLC, CE and IEF.

These procedures, however, do not allow completely missing and truncated chains to be accurately characterized.

ESI-MS of Tf is the preferred screening method due to its sensitivity (10 μL sample volume), analytical speed (10 min per sample) and relatively simple operation. Analysis is entirely automated and provides structural information, thus allowing discrimination between CDG-I and CDG-II defects. Despite these advantages, the instrument is expensive and antibody is needed. MALDI- and SELDI-TOF-MS are highly specific and sensitive fully automated methods that can be used to screen Tf-based CDG. In constrast to IEF, they are not, however, capable of detecting small mass differences which can contribute to false positivity due to protein polymorphisms.

False negativity of screening methods presents a considerable problem since some CDG-Ia patients with proven genetic and enzymatic defects developed a nearly normal Tf pattern [131–133]. In these cases, a suspicious clinical picture might be the only indication to warrant further investigation. In dubious cases with inconclusive screening results, the analysis of multiple serum glycoproteins (eg, Tf with aAT, TBG or ATIII) by IEF and re-testing with a different method (e.g. 2-D PAGE, which relies on a combination of charge and size) is recommended, especially when the clinical suspicion of CDG is high.

Apart from the underglycosylated Tf that lacks one or both *N*-glycans, all known subtypes of CDG I show other small changes, such as increased fucosylation and reduced branching of serum glycoprotein *N*-glycans [126]. Even a combined *N*- and *O*-defect cannot be completely excluded in some CDG types I (e.g. defect of glycophorin A, mucin-type glycoprotein in CDG Ig).

CDG type II patients might present high diversity of unknown defects in the *N*- and the *O*-linked glycosylation pathways and thus diagnosis cannot rely on Tf analysis by IEF or ESI-MS. For CDG type IIc, a loss of lectin binding to specific glycoconjugates is reported to be pathognomic. The absence of Bombay blood phenotype excludes CDG type IIc, while normal expression of sialyl-Lewis ^{X} antigen on the surface of leukocytes excludes CDG types IIc and IIf. In the type IIb, the unprocessed glycans are cleaved by alternative biosynthetic pathways, and cannot be found. Instead, an increase in specific tetrasaccharide in urine (via TLC) serves as a screening marker. Nevertheless, about 20% of CDG patients with abnormal Tf IEF patterns remain untyped.

Combined *O*-glycosylation defects may be distinguished by IEF of apoC-III and thus complement typical CDG screening methods.

3.2. Confirmatory procedures

Although analysis of serum Tf can detect altered glycosylation, it cannot pinpoint the defect. For confirmation of screening results and precise CDG subtyping, more complex analysis is required.

Comprehensive profiling of single glycoprotein isoform (e.g. by 2-D PAGE) provides a complex overview of the sample itself and may assist in deciding whether the glycan structure requires further detailed investigation. Further analysis of LLO, NLG and glycosylation sites on human serum proteins help to narrow the search for defective genes.

Currently, MS of Tf plays the central role in laboratory screening of CDG-I. Analysis of enzymatically-released (non-derivatized or permethylated) oligosaccharides by MALDI-TOF-MS is complementary to IEF for identification of unclear CDG types II. However, ability of this method for detailed structural analysis may be limited, since the positions of individual sugar linkage cannot be correctly localized. A more informative technique may be ESI-MS which can be coupled directly with

CE or (graphitized carbon-)LC. Various combinations of these formats with in-line immunoaffinity purification of Tf may provide a viable option.

Analysis of Tf that focuses on the Asn-630 glycosylation site might be preferred (vs Asn-432 or global glycans released from Tf) for detecting the decreased fucosylation of CDG-IIc (combined *N*- and *O*-defect) and the defective glycan structures in CDG-IIa, IId and IIe [113].

Recent demands for glycoprotein analysis have focused on site-specific features, namely protein sequence, glycosylation sites and glycan structures, since these are required to understand carbohydrate function in the local protein folding relevant to the overall glycoprotein functions.

Analyses of tryptic glycopeptides by MALDI-TOF-MS or nano LC/ESI MS/MS (giving the information about amino acid sequence as well as glycan structure) is not yet widely used in CDG diagnostics. However, it might play an important and decisive role in site-specific glycan profiling (and elucidate the carbohydrate function in the local protein folding, and other connections). MS of glycopeptide, derived from endoproteinase Asp-N digestion is helpful for defining the abnormality of CDG-IIx and for detecting other *O*-glycosylation defects.

Enzyme assays, preferably in leukocytes (to exclude possibly false high PMM residual activities in rapidly dividing fibroblasts [134]) can confirm the diagnosis of CDG Ia and Ib (while only fibroblasts are suitable for analysis of the other enzymes). Recently, a combined ACE/ESI-MS procedure is a promising diagnostic tool for the most frequent CDG type.

Molecular genetic methods play an important role in CDG diagnostics, even if some unusual, less frequent mutations might present a diagnostic challenge. Once the functional mutation is found, it is confirmed at the genomic level in the afflicted patient, in parents, and sometimes in siblings.

Reliable CDG typing is essential from the point of view that CDG Ib is treatable by oral mannose [32], whereas fucose administration is partially effective in type IIc [135]. Patients with CDG type Ia, however, still await confirmation of a prospective study of membrane-permeant derivatives of mannose-1-phosphate prodrugs [136].

With respect to overall high newborn and infant mortality ascribed to CDG, thorough peri-/post-mortem (clinical, histological, biochemical and genetic) investigation of fatal cases is very important for correct diagnosis and genetic counselling. Prenatal diagnosis by enzyme assay (types Ia and Ib) or mutation (if recognized) analysis is available for all affected families. Carriers may show slightly altered Tf pattern or lower PMM activity in leukocytes. Newborn screening based on analysis of a marker protein (usually serum Tf) should not be performed before three weeks of age to avoid false-positive results [62].

4. Algorithm of investigations

4.1. Suspicious signs of glycosylation disorders

4.1.1. Clinical symptoms

Since no general clinical guideline for CDG screening exists, it is recommended to screen any child with unclear multisystem dysfunction with various symptoms, e.g. psychomotor retardation, severe seizures, dysmorphic features, adducted thumbs, cyclic vomiting, diarrhea and protein-losing enteropathy, severe hypoglycemia, liver fibrosis, effusions, tromboembolic and hemorrhagic events, anemia, leukocytosis, chronic inflammatory and autoimmune diseases, peripheral neuropathy, episodes of hyperthermia or skin anomalies (wrincled skin or cutis laxa).

4.1.2. Laboratory abnormalities

Apart from lower levels/activities of serum glycoproteins (clotting factors, Tf, ATIII, aAT), many basic biochemical parameters may be abnormal in CDG, such as low serum glucose, cholesterol, albumin, and sodium, higher aminotransferases activity, or increased blood platelets.

4.2. Screening

Key points: CDG is divided into two groups. All CDG subtypes I (so far known) and the IIa, IId, IIe, IIg, and IIh are detectable by Tf analysis, while CDG IIb, IIc, and IIf reveal normal Tf pattern. Patients with the CDG type I, and the subtypes IIa, IIb, and IId present a normal apoC-III isoform distribution, whereas those with CDG IIc, IIe, IIf, IIg, and IIh show an abnormal apoC-III profile.

When suspicious CDG signs are recognized in a patient, Tf-isoforms analysis (by IEF, HPLC, CZE, etc.) should be ordered for detection of hyposialylated glycoproteins.

4.2.1. Positive Tf-based screening

Before the primary CDG defect is assigned, unusual Tf protein variants need to be ruled out by having precede the neuraminidase treatment and studying the patient's parents. Also secondary causes of increased CDT (usually pattern 1) should be excluded. Analysis of other *N*-glycoproteins (e.g. aAT, TBG, or hexosaminidase) may confirm a generalized CDG type I disorder.

CDG type I versus type II: Tf analytical pattern may present a rough differentiation tool for both main CDG types (type 1 and type 2 patterns, see Fig. 1). A helpful discriminator may also be the dissociation of serum aminotransferases activity

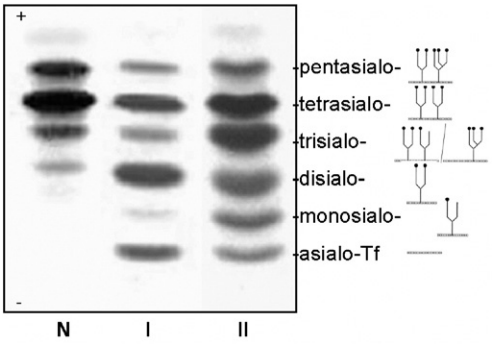

Fig. 1. IEF of serum Tf (N) Normal profile shows several bands with the highest intensity of the tetrasialo-Tf. (I) Type 1 pattern is characterized by decreased tetrasialo-, and increased asialo- and disialo-Tf bands (CDG types I). (II) Type 2 pattern may present various combinations of elevated asialo-, trisialo- and monosialo-bands, apart from the main disialo-Tf fraction (some CDG types II). Glycan structure of various Tf isoforms aside.

(↑AST and normal ALT, typical for the CDG type I, due to liver involvement?) and decreased platelet adhesion, found in some subtypes II. Elevation of plasma aspartylglucosaminidase is regarded as a useful marker for the CDG types I.

CDG subtyping: To differentiate between the individual CDG subtypes is not easy: when Tf-analytical pattern 1 is found, it may be helped by analysis of β-trace protein in cerebrospinal fluid (distinct pathology in the type Ia), serum α_1-antichymotrypsin (type Ic and Ia differentiation), or abnormal red cell-band 3 and glycophorin A with decreased serum IgG level, typical namely for CDG Ig.The CDG type Ib and Ih could be identified on the basis of clinical symptoms. In samples with Tf-analytical pattern of CDG type II, analysis of apoC-III may distinguish the combined subtypes IIc, IIe, IIf, IIg, and IIh from the other subtypes II.

4.2.2. Negative Tf-based screening

When normal Tf-IEF pattern together with CDG-suspicious clinical signs is found, TLC analysis of urine oligosaccharides (tetrasaccharide glc_3man_1) is indicated for the type IIb exclusion, while the Bombay blood phenotype and membrane bound sialyl-Lewis^X antigen in neutrophils are pathognomic for the CDG types IIc and IIf. However, neither CDG types I can be completely excluded on the basis of normal Tf-(IEF) screening result and further analyses of LLO and NLG should follow in suspicious cases.

4.3. Confirmation, detailed subtyping

CDG type Ia (type Ib) enzyme assays: When Tf screening pattern 1 has been found, the patient leukocytes should be isolated for enzyme assays, namely PMM for the most common type Ia, and PMI for the type Ib identification. Finding a deficient PMM specific activity, the diagnosis of the CDG type Ia is the conclusion. When the clinical symptoms call first for PMI assay and low enzyme activity is found, CDG type Ib is diagnosed and mutation analysis of the relevant gene then folows. In some laboratories, patients with Tf pattern 1 are first analysed for the locally most frequent PMM2 mutations; eventually, all exons of PMM2 are sequenced. When normal, the next step is the assay for PMM activity, since not all gene defects are easily detected by routine sequencing.

If the CDG types Ia or Ib are not confirmed, mutations in the gene ALG6 (CDG type Ic) should be checked for; when negative, cell lines should be obtained for LLO analysis, followed by a relevant enzyme assay (if developed) option and responsible-gene analysis for the other CDG I subtypes identification.

Detection of the Tf screening pattern 2 is indicative for NLG analysis and other investigations to suggest an affected step, followed by the CDG types IIa and IId specific enzyme assays, or leading to identification of COG7, COG1 or COG8 defects, typical for CDG types IIe, IIg and IIh, respectively.

Analysis of serum NLG from patients with normal Tf screening pattern but positive screening test for CDG type IIb (TLC of oligosaccharides) may be further confirmed by the appropriate enzyme test, while recognized membrane abnormalities, specific for types IIc and IIf lead to further investigations confirming the transport defects.

Combined *N*- and *O*-glycosylation disorder in CDG types IIc, IIe, IIf, IIg, and IIh can be detected by serum apoC-III screening analysis; hereafter, the structure of the protein-/peptide-*N*- and *O*-linked glycans should be assayed.

Various screening techniques have been used (several commercial kits are available) to detect abnormal glycosylation for the CDG diagnosis, exploiting the change in charge, and/or size, or lectin affinity of the glycoforms. However, most of them require preliminary purification or they are labor-intensive and low-throughput methods.

4.4. Modern screening and diagnostic strategies

An advanced approach to CDG diagnosis in several screening centers is based on a direct ESI-MS analysis. Generally suggested CDG screening starts with IEF and immunological detection of Tf in one portion of serum sample. In the next step, Tf is affinity-isolated from the second portion and goes on either by ESI-, MALDI-, or SELDI-TOF-MS analysis for the basic type I-type II differentiation; these methods can also be applied to infant blood cards. If the CDG type I was excluded, investigations for detailed type II identification should follow, e.g. by combined characterization of *N*-glycans (PNGase F-released) and *O*-glycans (chemically released by reductive elimination) from specific (Tf) or whole serum glycoproteins; based on (MALDI-linear TOF-) MS strategy, the structures of all components are defined [113,137]. Analysis of specially isolated glycopeptides makes already a part of some screening programs.

An overall proposed strategy for identifying disorders of glycosylation combines proteomics (2-D PAGE gives broad information about serum glycoproteins, suggesting potential disease targets and links to pathogenesis) with a detailed glycan analysis of a pool or of specific serum proteins from a patient and healthy controls. Digestion with arrays of specific exoglycosidases followed by automatic (BioGel P4) fractionating is a useful tool for further sequencing and final characterization of oligosaccharides [112]; thus, the defect severity or cell specificity of the recognized disorder could be assigned.

Otherwise slow process of effective, patient-specific genes identification in the DNA-based screening can be improved by cDNA-complementation studies, by a viral gene expression approach, or once perhaps by use of DNA microarrays/gene chips (that represent all known and potential defects).

5. Conclusions

At each step of CDG investigation, various techniques can be used as methods of choice, and are listed here, so that the reader, a potential user, can make his option mainly according to the accessibility of particular instrumentation. This study is not intended to be fully comprehensive; nevertheless it might give a practical view of the investigative procedures most commonly used in the diagnostics of CDG.

A survey of possible approaches to screening and diagnostics of CDG is presented with the intention (1) to give an idea to the physician about the clinical picture and other findings

(biochemical, histological, imaging, etc.) that are associated with this, still unfamiliar group of diseases, and (2) to provide clinical chemists with prompt information with respect to the references to various techniques here described in a perfunctory way, which are essential or might be helpful in the diagnostics. Also prospective procedures so far uncommom are mentioned. The proposed algorithm of the multi-step investigation rounds out the review.

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