Editorial Commentary

Integrating mass spectrometry-based plasma (or serum) protein N-glycan profiling into the clinical practice?

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Comment on: Chen J, Li X, Edmondson A, *et al.* Increased Clinical Sensitivity and Specificity of Plasma Protein N-Glycan Profiling for Diagnosing Congenital Disorders of Glycosylation by Use of Flow Injection-Electrospray Ionization-Quadrupole Time-of-Flight Mass Spectrometry. Clin Chem 2019;65:653-63.

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Congenital disorders of glycosylation (CDG) are clinically diverse inherited diseases affecting in particular the glycosylation of proteins. Since the first clinical description of CDGs in 1980 (1), a significant number of new CDG types have been reported with more than 130 today described (2). CDGs are rare essentially autosomal recessive disorders, with about 60% of them affecting protein N-glycosylation (3). CDGs are classically subdivided in 2 types if defects alter the synthesis (CDG-I) or the processing of N-glycans (CDG-II) in the Golgi apparatus after their '*en bloc*' transfer to the nascent protein. CDG patients can present very different and overlapping clinical symptoms, thus rendering both diagnostic and identification of causative genes difficult (4). Isoelectric focusing (IEF) of plasma/serum transferrin (Trf) is the preferred first-line method for CDG screening. In controls, Trf IEF patterns show the major tetra-sialo glycoform (4-sialo Trf) corresponding to Trf decorated by 2 complex bisialo -biantennary N-glycan chains. CDG-I-specific patterns typically show decreased 4-sialo and markedly increased levels of 2-sialo and 0-sialo Trf while CDG-II profiles might present 4-sialo, 3-sialo, 2-sialo, 1-sialo and 0-sialo Trf glycoforms. Trf glycoprofiling performed by IEF or other techniques such as capillary electrophoresis or high-performance liquid chromatography, might lack diagnostic sensitivity in some instances, thus generating some false negative results for some patients (5). Also, Trf-centric assays are not well suited to the diagnosis of CDGs with defective fucosylation due to the very low fucosylation level of the Trf complex-type N-glycans. The occurrence of some Trf sequence variants might also hinder CDG diagnosis (6). Therefore, there is a need for additional specific, sensitive, fast and (semi)quantitative analytical methods for improving CDG diagnostic, identifying new N-glycan biomarkers as well as monitoring disease progression or treatment efficiency [e.g., orally administered mannose for mannose phosphate isomerase (MPI)-CDG or galactose for phosphoglucomutase 1 (PGM1)-CDG] (7,8). In that context, the analysis of enzymatically released N-glycans from all the plasma (or serum) proteins, i.e., plasma (serum) N-glycomics, represents a viable alternative or complement to more traditional methods. Modifications of the total plasma N-glycan profile reflect alterations in the glycosylation present in one or more of the 24 highly abundant glycoproteins accounting for about half of the total plasma protein concentration (9). Although more suitable for CDG-II diagnosis, plasma N-glycomics also proved efficient for some type-I CDGs such as chitobiosyldiphosphodolichol beta-mannosyltransferase (ALG1)-CDG (10).

High-resolution mass spectrometry (MS) is one of

the most powerful techniques available for the sensitive detection and characterization of N-glycans showing qualitative and/or quantitative abnormalities associated with CDGs. Thus, several MS-based plasma N-glycomics studies have been successfully conducted using either matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) (11-14) or electrospray ionisation mass spectrometry (ESIMS) (15-17). Without any prior off-line or on-line fractionation step (e.g., using liquid chromatography), 40–60 distinct non-isomeric N-glycans can be routinely monitored depending on the conditions and samples considered. Chemical derivatization of enzymatically released N-glycans is often required for an improved detection sensitivity while several extraction/purification steps are also needed to make the sample compatible with MS analysis. Thus, a limitation of such MS-based approaches is that they often require long turnaround time (up to 48 h in certain cases) due to the complex nature of the associated sample preparation, which can also be synonymous with high analytical variability (especially for low-abundant N-glycan structures). The adoption of MS-based N-glycomics by clinicians as first-line and routine technique for CDG screening would imply improve method robustness, ease-of-use and speed of execution. Reliable absolute quantification of specific N-glycan biomarker candidates remains largely underdeveloped but might represent a relevant long-term objective for defining potential cut-off values for the clinical practice (18). Indeed, differences of ionization efficiencies across glycan structures and the lack of availability of standard compounds make absolute quantification of N-glycans difficult to perform by MS. Published MS-based approaches are often limited to relative quantification, i.e., the MS signal of a considered N-glycan is expressed as a percentage of the total signal intensity of all the detected N-glycan structures.

The paper of Chen and colleagues recently published in *Clinical Chemistry* (19) describes a new MS-based approach for CDG diagnostic that addresses some of the issues above mentioned, in particular regarding method throughput and robustness. N-glycans were enzymatically released from plasmatic glycoproteins upon a 5-min incubation following surfactant-aided protein denaturation. Resulting glycosylamines were labeled within another 5 min with a quinolone fluorophore, named RFMS or RapiFluor-MS (name under which it is marketed), also incorporating a tertiary amine side chain which significantly enhances detection sensitivity in the positive ESI mode (20). After being extracted by hydrophilic interaction chromatography (HILIC), derivatized N-glycans were then directly analyzed by flow injection-ESIMS using a high-resolution quadrupole-time-of-flight instrument (QTOF). The process of going from glycoprotein to extracted, labeled N-glycans was accomplished in less than 60 min, while MS acquisition would require a few additional minutes. Thus the plasma N-glycomics method implemented by Chen *et al.* is characterized by one of the fastest (if not the fastest) time-to-result (19). In addition, their protocol also makes use of a stable isotope-labeled sialylated glycopeptide as an internal standard for sample normalization purposes and for the (semi)quantification of N-glycans. Under these conditions, about 45 N-glycan structures were detected in human plasma while their detection proved linear over 2 orders of magnitude when assayed using a serial dilution of a plasma sample from an alpha-1,3-mannosyltransferase (ALG3)-CDG patient. In parallel, purified human Trf was used to generate calibration curves for the tentative (semi)quantification of some 28 N-glycans. Since the concentration of only the most abundant biantennary bisialylated Trf N-glycan was known, this was the sole N-glycan quantified in human plasma. The other 27 N-glycans were only semiquantified, some of them suffering from strong ionization suppression effects precluding their reliable quantification. Chen *et al.* then demonstrated the reproducibility of their approach for measuring relative abundances of all N-glycans, with coefficients of variation <15%. N-glycans also proved stable in both plasma and serum samples stored at room temperature for up to 48 h. Thus, the implemented and validated plasma N-glycomics approach demonstrated fast turnaround time and high robustness, which are prerequisites for clinical diagnosis of CDGs. A set of plasma samples from 19 CDG-I and CDG-II patients was used to biologically evaluate the relevance of the assay.

On the basis of a previous study from the same team (21), authors focused on small ('Man0', 'Man1' and 'Tetra') and polymannose linear and branched circulating N-linked glycans ('Man2' to 'Man9') as clinical sensitive biomarkers for screening CDG subtypes. By very accurately detecting and by semi-quantifying these low abundance (and stable) saccharides, they were able to differentiate 6 phosphomannomutase 2 (PMM2)-CDGs from 6 other CDG-I patients sharing abnormal but undistinguishable Trf patterns. In this non-PMM2-CDG group (n=6), they found consistent features relatively to enzymatic defects of ALG1-CDG (n=1), ALG3-CDG (n=1) and alpha-1,2-

mannosyltransferase (ALG9)-CDG (n=1) patients. For the 3 remaining CDG-I patients with defects in subunits of the translocon protein complex [oligosaccharyltransferase complex catalytic subunit B (STT3B)-CDG, dolichyl-diphosphooligosaccharide—protein glycosyltransferase non-catalytic subunit (DDOST)-CDG, signal sequence receptor subunit 4 (SSR4)-CDG], they measured similar polymannose N-glycan quantitative differences, thus potentially interesting for diagnosis purposes.

Concerning CDG-II patients, one PGM1-CDG patient exhibiting abnormal but unclear Trf profile, was orally treated by D-galactose supplementation during a 3-month period. His N-glycan profile improved although not fully normalized, with some specific N-glycan abundances evolving in a dose-dependent manner. In 3 solute carrier family 35 member A2 (SLC35A2)-CDG patients, N-glycan profiles overall appeared abnormal with increased levels of monogalactosylated or agalactosylated N-glycans, whereas 2/3 cases had normal Trf profiles. Last, samples from patients with genetic deficiencies in carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD; n=1) and archain 1 (ARCN1; n=2), were analyzed upon the hypothesis of likely, but not yet characterized, serum N-glycans defects. As expected, discrete but potentially diagnostic quantitative N-glycan abnormalities were highlighted using the implemented MS-based assay.

From the clinical biochemist's point of view, this fast and semi-quantitative MS-based ESI-QTOF technique appears promising since it clearly demonstrates high sensitivity and specificity levels allowing the detection of potential new N-glycan biomarkers of CDGs. Of major interest, potential N-glycan signatures are proposed for the more frequent CDG, i.e., PMM2-CDG, which could greatly accelerate and simplify its screening and diagnosis. Nevertheless, in the presence of a 'borderline' adult case, these results related to only 6 PMM2-CDG patients should still be considered as preliminary and need further validation. For SLC35A2-CDG, i.e., genetic defect in the uridine diphosphate (UDP)-galactose transporter, reported results are also promising since this potentially galactose treatable CDG is frequently missed using classical Trf analysis. Indeed, Ng *et al.* recently reported normal Trf pattern in 65% of patients from a large cohort (22). In view of these results, it would be very interesting to apply this method to other 'tricky' and potentially treatable CDGs notably including those affecting the sialic acid/N-acetylglucosamine pathways such as UDP-N-acetyl-glucosamine-2-epimerase/N-acetylmannosamine kinase (GNE) myopathy, N-acetylneuraminate synthase (NANS)-CDG or phosphoglucomutase 3 (PGM3)-CDG (23). Indeed, in GNE myopathy, the lack of specific biomarkers to monitor disease progression and response to sialic acid treatment has been highlighted as a critical point contributing, among others, to the recent failure of a phase 3 clinical trial (24). Application of this sensitive and semi-quantitative N-glycomics approach to MAN1B1-CDG could be interesting since this relatively frequent CDG was associated to very discrete or even absent abnormalities when using classical serum N-glycans profiling after peptide-N-glycosidase F (PNGase F) enzymatic release (13).

In the field of CDG therapy monitoring, proposed N-glycan levels/ratios related to one PGM1-CDG patient under galactose are also promising and have to be firmly validated on additional samples. Furthermore, it would be of great interest to compare their performances against those of 'glycan-indexes' recently reported by Abu Bakar *et al.* (25) deduced from MS-based Trf glycoprofiling and plasma N-glycomics. Also, the presented method could be usefully applied to MPI-CDG patients under oral mannose therapy for which Trf IEF has been shown to be poorly informative regarding to treatment response (7).

Overall, the method described by Chen *et al.* paves the way to the integration of N-glycomics approaches in the clinical practice for diagnosis of glycosylation disorders. In the context of CDGs that are very rare metabolic disorders, the economic impact relative to the routine use of such commercial 'Rapifluor-MS™ N-Glycan kit' (Waters, Milford, MA) still needs to be evaluated. Similarly to previously reported MS-based plasma N-glycomics approaches, the present method can still benefit from methodological improvements regarding absolute quantification as well as regarding the separation of isomeric N-glycan biomarkers that might exhibit distinct diagnostic characteristics.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest

to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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