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Regular paper

Transferrin isoform analysis from dried blood spots and serum samples by gel isoelectric focusing for screening congenital disorders of glycosylation

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Congenital disorders of glycosylation (CDG) are a growing, heterogeneous group of genetic disorders caused by a defect in the glycoprotein synthesis. The first and still widely used method for routine CDG screening was isoelectric focusing (IEF) of serum transferrin. Dried blood spot (DBS) testing is commonly used in newborn screening procedures to detect inborn errors of metabolism. The aim of this study was to demonstrate the reliability of the IEF method in DBS testing. Dried blood spot testing can help in the postmortem diagnosis of CDG disorders when other material is unavailable. The patterns and concentrations of transferrin isoforms in serum and DBS are comparable, and slight differences do not affect interpretation of results.

Keywords: isoelectric focusing, dried blood spot, congenital disorders of glycosylation, transferrin isoforms

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Abbreviations: CDG, congenital disorders of glycosylation; IEF, isoelectric focusing; DBS, dried blood spot; Tf, transferrin; GlcNAc, N-acetylglucosamine; Dol-P, dolichol phosphate; LLO, lipid-linked oligosaccharide structure; Glc, glucose; Man, mannose; Sia, sialic acid; PMM2-CDG, phosphomannomutase 2 deficiency; MPI, mannose phosphate isomerase; VMA21-CDG, vacuolar ATPase assembly factor; ALG3-CDG, alpha-1,3-mannosyltransferase deficiency

INTRODUCTION

Congenital disorders of glycosylation (CDG) are a heterogeneous group of genetic disorders caused by a defect in the glycoprotein synthesis (defects in protein N-glycosylation, and O-glycosylation), or glycosphingolipid, glycosylphosphatidylinositol anchor glycosylation defects and multiple glycosylation pathway defects (Jaeken *et al*., 2017; Francisco *et al*., 2019).

From the first report by Jaeken and others in 1980, over 150 CDG subtypes, have been described (Jaeken *et al.,* 1980; Verheijen *et al*., 2020). Disorders of the N-glycosylation pathway are detected by analysis of serum transferrin isoforms using different methods (Wolking *et al*., 2019). The methods routinely used for CDG screening include isoelectric focusing (IEF), capillary zone electrophoresis (CZE) and high-performance liquid chromatography (HPLC).

Transferrin isoelectric focusing was introduced in 1984 and is still widely applied and considered as the gold standard for CDG screening (Bruneel *et al*., 2020; Jaeken *et al*., 1984). Mass spectrometry (MS) techniques are currently giving more detailed insights into the glycan structural abnormalities (van Scherpenzeel *et al*., 2016). Electrospray (ESI) MS and matrix assisted laser desorption ionization (MALDI) MS are useful for underglycosylation analyses of intact serum transferrin (Tf). MALDI MS analysis of N-linked glycans released from total plasma or targeted glycoproteins, is the mainstream tool to explore abnormal glycosylation in CDG-II patients (Sturiale *et al*., 2011).

N-glycosylation of proteins

The N-glycan synthesis starts on the cytoplasmic side of the endoplasmic reticulum membrane. Two N-acetylglucosamine (GlcNAc) residues are transferred to dolichol phosphate (Dol-P) using UDP-GlcNAc as GlcNAc donor. This process is catalyzed by two enzymes: GlcNAc-1-P transferase and chitobiose synthase. Thus formed GlcNAc2-P-P-Dol structure, is extended by five mannosyltransferases using GDP-mannose as the donor substrate. Next the formed Man5GlcNAc2 structure is translocated to the lumenal side. A flipping enzyme catalyzes this process. There, further elongation of the oligosaccharide chain takes place by mannosyltransferases and glucosyltransferases to a Glc3Man9GlcNAc2 oligosaccharide. Next, lipid-linked oligosaccharide structure (LLO) is transposed in N-linkage to an asparagine residue of nascent protein by oligosaccharyl transferase (OST). After the removal of glucose (Glc) residues by glucosidases and one mannose (Man) by mannosidase, the glycoprotein structure is transferred to the Golgi by vesicular transport. In the Golgi, mannosidases cleave mannoses and GlcNAc-transferases attach GlcNAc. Both antennas are then elongated by the addition of galactose (Gal) and sialic acid (Sia) residues to form the Sia2Gal2GlcNAc2Man3GlcNAc2 structure. Fucosyltransferase VIII attaches a fucose residue to some of the glycoproteins. In this way, several types of glycans are created based on a common core consisting of two N-acetylglucosamine residues and three mannose residues. The glycoproteins are then transported from the Golgi to their destinations. Defects related to the LLO chain synthesis and its transfer to a protein are termed CDG type I glycosylation disorders. Defects in processing of the protein-bound glycans are classified as CDG type II (Marquardt *et al*., 2003; Kornfeld *et al*., 1985).

Transferrin

Serum transferrin (Tf) is used for routine screening of CDG with an N-glycosylation defect. This glycoprotein

is synthesized in the liver, and it's function is iron transport. Tf is composed of a single polypeptide chain that carries two Asn-linked complex-type N-glycan chains (Brunnel *et al*., 2020). Depending on the structure of glycans, there are several isoforms of transferrin. In healthy people, the serum has five fractions of Tf with a predominance of tetrasialotransferrin, with two sialic acid residues on each of the two glycans. The disialotransferrin, trisialotransferrin, pentasialotransferrin and hexasialotransferrin, containing glycans with one or three sialic acid residues, are present in much smaller amounts. In CDG-I, the lack of N-glycan chains leads to a decrease in the tetrasialo- and a marked relative increases in the disialo- and asialotransferrin isoforms. In CDG-II, deficient N-glycans lead to a more or less combined increase in the trisialo-, disialo- and asialotransferrin isoforms by trimming processing (Brunnel *et al*., 2020).

Abnormal transferrin isoforms are also observed in untreated fructosemia and galactosemia in genetic diseases causing secondary hypoglycosylation. Patients with these diseases typically present with an abnormal transferrin pattern of type I, which is completely normalized or reaches normal values during dietary treatment (Adamowicz *et al*., 2007; Pronicka *et al*., 2007). Abnormal profile suggestive for type I is also observed in case of increased alcohol intake (Jaeken *et al*., 2017).

In some neonatal cases, it has been reported that the serum transferrin cathode fractions are slightly elevated, (mainly asialo-, monosialo- and sometimes also disialotransferrins), resembling a mild type II pattern (Peanne *et al*., 2018). Hypoglycosylation has been observed in patients with impaired liver function and infections with neuraminidase-producing microorganisms (Jaeken *et al*., 2017; Jansen *et al*., 2020, Bogdańska *et al*., 2021).

MATERIALS AND METHODS

Sample collection

Serum and DBS samples were collected from 12 patients with congenital disorders of glycosylation: eight from phosphomannomutase 2 deficiency (PMM2-CDG) patients, two from mannosephosphate isomerase deficiency (MPI-CDG) patients, one from vacuolar ATPase assembly factor (VMA21-CDG) patient and one from alpha-1,3-mannosyltransferase deficiency (ALG3-CDG) patient; two samples were from patients with transferrin polymorphism and 36 from healthy control group patients. Samples were stored at –20°C.

Dried blood samples were applied to filter paper (Whatman 903) and dried at room temperature for 24 h before being stored at –20°C.

Preparation of dried blood spot samples

Preparation of 48 dried blood spot samples was carried out according to the method described by Wolking et al (Wolking *et al*., 2019). Five discs with 3 mm diameter were punched out from dried spot cards (filter paper Whatman 903). Then, 50 µl of ultrapure water were added and incubated over night at about 7°C. Next, 10 µl of eluate were mixed with 1.25 µl of 10 mM iron (III) citrate and incubated at room temperature for 10 min. Then, 1.25 µl of 0.1 M $NaHCO_3$ was added and mixed (Wolking *et al*., 2019).

Preparation of serum samples

Serum samples were prepared by the method described by van Eijk et al. 20 µl of serum were mixed with 80 µl of 0.9% NaCl. Next, 2 µl of 10 mM Fe(III) citrate and 2 µl of 0.1 M $NaHCO_3$ were added and incubated for half an hour at room temperature (van Ejik *et al*., 1983).

Isoelectric focusing (IEF)

Transferrin isoform analysis from dried spot cards and serum was analyzed by isoelectric focusing agarose gel electrophoresis according to the method described by van Eijk *et al*., Stibler *et al*., Jaeken and others (van Ejik *et al*., 1983; Stibler 1979; Jaeken *et al*., 1993). IEF is a very efficient fine separation technique of Tf glycoforms (Brunnel *et al*., 2020).

Preparation of a 1% agarose gel

First, 45 mg of agarose was added to 4.5 ml of distilled water and incubated in a boiling water bath for 10 minutes. Next, 230 µl of ampholines in a pH range of 5.0–7.0 was added. After mixing, the liquid gel was poured out over a plastic plate (Gel- Fix) placed on an LKB table heated to 50°C (van Ejik *et al*., 1983; Stibler 1979; Jaeken *et al*., 1993).

Electrofocusing and immunofixing

Electrode wicks were soaked with electrode solutions: anode – 0.04 M glutamic acid, cathode – 0.5M NaOH, and then were placed on agarose gel in the Multiphor apparatus. The gel ampholine gradient was obtained by pre-focusing, which was carried out for 15 minutes at a constant current of 1.5 W. Serum samples (1 µL) were applied to the gel, 2 cm from the cathode, and separated for 20 minutes. During this period, the current decrease occurred in the range of 6 mA to 3 mA, and the voltage increased in the range of 300 V to 500 V. After 20 minutes, the power was turned off, the electrode wicks were removed and 200 µl of polyclonal rabbit anti-human transferrin antibodies were applied to the gel surface to form transferrin-antibody complexes. The gel was transferred to a moist chamber where it remained for 20 minutes. After this time, the antibodies from the gel surface were rinsed with 0.9% NaCl, and the gel was placed in 0.9% NaCl overnight. The next day, the gel was rinsed with distilled water for 30 minutes (van Ejik *et al*., 1983; Stibler 1979; Jaeken *et al*., 1993).

Drying and staining the gel. After removing the gel from the water, it was dried with a 0.5 cm layer of filter paper for 30 minutes using a Paragon blotting equipment and then dried for 5 minutes with a hair dryer until a transparent film was obtained. The gel was stained for 10 minutes in a 0.5% Coomasie Brillant Blue solution (500 mg Coomasie Brillant Blue in 100 ml of destaining solution). To remove excess stain, the gel was placed in a destaining solution (350 ml ethanol, 100 ml acetic acid, 650 ml distilled water) three times for a few minutes until background color was removed and transferrin bands were clearly visible (van Ejik *et al*., 1983; Stibler 1979; Jaeken *et al*., 1993).

Quantification of IEF results

The percentage of transferrin fractions was assessed using a densitometer (Beckman).

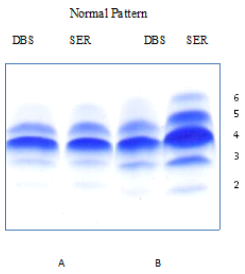

Figure 1. IEF of transferrin from serum and DBS samples
(**A, B**) typical exemplars of normal pattern in the control group; 2, disialoTf; 3, trisialoTf; 4, tetrasialoTf; 5, pentasialoTf; 6, hexasialoTf; DBS, dried blood samples; SER, serum

RESULTS

Transferrin isoforms were determined in patients of the control group (n=36) (see Fig. 1). The relative amounts of isoforms and the mean of the DBS and serum results are presented in Table 1. For all patients, there was a difference in the relative amounts of isoforms in DBS when compared to serum. The most significant difference was observed for the disialotransferrin fraction, where values higher up to 14.7% occurred in the serum. This material also showed higher values for trisialotransferrin (by 1.25%) and for hexasialotransferrin (by 1.5%). In the case of the tetrasialo- and pentasialotransferrin fractions, the values were higher for DBS by 0.95% and 0.5%, respectively. Taking into account the mean values, the isoform values differed very slightly (by 0.1%) for tri-, pentasialo- and hexasialotransferrins. In the case of disialo- and tetrasialotransferrin the difference is larger (higher by 0.6%). There were no asialo- and monosialotransferrins in dried blood spots, similar to results obtained for the serum. The concentrations of transferrin isoforms in the serum and DBS are comparable, and the differences do not affect the clinical interpretation of the results.

Analysis of a dried blood spot sample from the patient with PMM2- CDG, ALG3-CDG and MPI-CDG shows patterns consistent with increased disialo- and asialotransferrins and increased tetrasialotransferrin. The patient with VMA21-CDG type shows a usual type II pattern with increased asialo-, monosialo- disialo- and trisialotransferrins and increased tetrasialotransferrin (see Table 2, Fig. 2).

There are some slight but significant differences between dried blood spot and serum samples from patients with milder outcomes in type I. In DBS, the lack of the asialo- fraction and a slight disialotransferrin presence do not indicate CDG type I, affecting the clinical interpretation.

DISCUSSION

Dried blood spot testing is used in newborn screening procedures widely available worldwide, aiming to detect inborn errors of metabolism (Winter *et al.*, 2018; Elliott *et al.*, 2016). There are only single reports in the literature regarding the IEF methods using dried spot cards for detectinon of underglycosylated serum transferrins (Bean *et al.*, 1996; Wolking *et al.*, 2020).

In this study, DBS from PMM2-CDG, MPI-CDG, VMA21-CDG, ALG3-CDG and from patients with transferrin polymorphism was used. Determining the transferrin isoforms by the IEF method in dried blood spots and serum samples from patients with PMM2-CDG, ALG3-CDG and MPI-CDG, a characteristic pattern of type I CDG with a clearly elevated isoform of asialo- and disialotransferrins and decreased tetrasialotransferrin can be observed. In the case of a mild PMM2-CDG pattern, slightly increased serum asialo- and disialotransferrins can be observed by the IEF method. In DBS using the IEF method, we observed

Table 1. Relative amounts of transferrin isoforms in the DBS and serum in the control group (normal pattern).

	Range (n=36) SER %	Mean SER %	Range (n=36) DBS %	Mean DBS %
AsialoTf	0		0	
MonosialoTf	0		0	
DisialoTf	3.5–4.1	3.8	2.8–3.6	3.2
TrisialoTf	5.3–13	9.1	5.8–12.8	9.0
TetrasialoTf	55.1–66.9	61.0	58.4–66.0	61.6
PentasialoTf	16.4–25.3	21.3	19.6–24.0	21.4
HexasialoTf	3.4–6.4	4.8	3.7–5.8	4.7

Table 2. Transferrin isoforms in DBS and serum in CDG type I and CDG type II patients.
Results from single measurements. A,B,C, CDG type I: A, patient PMM2-CDG mild pattern; B, patient PMM2-CDG; C, patient MPI-CDG on treatment; D, CDG type II, patient VMA21-CDG.

	SER % Patient A	DBS % Patient A	SER % Patient B	DBS % Patient B	SER % Patient C	DBS % Patient C	SER % Patient D	DBS % Patient D
AsialoTf	1.1	0	9.3	7.6	10.3	9.5	0.5	0.8
MonosialoTf	1.5	0	1.4	2.2	1.9	2.1	1.8	3.6
DisialoTf	7.9	6.5	31.9	29.9	28.7	22.5	15.9	14.1
TrisialoTf	11.1	9.3	8.3	10.8	7.6	8.0	34.3	32.3
TetrasialoTf	52.9	54.0	36.9	35.0	38.6	42.1	40.1	39.1
PentasialoTf	21.5	24.6	10.2	12.0	10.7	13.2	7.2	7.7
HexasialoTf	4.0	5.7	2.2	2.5	2.2	2.6	0.2	2.3

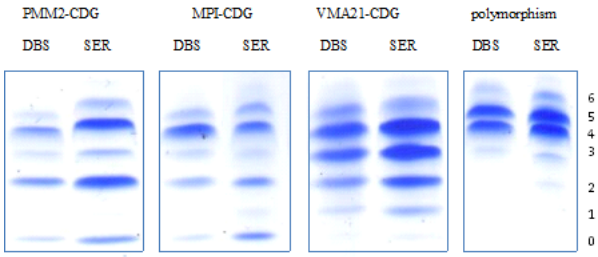

Figure 2. IEF of transferrin from serum and DBS samples from patients with CDG type I, CDG type II and transferrin polymorphism.
0, asialoTf; 1, monosialoTf; 2, disialoTf; 3, trisialoTf; 4, tetrasialoTf; 5, pentasialoTf; 6, hexasialoTf; DBS, dried blood samples; SER, serum

that the isoforms are almost normal. Only a slightly elevated disialotransferrin indicates a minor hypoglycosylation. Lack of the asialo- fraction and a slight presence of disialotransferrin in DBS do not indicate CDG type I, which may have an impact on the clinical interpretation. In this case, it is necessary to determine transferrin isoforms in the serum.

In the serum IEF method for patients with type II CDG we observe a characteristic pattern with increased asialo-, monosialo-, disialo- and trisialotransferrins and decreased tetrasialotransferrin. The transferrin isoform pattern is similar in DBS.

Human transferrin can show genetic polymorphisms (Scherpenzel *et al.*, 2016). A profile characteristic of the transferrin polymorphism is evident in DBS, the same as in the serum (see Fig. 2).

CONCLUSIONS

This work aimed to demonstrate the reliability of the IEF method in DBS testing.

This method can be easily applied in laboratories performing IEF in serum.

Dried blood spots collected routinely for neonatal screening and stored at freezing temperatures may aid the postmortem diagnosis of CDG disorders.

DBS isoform profile obtained by the IEF method may be insufficient in the case of mild PMM2-CDG, and a serum assay may be necessary.

Conflict of interest

All authors declare no conflict of interest.

Ethics approval

Ethical approval was granted by the Bioethical Committee of the Children's Memorial Health Institute, No. 23/KBE/2020, Warsaw, Poland.

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