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Two-dimensional electrophoresis highlights haptoglobin beta chain as an additional biomarker of congenital disorders of glycosylation

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ABSTRACT

Congenital disorders of glycosylation (CDGs) are rare inherited disorders affecting glycosylation of proteins and lipids and sharing very heterogeneous multivisceral symptoms. The biochemical screening of these diseases is currently limited to electrophoresis or HPLC separation/quantification of serum transferrin glycoforms and is relatively frequently hampered by genetic polymorphism. Further, it has been shown that transferrin glycosylation can be very poorly affected in confirmed CDGs. We developed a fast and simple two-dimensional (2-DE) Western-blot analysis applied to the simultaneous detection of various serum glycoproteins, i.e. haptoglobin, α1-anti-trypsin, transferrin and α1-acid glycoprotein, and applied it to a large cohort of CDGs and secondary glycosylation disorders. When separated using 2-DE, haptoglobin β glycoforms showed clear abnormalities in all interpretable CDG type I and CDG type II patterns. Although secondary glycosylation defects such as alcoholism, untreated fructosemia and bacterial neuraminidase remain to be excluded, we showed that 2-DE pattern of haptoglobin β glycoforms thus constitute a very reliable additional biomarker of all types of CDGs. Coupled with common screening techniques and glycans mass spectrometry, it can orientate and facilitate the way towards CDG molecular diagnostic.

1. Introduction

Glycosylation is a post-translational modification involving numerous molecular partners (nucleotide sugars, enzymes, transporters, lipid anchor, tethering factors, vesicular ATPases…) within the secretory pathway in endoplasmic reticulum (ER) and Golgi apparatus (GA) [1,2]. Depending on the oligosaccharide attachment site, i.e. amine of Asn or hydroxyl of Ser/Thr, glycosylation can be mainly subdivided into *N*- and *O*-glycosylation, respectively. Congenital disorders of glycosylation (CDG) are rare inherited diseases sharing heterogeneous symptoms with variable severity [3]. They are classically sub-grouped as type I (CDG-I) or type II (CDG-II). In CDG-I, the defect alters the lipid linked oligosaccharide (LLO) synthesis or its transfer to nascent proteins leading to unoccupied *N*-glycosylation sites with significant changes in both glycoproteins electric charge and molecular weight (Mw). In CDG-II, the defect alters the maturation of protein-linked oligosaccharide leading to incomplete or abnormal *N*-glycan structures with mainly significant charge modifications [4]. CDG screening is usually performed using isoelectric focusing (IEF) or capillary electrophoresis (CE) or HPLC of serum transferrin (Trf) [5–7] based on the separation of its *N*-glycoforms according to charge i.e., to the number of terminal sialic acids (SA). PAGE-SDS followed by Western-blot (PAGE-WB) of various serum glycoproteins has also been described as a valuable screening method for CDG-I since detecting associated Mw variations [8,9]. IEF,

Abbreviations: 2-DE, two-dimensional electrophoresis; AAT, α-anti-trypsin; AGP, α1-acid glycoprotein; ALG1, mannosyltransferase 1; ALG3, endoplasmic reticulum (ER) mannosyltransferase VI; ALG8, glucosyltransferase 2; ATP6V0A2, ATPase H + transporting V0 subunit a2; CDG, congenital disorder of glycosylation; CE, capillary electrophoresis; COG, conserved oligomeric golgi complex; DPGAT1, dolichol phosphate *N*-acetylglucosamine-1 phosphate transferase; DPM1, dolichol phosphate mannose synthase; DOLK, dolichol kinase; ER, endoplasmic reticulum; GA, Golgi apparatus; Hpt, haptoglobin; HUS, hemolytic uremic syndrom; IEF, isoelectric focusing; LLO, lipid linked oligosaccharide; MGAT2, *N*-acetylglucosaminyltransferase 2; PMI, phosphomannose isomerase; PMM2, phosphomannomutase 2; SA, sialic acid; SLC35A1, solute carrier family 35 member A1; Trf, transferrin

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HPLC and CE of Trf share the shortcoming to being restricted to one glycoprotein, commonly subjected to genetic polymorphism (0.5–2.0%) [10], and whose glycosylation is sometimes poorly affected in CDG. Indeed, several studies reported (*i*) screening difficulties related to Trf variants-associated charge heterogeneity [11–13] as well as (*ii*), cases of confirmed CDGs with normal or very discretely abnormal Trf profiles [14–16]. Regarding PAGE-WB, it is not subjected to protein variants interference but does not allow detecting the majority of CDG-II [8].

We previously showed that after separation of serum proteins according to charge and to Mw using two-dimensional electrophoresis (2-DE), it was possible, after electric transfer, to specifically detect on the same nitrocellulose sheet both glycoforms of serum haptoglobin β chain (Hpt), transferrin (Trf), α1-anti-trypsin (AAT), and α1-acid glycoprotein (AGP) [17]. When applied to CDG-I cases, we obtained encouraging results showing abnormal protein spots with decreased Mw (nonoccupancy of *N*-glycosylation sites) and acidic p*I* (loss of SA) by comparison with controls. In this work, we present a simplest and much faster 2-DE procedure and resume our two-years experience about its use besides routine CDG screening using CE of Trf. A large cohort of samples from confirmed CDG-I, CDG-II and PGM1-CDGs (described as 'mixed' CDGs) [18] were analyzed together with (*i*) samples with secondary glycosylation defects and (*ii*), samples with Trf polymorphism. In all CDG cases and studied secondary glycosylation defects, abnormalities affecting at least one glycoprotein were observed on 2-DE patterns. In Trf polymorphism cases, the patterns of others studied glycoproteins were systematically normal. Apart from one hemolyzed serum where undetectable, 2-DE profiles of haptoglobin β chain were interpretable and markedly altered in all CDGs, highlighting haptoglobin as a reliable and useful additional marker for these inherited diseases.

2. Material and methods

2.1. Samples

Tested samples i.e. serum, plasma or blood spotted on a Guthrie card, were sent to our lab for CDG screening or for chronic alcohol consumption-related 'carbohydrate deficient transferrin' (CDT) analysis.

They originated as follows: 14 non-CDG controls (negative Trf CE-based CDG screening). CDG-I samples: 5 PMM2-CDG; 3 PMI-CDG; 1 ALG3-CDG; 2 ALG8-CDG; 1 ALG1-CDG; 1 DPGAT1-CDG; 1 DPM1-CDG and 1 DOLK-CDG. CDG-II samples: 1 MGAT2-CDG; 5 COG-CDG (1 COG1, 3 COG5 and 1 COG7); 5 ATP6VOA2-CDG, 2 SLC35A1-CDG and 6 CDG-IIx (i.e. awaiting for molecular diagnosis; CDG-II Trf CE pattern). Previously described as 'mixed' CDG-I/CDG-II [18], 4 PGM1-CDG were also analyzed.

Samples with well-documented secondary glycosylation defects were analyzed: 1 untreated hereditary fructose intolerance, 3 *S. pneumoniae*-related hemolytic and uremic syndroms (HUS) [19] and 2 chronic alcohol abuses (ERNDIM CDG quality controls). Sixteen serum samples with transferrin polymorphism, as confirmed after neuraminidase treatment, were also analyzed.

2.2. Two-dimensional electrophoresis (2-DE) and Western-blot of glycoproteins

The 'new' 2-DE procedure we used is detailed in Supplementary File 1. For serum/plasma, 2 μL of 10-fold diluted sample in deionised water were analyzed; for blood spot on Guthrie card, one circular punched spot was eluted in 100 μL of deionised water and 10 μL of the eluate was used. 2-DE was carried out as described by the manufacturer (Life Technologies) using ZOOM Strip pH 4–7 for the first dimension and 4–12% NuPAGE Bis-Tris gels for the second dimension. After the first dimension, IEF gels can be stored at − 80 °C before subsequent steps. Proteins were transferred to nitrocellulose (100 V, 1 h) and glycoforms of Trf, AAT, Hpt and AGP were both revealed on the same sheet using a mix of rabbit primary antibodies and HRP-linked anti-rabbit IgG secondary antibody. More precisely, antibodies dilutions (v/v) in TTBS were as follows: anti Trf (Siemens): 1/4000; anti-Hpt (Dako): 1/5000; anti-AAT (Siemens): 1/10000; anti-AGP (Dako): 1/2000 and secondary anti-rabbit IgG: (GE healthcare): 1/5000. Lastly, 2-DE profiles were acquired using Chemidoc XRS camera system from Bio-Rad.

3. Results

3.1. The 'new' 2-DE procedure accelerates serum proteins glycosylation analysis

By greatly accelerating IEF gel rehydration and migration steps, the presented 2-DE/Western-blot method allowed the fast separation and accurate identification of various protein glycoforms from less than 1 μL of pure serum/plasma. Indeed, separation and reliable antibody-based detection of Hpt β chain, AAT, Trf and AGP glycoforms could be achieved on the same nitrocellulose sheet (6 sheets per run) in less than 12 h with minimal manipulations compared to around 48 h for the previous 'tricky' technique (Supplementary File 1).

3.2. 2-DE profile of control samples

As illustrated Fig. 1A, typical 2-DE control pattern showed several glycoforms of the four investigated *N*-glycoproteins i.e. Hpt, AAT, Trf and AGP. All 4 glycoproteins could be localized at their expected calculated p*I*/Mw values and up to 14 glycoforms could be individualized for Hpt, up to 6 for AAT and up to 5 for Trf. Concerning Trf, because of p*I* values close to the upper limit of the used pH gradient (i.e.

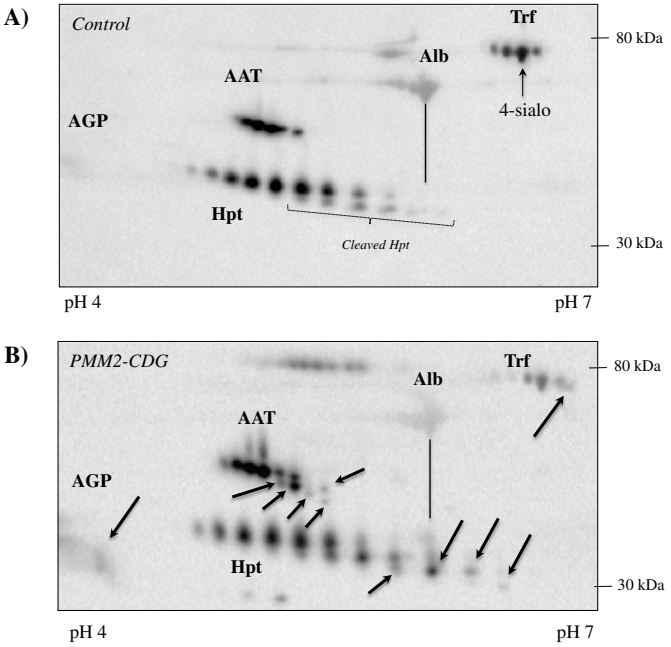

Fig. 1. Two-dimensional Western-blot profiles of control and CDG type I (PMM2-CDG).p*I* range: 4.0 to 7.0; Mw range: 20 kDa to 100 kDa. AGP: glycoforms of α1-acid glycoprotein; AAT: glycoforms of α-anti-trypsin; Hpt: glycoforms of haptoglobin β chain; Trf: glycoforms of transferrin; Alb: isoforms of non-glycosylated albumin. In control (A), AGP glycoforms are non-detectable or very poorly resolved because of out-of-range p*I* values. Hpt β chain can be subdivided into entire protein glycoforms (upper train of spots) and cleaved protein glycoforms (lower train of spots). For Trf, the major tetra-sialo glycoform (vertical arrow) can be systematically detected. No entire Hpt spot can be detected just under the major Alb spot. In PMM2-CDG (B), various additional abnormal glycoforms (plain arrows) can be detected showing modifications in Mw (underglycosylation) coupled to cathodical shift (loss of sialic acid residues).

7.0 pH-units), this glycoprotein was sometimes poorly resolved with frequent IEF gels batch-to-batch variability. Nevertheless, the major spot (arrow) corresponding to the tetra-sialo (4-sialo) glycoform carrying two complete *N*-glycan chains was systematically detectable. Concerning AGP glycoforms, they were usually invisible or very poorly detectable ('smear') in controls as a result of out-of-range acidic p*I*. For Hpt, two groups of spots could be distinguished corresponding respectively to entire β chain (upper train of spots) and to cleaved β chain (lower train of spots). Lastly, protein isoforms of abundant and non-glycosylated albumin (Alb) were also stained in the background constituting a useful landmark for further pattern interpretations.

3.3. 2-DE profiles of CDG-I samples

In the 15 CDG-I samples, whatever the concerned glycoprotein, all observed additional spots (Fig. 1B) systematically showed lower Mw and less acidic p*I* (cathodical shift) by comparison with controls. Apart from one PMM2-CDG hemolyzed case where non-detectable (not shown), Hpt was affected in all CDG-I cases (with up to 4/5 additional spots). AAT was clearly altered in 14/15 CDG-I cases (no visible abnormality in DPM1-CDG) with up to 7 additional spots found in the ALG1-CDG case (Supplementary File 2). Under our conditions (as mentioned above), Trf was visibly affected in 9/15 CDG-I cases while AGP, frequently poorly detectable and/or interpretable, showed clear abnormalities in only 5/15 CDG-I cases. Detailed interpretation of each CDG-I pattern is summarized in Supplementary File 3.

3.4. 2-DE profiles of CDG-II samples

In the 19 CDG-II samples, observed abnormal additional spots all shared less acidic p*I* (cathodical shift) than controls (Fig. 2). With exception of peculiar MGAT2-CDG (described below), CDG-II-associated abnormalities were essentially restricted to p*I* without marked Mw differences (Fig. 2A to C). Hpt patterns were disturbed in 19/19 cases with the systematic detection, at the right of the upper Hpt glycoforms group (complete Hpt), of up to three additional spots. As a hallmark, one spot among the upper group was systematically clearly detected just under the albumin landmark isoform in CDG-II by contrast with controls. For AAT, up to 3 discrete additional spots were observed in 17/19 cases (no visible abnormality in two ATP6-V0A2-CDG). Elevated levels of hyposialylated Trf glycoforms (mainly 3-sialo) were observed in 17/19 CDG-II cases (no visible abnormality in two ATP6-V0A2-CDG cases; one being common to the two AAT 'negative' cases). AGP was detected and showed clear abnormalities in 10/19 cases. Patterns from the 6 CDG-IIx showed abnormalities (not shown) similar to those retrieved in COG and ATP6V0A2-CDGs. In MGAT2-CDG, a marked p*I*/Mw shift was observed for all glycoproteins (Fig. 2D). Detailed interpretation of each CDG-II pattern is summarized in Supplementary File 4.

3.5. 2-DE profiles of PGM1-CDG, secondary glycosylation defects and transferrin polymorphisms

As illustrated Fig. 3B, 2-DE patterns of all PGM1-CDGs shared marked abnormalities (especially on Hpt and AAT) combining those retrieved in CDG-I (plain arrows) and in CDG-II (dotted arrows). Concerning secondary glycosylation defects, HUS patterns (Fig. 3C) showed a massive cathodical p*I* shift notably strongly affecting AAT and AGP with frequently poorly detectable Hpt glycoforms (2/3 patients). In chronic alcoholism (Fig. 3D) and hereditary fructose intolerance (not shown), slight CDG-I abnormalities notably affected Hpt and AAT. Lastly, in the 16 cases of transferrin polymorphism, others detectable glycoproteins showed normal glycosylation patterns (Supplementary File 5).

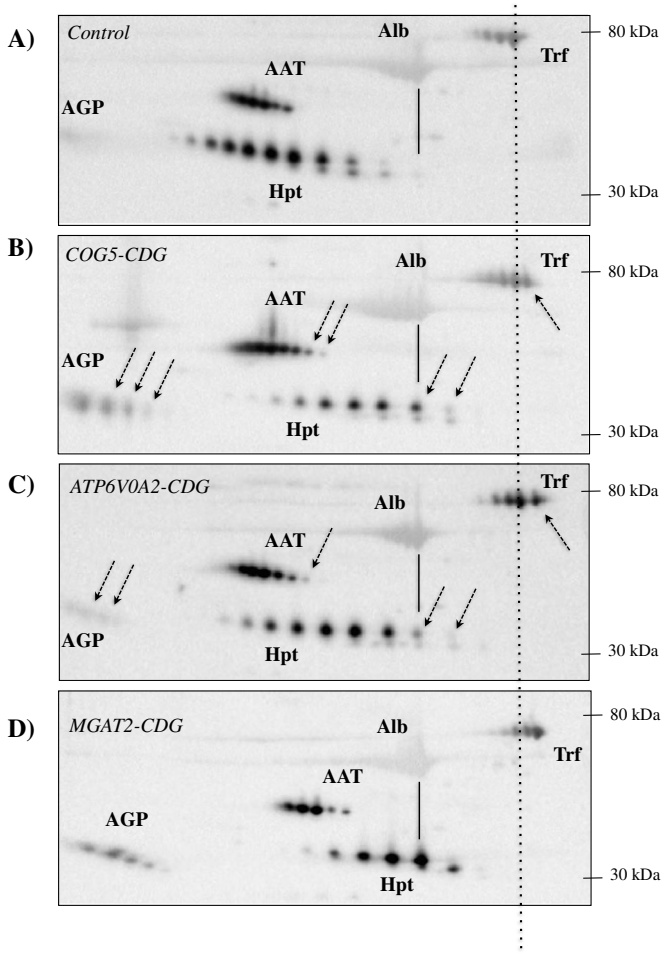

Fig. 2. Two-dimensional patterns of CDG type II.By comparison with control (A), 2-DE profiles of the most common CDG-II i.e. COG-CDG (B) and ATP6V0A2-CDG (C) show additional abnormal spots (dotted arrows) with p*I* cathodical shift. As a hallmark, one Hpt spot can be detected just under the major Alb spot. In MGAT2-CDG (D), 2DE-pattern shows marked p*I* and Mw variations in accordance with the absence of terminal GlcNAc-Gal-SA upon one-in-two *N*-glycan antennas.

4. Discussion

2-DE has already been undertaken in the field of CDG but as a relatively long and tricky technique poorly suited to laboratory routine. Furthermore, described 2-DE applications generally suffered from the use of nonspecific proteins labeling (silver nitrate, Coomassie, fluorescence, lectins…) theoretically necessitating additional identification steps [20,21]. We presented here a simple, fast and efficient procedure which considerably accelerates 2-DE separation and antibody-based specific detection of multiple circulating glycoproteins. When applied to CDG-I cases, it showed p*I*/Mw variations corresponding to the loss of entire *N*-glycan chains related to defect in the LLO biosynthesis pathway or in the glycan transfer on nascent proteins. Under the used conditions, entire Hpt β chain was the more sensitive CDG-I marker showing typical abnormalities in 14/15 cases, the remaining one corresponding to a sample with undetectable Hpt level related to intravascular hemolysis. AAT also shared good sensitivity towards CDG-I (14/15 CDG-I cases) but showed normal patterns in one case (DPM1-CDG) without any AAT concentration-related bias. In the ALG1-CDG case, AAT profile was strikingly altered with seven abnormal spots sharing unusual relative intensity by comparison with others CDG-I (Supplementary File 2). Although right now limited to one case, this putative ALG1-CDG peculiarity will be further investigated on additional samples since possibly related to recently described circulating

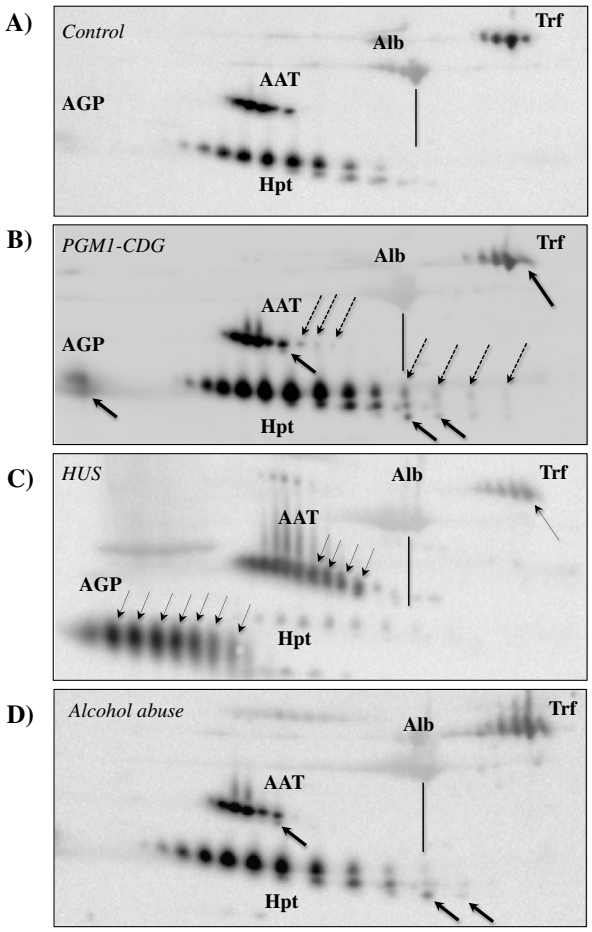

Fig. 3. Two-dimensional patterns of PGM1-CDG and secondary glycosylation defects.By comparison with control (A), 2-DE profile of PGM1-CDG (B) shows mixed abnormalities suggestive of CDG-I (plain arrows) and CDG-II (dotted arrows). In hemolytic and uremic syndrom (HUS; C), a massive cathodical shift (arrows) can be observed for AGP, AAT and Trf in relation to bacterial neuraminidase. In chronic alcohol abuse (D), discrete CDG-I abnormalities (arrows) can be observed for Hpt and AAT.

N-linked xeno-tetrasaccharide NeuAc-Gal-GlcNAc2 in this CDG [22,23]. Trf and AGP showed poor sensitivity towards CDG-I (9/15 and 5/15, respectively) that can be mostly explained by out-of-range p*I* values.

In CDG-II, if considering all glycoproteins, the sensitivity of the technique appeared clearly better than in CDG-I. Hpt patterns were unambiguously altered in all tested cases (19/19) with one to four additional spots corresponding to the loss of negative charges carried by terminal SA. AAT patterns were affected in 17/19 cases with two negative ones in ATP6V0A2-CDG patients. Trf and AGP patterns were clearly abnormal in 17/19 and 10/19 cases, respectively. For Trf, the two negative cases also corresponded to ATP6V0A2-CDG profiles, one of them being common to AAT negative patterns. In MGAT2-CDG, 2DE-pattern appeared 'diagnostic' showing typical marked p*I* and Mw variations in accordance with the absence of terminal GlcNAc-Gal-SA upon one-in-two *N*-glycan antennas [24]. This MGAT2-CDG peculiarity is related to the defect of a specific glycosyltransferase that can be differentiated from COG-CDG and ATPV0A2-CDG where glycosylation defects result from disturbance in GA dynamics and/or pH homeostasis [2,25]. Apart from MGAT2-CDG, no pattern appeared specific towards any CDG-II subtype. Nevertheless, in our CDG-II cohort, ATP6V0A2-CDG apparently shared the more discretely disturbed profiles.

In this study, PGM1-CDGs have been considered as an individual group since they have been described as 'mixed' CDG-I/CDG-II when analyzed using Trf IEF and MS techniques [18]. Our results strongly corroborated these data notably showing Hpt and AAT mixed abnormalities in the 4 tested PGM1-CDGs.

In the field of CDG, we showed here that Hpt entire β chain glycoforms constitute a very sensitive CDG biomarker since corresponding patterns were clearly abnormal in all studied types and subtypes unless having insufficient Hpt concentration. When separated according to Mw only, Hpt β chain has already been described as an interesting marker for CDG-I [8,26] but was inefficient to screen for CDG-II (apart from MGAT2-CDG) where abnormalities are commonly restricted to charge (i.e. sialylation) differences. Concerning Hpt glycoforms IEF separation according to charge, it has never been described in CDG probably because of interfering presence of cleaved β chains and of non-glycosylated Hpt α chains [27]. Since these two forms shared lower Mw than complete β chains, 2-DE, by combining Mw and p*I* separation, allowed overcoming this pitfall. Using more appropriate pH gradient for Trf glycoforms in the first dimension (3.0–10.0 or 5.0–8.0), future work will be dedicated to compare CDG screening performances of Hpt vs. Trf on the same sheet. AAT patterns also showed good specificity for all types of CDG (35/38) in accordance with one publication reporting it as a useful CDG marker [28]. In case of abnormal Trf profiles or in doubtful cases, MS techniques applied to purified Trf or to whole glycans can be used in order to confirm and characterize glycosylation defects. Such MS-based approaches were shown to be helpful for CDG-II subtyping but also 'diagnostic' in ALG1-CDG, PGM1-CDG and in some CDG-II e.g. SA transporter deficiency SLC35A1-CDG [18,23,29,30]. Our results suggest that presented 2-DE patterns, by fastly/simply evaluating the glycosylation status of multiple serum glycoproteins could be useful as an orientation tool for accurate MS-based glycans analysis.

In *S. pneumoniae*-related HUS, we previously showed using an appropriate pH gradient that 2-DE patterns of Trf were dramatically affected in relation to bacterial neuraminidase [19]. While Hpt appeared frequently poorly detectable as a consequence of hemolysis (2/3 HUS cases), we showed here marked hyposialylation of Trf, AAT and AGP in HUS. These results illustrate the necessity to consider potential neuraminidase interference when screening for CDG.

Hypoglycosylated forms of Trf, i.e. 'CDT' for 'carbohydrate deficient Trf' currently constitute the more reliable marker for chronic alcoholism [31]. In two cases, we showed discrete glycosylation abnormalities on Hpt and AAT highly evokable of the loss of entire *N*-glycan chains. Further work should be considered in evaluating the practicability and sensitivity/specificity of 2-DE-determined 'carbohydrate deficient Hpt' or 'carbohydrate deficient AAT' in chronic alcoholism. Notably, these two biomarkers could be potentially helpful in relatively frequent Trf polymorphism cases where accurate CDT determination is frequently disturbed [32,33].

Trf polymorphisms can also seriously complicate CDG screening since they frequently mimic abnormal IEF/HPLC/CE profiles [11,12]. Based on the analysis of 16 non-CDG samples with Trf polymorphism, we showed that associated normal AAT and Hpt 2-DE patterns allowed to unambiguously identify them.

5. Conclusions

By using a fast and simple 2-DE method coupled to antibody-based detection of multiple serum glycoproteins, we showed that the glycoforms pattern of haptoglobin entire β chain constitute a very reliable biomarker towards all types of CDG. It could be helpful for CDG screening and confirmation notably when Trf profiles are doubtful, non interpretable, or subjected to protein polymorphism.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2017.04.022.

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