Screening Using Serum Percentage of Carbohydrate-Deficient Transferrin for Congenital Disorders of Glycosylation in Children with Suspected Metabolic Disease

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BACKGROUND: Diagnoses of congenital disorders of glycosylation (CDGs) are based on clinical suspicion and analysis of transferrin (Tf) isoforms. Here we present our experience of CDG screening in children with a suspected metabolic disease by determination of serum percentage of carbohydrate-deficient transferrin (%CDT) in tandem with isoelectric focusing (IEF) analysis of Tf and α₁-antitrypsin (α₁-AT).

METHODS: We performed approximately 8000 serum %CDT determinations using %CDT turbidimetric immunoassay (TIA). In selected samples, IEF analysis of Tf and α₁-AT was carried out on an agarose gel (pH 4–8) using an electrophoresis unit. The isoforms were detected by Western blotting and visualized by color development. We performed neuraminidase digestion of serum to detect polymorphic variants of Tf.

RESULTS: We established a cutoff value for serum %CDT of 2.5% in our pediatric population. Sixty-five patients showed consistently high values of serum %CDT. In accordance with Tf and α₁-AT IEF profiles, enzyme assays, and mutation analysis, we made the following diagnoses: 23 CDG-Ia, 1 CDG-Ib, and 1 conserved oligomeric Golgi 1 (COG-1) deficiency. In addition, we identified 13 CDG-Ix non Ia, non-Ib; 3 CDG-Ix; and 9 CDG-Ix cases, albeit requiring further characterization; 9 patients with a secondary cause of hypoglycosylation and 6 with a polymorphic Tf variant were also detected.

CONCLUSION: The combined use of CDT immunoassay with IEF of Tf and α₁-AT is a useful 1st-line screening tool for identifying CDG patients with an N-glycosylation defect. Additional molecular investigations must of course be carried out to determine the specific genetic disease.

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Congenital disorders of glycosylation (CDGs)3 are a group of genetic diseases caused by defects in the biosynthesis of the glycans moiety of glycoproteins and glycolipids (1). To date, 30 CDG hypoglycosylation defects have been identified, including 17 N-glycosylation defects (13 CDG-I or N-glycan assembly defects and 4 CDG-II or N-glycan processing defects), 6 O-glycosylation defects, 5 combined N- and O-glycosylation defects, and 2 lipid glycosylation defects (2–4, 5–9).

Except for the dominantly inherited multiple exostoses syndrome, the known CDGs are autosomal recessive transmitted and the clinical spectrum is highly heterogeneous, ranging from severe multisystem to monooorgan disease. CDG Ia is caused by phosphomannose mutase (PMM) deficiency (10), and except for hereditary multiple exostoses, is the most commonly encountered type, affecting mainly the nervous system (neurological form) with variable involvement of other organs such as the heart, liver, gastrointestinal tract, and gonads (multisystemic form) (11). Among the CDG-I group, CDG-Ib is peculiar because it is characterized by hepatointestinal symptoms without nervous system involvement (12).

N-glycosylation defects involve deficient synthesis of N-glycans of several serum glycoproteins, which results in deficient incorporation of sialic acid, the terminal negatively charged sugar (13–16). The incomplete addition of sialic acid to a proportion of serum trans-
ferrin (Tf) molecules is the most commonly used marker for initial diagnosis of a CDG. Tf, one of the predominant serum glycoproteins, carries 2 bi- or triantennary carbohydrate chains, each terminated with 2 or 3 sialic acid residues. The least sialylated isoforms of Tf, with 0 (asialoTf), 1 (monosialoTf), and 2 (disialoTf) sialic acids, are usually referred to as carbohydrate-deficient transferrin (CDT) (17). Because sialic acid has a negative charge, the isoelectric focusing (IEF) patterns of hyposialylated Tf show, in most CDG patients, a cathodal shift that can be caused either by a complete absence of carbohydrate chains (type 1) or by structurally abnormal carbohydrate chains (type 2). Therefore, analysis of Tf glycosylation status could indicate a glycosylation abnormality but not the specific defect. In addition to CDG, other inherited or acquired pathological conditions—such as hereditary fructose intolerance (HFI) (18), galactosemia (19), cystic fibrosis (20), achondroplasia (21), decreased ferritin concentrations (22), severe liver pathology (cirrhosis, chronic viral hepatitis, and hepatocarcinoma) (23–25), hemolytic uremic syndrome (HUS) associated with Streptococcus pneumoniae infection (26), combined pancreas and kidney transplantation (17), chronic pulmonary obstructive disease (27), or genetic Tf variants (28)—may cause hypoglycosylation.

We present our results of CDG screening in 7910 children with suspected metabolic disease. We determined the plasma/serum %CDT in tandem with IEF analysis of Tf and α1-antitrypsin (α1-AT). Fifty patients had been tentatively classified as suffering a CDG; in half of these cases the deficiencies were genetically confirmed. In another 15 cases the abnormalities found were attributable to a secondary alteration. We have focused our efforts on resolving primary and secondary N-glycosylation defects.

Materials and Methods

STUDY PARTICIPANTS AND DIAGNOSTIC FLOW CHART
To establish references values for the different sample types, sex, and age groups, we measured %CDT in 250 serum samples, 253 heparin or citrate-plasma, and 125 EDTA-plasma samples from healthy controls and patients in whom infectious, liver, and metabolic disease were excluded. Plasma or serum samples from 7910 pediatric patients (newborn to 16 y, mostly white) from mainly nephropediatric, neonatology, and intensive care services of various Portuguese and Spanish hospitals were referred to the laboratories in Porto (IGM) and Madrid (CEDEM) for metabolic disease screening. Patients presented with neurological disorders that were isolated or with additional symptoms compatible with metabolic disorder. Samples were received frozen or in a refrigerated storage container and stored at −20°C until analysis. We measured %CDT with an immunoturbidimetric method (29), and %CDT values higher than the established cutoff value were confirmed in a 2nd serum sample. Confirmed increase results were evaluated with regard to the clinical history of the patient, and IEF analysis of Tf and α1-AT was subsequently performed. In some cases, neuraminidase digestion of the serum and a family study were performed to rule out a polymorphic variant of Tf.

REAGENTS
We obtained agarose IEF, ampholines pH 4-6 and 5-8, and gel bond film for agarose from Amersham Bioscience; rabbit antihuman Tf antibody from the reagent set %CDT TIA (turbidimetric immunoassay) (Bio-Rad); rabbit antihuman α1-AT antibody from Dako; phosphatase alkaline–conjugated goat antirabbit antibody from Sigma; and nitroblue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP) solution and neuraminidase from Clostridium perfringens from Roche. All reagents were of analytical grade.

%CDT ANALYSIS
We determined %CDT by use of %CDT TIA Bio-Rad microtiter plate version, with measurements made according to the manufacturer’s instructions. This assay measures the sum of the Tf variants containing 0–2 sialic acid residues relative to the amount of total Tf. Samples were initially saturated with Fe3+ to avoid charge variations resulting from a variable degree of transferrin iron saturation that might affect chromatographic elution. The CDT isoforms were separated on ion-exchange chromatography microcolumns; eluted CDT isoforms were then quantified using anti-Tf antibodies. We measured the total concentrations of serum Tf separately using the same anti-Tf antibody. These measurements were carried out on a 680X microplate reader (Bio-Rad) at 405 nm.

STATISTICAL METHOD
Data are expressed as the mean (SD). Comparisons between groups were carried out using the SPSS program (version 12.0 for Windows). A p-value <0.005 was considered statistically significant.

IEF with western blotting of serum TF and α1-AT
For Tf analysis, we incubated serum aliquots at room temperature for 30 min with an Fe(III) solution buffer at a 1:5 ratio to saturate the transferrin with iron. The mixture was diluted 1:50 with distilled water. For α1-AT analysis, we incubated serum aliquots at room temperature for 30 min in a buffer solution containing 25 mmol/L dithiothreitol and 12.5 mL/L Tween-20. The mixture was diluted 1:100 with distilled water. We carried out IEF of these serum glycoproteins ac-
According to a published method (16) with the following modifications. Agarose gel of 0.5-mm thickness was molded on a gel bond–agarose film. The gel was prepared by dissolving 0.012 mg/L agarose, 0.145 mg/L sorbitol, 1:10 (vol:vol) glycerine, 1:20 (vol:vol) phar-malyte 4–6.5 and phar-malyte 5–8 in deionized water. Electrode solutions consisted of 1 mol/L NaOH and 0.05 mol/L SO4H2 at the anode and cathode, respectively. We applied 3 μL of the diluted samples to the gel on a MultiPhor II electrophoresis unit (Amersham Bioscience). IEF was performed at 1500 Vh at 10° C. After transfer to 0.2-μm nitrocellulose membranes, detection of TF or α1-AT isoforms was performed using rabbit antihuman TF antibody or rabbit antihuman α1-AT antibody, respectively, both diluted 1:250. Once the primary antibodies were removed and washed, phosphatase alkaline–conjugated goat antirabbit antibody (dilution 1:2500) was added. Membranes were stained with an NBT/BCIP solution.

To discard a polymorphic variant of TF, selected samples were digested with neuraminidase from Clostridium perfringens to remove the negatively charged terminal sialic acid residues. Plasma/serum samples and 10 kU/L neuraminidase in 0.1 mol/L Tris-ClH, pH 7, 10 mmol/L Fe citrate in 0.5 mol/L NaHCO3 were incubated in a ratio of 1:2:1 at 37 °C for 24 h. The mixture was diluted 1:300 with distilled water and loaded onto a gel.

**Results**

**ESTABLISHMENT OF REFERENCE %CDT VALUES**
We grouped blood samples according to the type of anticoagulant used and the age and sex of the children to obtain %CDT reference values for our pediatric population. No significant differences were found between serum values and heparin/citrate plasma values (Fig. 1a). The values for EDTA-plasma were significantly higher (P < 0.005), however, with a mean %CDT value of 4.1%, because EDTA may disturb in vitro iron–Tf saturation (30). We confirmed that EDTA sampling is related to higher %CDT by checking for values within the reference interval in a new requested serum sample. On the other hand, no differences were found with regard to sex (P = 0.498) (Fig. 1b) or age when we compared 2 groups of children ages 1–6 months or 6 months to 16 years (P = 0.143) (Fig. 1c). Nevertheless, the %CDT values for neonates were slightly higher (P = 0.03), with mean %CDT values of 2.05 (Fig. 1c). Statistical analysis of results, excluding those from neonates (<1 month), revealed that normal %CDT values ranged from 0.6% to 3.5% and the mean (SD) was 1.77 (0.45). We established a cutoff value for %CDT of 2.5% based on the 95th percentile of reference value distribution (Fig. 1d).

**CDG CASES**
For selection of possible patients with N-glycosylation defects, we asked for a new serum sample or we performed Tf IEF analysis in cases presenting a %CDT value >2.5%, even in those with values of 2.6% to 3.5% that were in the reference interval. Sixty-five patients had consistently high values of serum/plasma %CDT, ranging from 3.4% to 40.4%. A review of their clinical histories and laboratory results, including TF and α1-AT IEF analysis, led us to tentatively classify 50 cases as CDG and 15 as having a secondary glycosylation alteration or a TF protein variant. Serum IEF-Tf allowed us to classify 40 of these 50 as probable CDG-I patients (type 1 Tf IEF profile with increased amounts of asialo- and disialo-Tf; a representative example is shown in Fig. 2, lane 3) and 10 as probable CDG-II patients (type 2 asialo TF IEF profile with increased amounts of asialo-, monosialo-, and disialo-Tf (31); a representative example is shown in Fig. 2, lane 4). Of the 40 CDG-I patients, 23 showed a deficiency of PMM activity in fibroblasts [values ranging from 0.64–2.9 μU/mg protein; control value 3.77 (0.86)] and PMM2 mutations, and 1 a deficiency of phosphomannose isomerase (PMI) activity in fibroblasts [2.6 μU/mg protein; control value 15.9 (3.8)] and PMI mutations. Thirteen unresolved CDG-I patients showed normal PMM and PMI activity, and for the 3 remaining unresolved CDG-I cases no whole blood or fibroblast samples were available for further enzymatic and genetic studies. All of these suspected CDG-I patients had suggestive clinical signs and a clearly abnormal IEF-Tf profile in at least 2 serum samples. Of the samples from 10 probable CDG-II patients, 1 sample also showed on IEF a cathodal shift of the O-glycosylated apolipoprotein CIII (not shown), and conserved oligomeric Golgi complex subunit 1 (COG-1) deficiency was later diagnosed in that sample donor (4). The other 9 patients presented a wide range of symptoms including multisystem disease and hepatic, neurological, or myopathic disease. All of the unresolved CDG-I and -II cases also displayed an altered isoform profile for the glycoprotein α1-AT (a representative example is shown in Fig. 3, lanes 1 and 4), pointing to a generalized hypoglycosylation and requiring further characterization.

**ALTERATIONS SECONDARY TO OTHER DISEASES**
We detected increased serum %CDT (from 5.2% to 16.4%) in 9 cases with a hypoglycosylation secondary to cystic fibrosis, Epstein-Barr virus (EBV) infection, HFI, and galactosemia. Patients with cystic fibrosis (serum %CDT value 6.2%; Fig. 4, lane 3) or EBV infection exhibited a type 2 Tf IEF profile, and patients with HFI or galactosemia a type 1 profile (Fig. 4, lanes 4 and 6). With dietary treatment, serum Tf IEF and %CDT val-
ues normalized in both HFI and galactosemia patients (Fig. 4, lanes 5 and 7).

**POLYMORPHIC VARIANTS OF TF**

The remaining 6 patients with a presumed secondary alteration of glycosylation had polymorphic variations of Tf confirmed by neuraminidase digestion of serum samples from patients and their parents. In these patients serum %CDTs ranged from 3.4% to 9.1%. Four different serum Tf IEF profiles were found to be associated with these transferrin variants: CDG-Ia with a double band at the disialoTf position (Fig. 5, lane 5), a prominent band at the trisialoTf position (Fig. 5, lane 7), a prominent band at the position of asialoTf (Fig. 5, lane 9), and a pattern characterized by an anodal shift (decreased tetra- and pentasialoTf and strong hexa- and heptasialoTf bands) (Fig. 5, lane 11). The latter was associated with a %CDT within the reference

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**Fig. 1.** Statistical analysis of serum/plasma %CDT reference values in the pediatric population. Comparison of mean values for different groups of samples. a. Serum and plasma ($P = 0.200$) or plasma-EDTA samples ($P < 0.005$). b. Serum correlated to sex ($P = 0.498$). c. Serum correlated to age (≤1 month ($P = 0.03$) or 1–6 mo or 6 mo to 16 y ($P = 0.143$)). d. Range, mean (SD), median and percentile distribution of values. Statistical analysis was performed with SPSS 12.0.
Fig. 2. Serum Tf isoform profiles of transferrin from CDG patients and controls.

Lanes 1 and 2: normal profile of a control individual; lane 3: a type 1 profile that corresponded to a CDG-Ia patient; lane 4: a type 2 profile that corresponded to a CDG type II patient. Numbers at left indicate the number of sialic acid residues for each transferrin isoform.
interval. In all cases, a double band appeared at the asialoTf concentration after neuraminidase digestion. The parents showed Mendelian inheritance of these polymorphic variants, with high %CDT serum concentrations similar to those of their children, with the exception of the father of the CDG-Ia patient, who showed a normal value (2.1%).

Discussion

We have quantified the serum %CDT value as a 1st step in looking for defects in protein N-glycosylation among patients with a suspected metabolic disease. Further diagnostic workup by Tf IEF analysis confirmed abnormal serum %CDT results. Tf is the most widely used screening method because its reliability and easy interpretation; moreover, it permits orientation to N-glycan assembly (CDG-I) or processing (CDG-II) defects (32). The last step in our screening flow chart was to analyze serum H9251 1-AT, as a complement to selective screening with analysis of Tf, to confirm a generalized hypoglycosylation (14). This was particularly helpful in patients with a combination of a CDG defect and a rare Tf variant.

With our screening strategy, we tentatively classified 50 patients as having a CDG among 7910 serum/plasma samples analyzed. Twenty-five were genetically confirmed [23 CDG type-Ia, 1 CDG type Ib, and 1 conserved oligomeric Golgi 7 (COG7) deficiency]; in addition, 16 patients with an unsolved CDG defect displaying a typical type I TfIEF profile were classified as CG-Ix. Nine patients with the asialo type 2 Tf IEF profile were also identified. But as long as the primary defect in these other 25 unsolved CDG patients has not been determined, the significance of these abnormalities remains unknown. To elucidate the primary defect, further diagnostic investigations will be necessary, including apolipoprotein C-III IEF analysis to detect combined N- and O-glycan biosynthesis defect, determination of dolichol-linked oligosaccharides, analysis of N-glycan structures linked to glycoproteins, and mutation analysis.

To quantify serum carbohydrate-deficient transferrin, we used the commercial %CDT TIA set, which principally measures asialo-, monosialo-, and disialoTf instead of the absolute CDT values (U/L) obtained from a CDTect test. The former has the advantage of expressing CDT concentrations as percentages of total Tf to compensate for variations in total transferrin concentration. We initially established a cutoff value of 2.5% for our pediatric population and found no differences in the plasma/serum %CDT values between sexes or age groups (>1 month). In children younger than 1 month, however, we found a wide range and a higher mean of %CDT reference values, probably reflecting the increase in hypo-
sialylated fractions in children during the 1st days of life (33). In our hands, measurement of serum %CDT was a rapid and easy technique to promptly detect glycosylation alteration.

Our screening strategy has limitations. Considering that certain CDG subtypes do not result in a hyposialylation of Tf (CDG-IIb, IIc, and III) (34–36) and that some confirmed CDG-la patients have been reported to have normal serum glycoproteins (37, 38), we cannot exclude the possibility that some cases were missed. Therefore, in clinically suspected cases CDG diagnosis should be reviewed with other biochemical findings. Secondary causes of abnormal glycosylation, such as some inherited or acquired diseases and Tf protein variants, may also yield higher serum %CDT values and abnormal Tf-IEF profiles.

We detected abnormal Tf results in some patients with inherited diseases (galactosemia, HFI, and cystic fibrosis). A female patient with cystic fibrosis showed high %CDT as well as a type 2 Tf-IEF profile, supporting the hypothesis that altered function of cystic fibrosis transmembrane conductance regulator (CFTR) affects the compartmentalization of glycosyltransferases in the Golgi, thereby disturbing protein glycosylation (39). Moreover, we have found several types of Tf polymorphisms that hampered efforts to reach a conclusive diagnosis in sick children. The most common polymorphism in human serum is TfC (>99%), whereas TfB (lower pI) and D (higher pI) variants, which possess a different primary structure but a normal set of carbohydrate chains, occur less often (28). After neuraminidase treatment, all homozygote Tf variants present 1 band at the asialoTf level, whereas heterozygote variants show 2 bands, based on the particular subtype. We detected a high %CDT value due to the rare TfC1D4–5 variant in a newborn presenting with metabolic ketoacidosis and hyperammonemia, who showed an abnormal TfIEF profile with a considerable but isolated increase in the asialoTf band (40). The patient did not show any abnormality of the α1-AT IEF pattern and was finally diagnosed with a holocarboxylase synthetase deficiency. Another complicated case was a 14-month-old girl presenting with hypotonia and motor delay since the neonatal period. She had a heterozygous CB TF phenotype, a condition that regularly yields low CDT values, but she showed consistently high serum %CDT values together with an abnormal α1-AT IEF profile, findings that were finally attributed to a generalized disturbance in N-glycosylation caused by CDG-Ia syndrome. In this case, the abnormal α1-AT analysis facilitated further diagnostic studies. Thus, reliable identification of Tf variants and the analysis of other N-glycosylated proteins provide useful information regarding CDG (14).

In conclusion, in screening some 8000 patients with suspected metabolic disease, we identified 50 patients with suspected CDG syndrome; in half of them, the basic defect could be identified. Immunoassay of CDT combined with the IEF of Tf and α1-AT techniques offers a valuable 1st-line screening tool for identifying patients with a CDG associated with N-glycoprotein hypoglycosylation.

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