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A new strategy implementing mass spectrometry in the diagnosis of congenital disorders of N-glycosylation (CDG)

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Abstract

Objectives: Congenital disorders of N-glycosylation (CDG) are a large group of rare metabolic disorders caused by defects in the most common post-translational modification of proteins. CDGs are often difficult to diagnose as they are manifested with non-specific symptoms and signs. Analysis of serum transferrin (TRF) isoforms, as the classical procedure used to identify a CDG patient, enables to predict pathological steps in the N-linked glycosylation process.

Methods: We devised a new strategy based on liquid chromatography-mass spectrometry (LC-MS) for the analysis of TRF isoforms by combining a simple and fast sample preparation with a specific chromatographic cleanup/separation step followed by mass-spectrometric measurement. Single TRF isoform masses were obtained through reconstruction of multiply charged electrospray data collected by quadrupole-MS technology. Hereby, we report the first analyzed serum samples obtained from 20 CDG patients and 100 controls.

Results: The ratio of desialylated isoforms to total TRF was calculated for patients and controls. CDG-Type I patients showed higher amounts of bi-sialo isoform (range: 6.7–29.6%) compared to controls (<5.5%, mean percentage 3.9%). CDG-Type II pattern showed an increased peak of tri-sialo isoforms. The mean percentage of tri-sialo-TRF was 9.3% (range: 2.9–12.9%) in controls, which was lower than that obtained from two patients with COG5-CDG and MAN1B1-CDG (18.5 and 24.5%). Intraday and between-day imprecisions were less than 9 and 16%, respectively, for bi-sialo- and less than 3 and 6% for tri-sialo-TRF.

Conclusions: This LC-MS-based approach provides a simple, sensitive and fast analytical tool for characterizing CDG disorders in a routine clinical biochemistry while improving diagnostic accuracy and speeding clinical decision-making.

Keywords: congenital disorders of N-glycosylation (CDG); inborn error of metabolism; liquid chromatography-mass spectrometry (LC-MS); *N*-glycosylation; online trapping and clean-up; spectral deconvolution; transferrin isoforms.

Introduction

Congenital disorders of N-glycosylation (CDG) cause severe multiorgan and multisystem disorders that are manifested as early as the first months of life [1]. Making a diagnosis of CDG is often challenging due to the low specificity and broad spectrum of symptoms they are manifested with

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[2–4]. CDGs are usually suspected in children who exhibit hypotonia, failure to thrive, skeletal abnormalities, developmental delay, ataxia, coagulopathy, seizures and cerebellar hypoplasia. However, it is often difficult to suspect a CDG based on clinical presentation alone. Although treatment for most CDG types is largely supportive, an early diagnosis would at least avoid unnecessary investigations and provide an opportunity for some patients to be included in currently ongoing clinical trials [5, 6].

The first diagnostic step to detect disorders of protein N-glycosylation is a biochemical assay with analysis of serum transferrin (TRF), a glycoprotein with different isoforms depending on the number of sialic acid residues present on its oligosaccharide chains. Normal human serum contains tetra-sialo-TRF, a predominant TRF glycoform (usually 75–80% of total TRF) with two bi-sialylated biantennary glycans. The penta-sialo, hexa-sialo and tri-sialo glycoforms can also be found in healthy subjects [7]. Genetic variants impacting N-linked glycosylation lead to an altered TRF pattern with isoforms lacking sialic acid residues [8, 9].

Hyposialylated or desialylated isoforms of TRF can also occur in other secondary glycosylation defects like fructosemia, galactosemia or secondary to alcohol abuse [10–13].

Once biochemical testing identifies an abnormal pattern of TRF, diagnosis must be pursued further with the molecular analysis to establish the specific type of CDG. However, despite a negative biochemical analysis of TRF, if clinical suspicion is strong (particularly in regard to CDG type II) molecular genetic testing should also be performed [14].

Various methods have been proposed for measuring the variously glycosylated forms of TRF. Isoelectric focusing is the most commonly used diagnostic test and is considered as the reference method though HPLC is widely used in alcohol-abusers for targeting changes in serum TRF glycoforms [15].

Proteomic research, boosted by mass spectrometry, has prompted new diagnostic approaches. The high scanning speed associated to high resolution as offered by the hybrid instrumentation (Quadrupole-Tof or Quadrupole-Trap configurations) has been pivotal for elucidating protein structure, including the study of any occurred modification (post-translational or not).

After more than two decades the interest is now shifting on how to exploit mass spectrometry in the clinical context. One area of interest focuses on measuring biomarkers in biological fluids. A way for protein characterization in some specific applications is to measure the undigested proteins and, thanks to the high resolution of the mass spectrometric measurement, to estimate structural modifications through the resulting molecular weight of the intact protein.

Adequate mass resolution and good chromatographic separation are compelling for achieving reliable results in detecting CDG defects. MS-based tests can analyze the variations in glycosylation of the intact protein. Some methods employed high-resolution MS-like Maldi-TOF [16–18], while others rely on MS-quadrupole nominal resolution [19–21]. There are many advantages associated with these approaches, including time and labor saving, with the burden of protein digestion being avoided, and no external standards necessary since result interpretation rely on measuring ratios. However, these methods are somehow hampered by a series of sample processing, clean up and concentration steps which appear to unsuitable for implementation in large routine laboratory.

A critical point concerns how to make the characterization by mass spectrometry reliable for an intact protein surrounding by other matrix components. The target protein should be conveniently free of contaminants including other proteins, especially if they fall in the same molecular weight range or at a concentration close to, or higher than, those investigated. Some examples were immunoaffinity application [16–19, 21] or ion exchange chromatography [20] used for selective purification of TRF. Nevertheless, these approaches are labor-intensive and time-consuming.

In order to address this and other issues, we have developed a new online trapping- and cleanup-liquid chromatography-mass spectrometry (LC-MS)-based

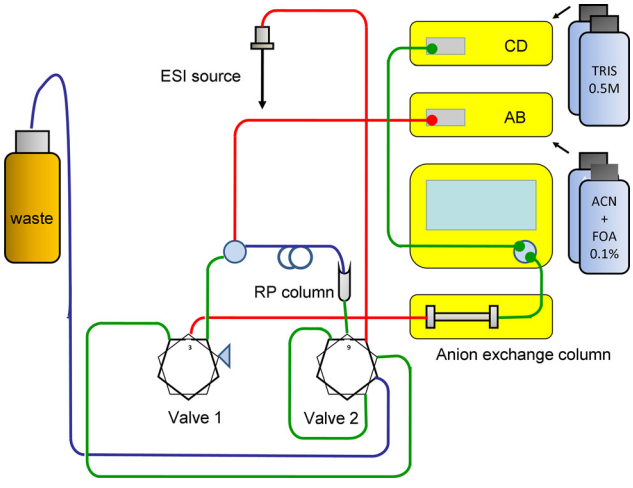

Figure 1: Schematic diagram of 2D-LC-MS configuration.
Anion exchange column fed by binary pump AB provides the 1st chromatographic dimension for isolating the TRF isoforms. Reverse-phase column implements through pump CD the cleanup of the trapped isoforms before entering in the mass spectrometer via the ESI source. Valve 1 is intended for cutting-up the target from the 1st chromatographic step meanwhile valve 2 manages the solvents delivering for either the cleaning-up and the subsequent elution (2nd step) to the MS of the collected target.

method aiming at simplifying both sample preparation and analytical measurement, as required by a routine clinical biochemistry laboratory. A fast and easy sample preparation/workflow set-up is pivotal for a rapid turnaround response to a clinical query. Equipment investment and running costs must be also affordable in this context.

Materials and methods

Chemical and reagents

Water, acetonitrile (LC-MS grade) were supplied by Biosolve (Chebios, Rome, Italy) while analytical grade formic acid, TRIS and ammonium formate were from Sigma-Aldrich (Milan, Italy). In developing the method, human apo-TRF from Sigma-Aldrich was used to confirm identity of the targeted sample TRF.

Samples

This study was approved by the Institutional Ethics Board of Meyer Hospital of Florence (No. 137/2020). Informed consent was obtained from all patients or their legal guardians prior to their participation in this study.

Serum samples from 100 healthy donors were collected after anonymisation for assessment range depending on the normal profile. To test the method sensitivity in identifying the altered TRF isoforms, serum samples were obtained from 20 patients genetically confirmed as suffering CDG (CDG-Type I: nine PMM2-CDG, two MPI-CDG, one ALG8-CDG, two ALG6-CDG, one ALG9-CDG; CDG-Type II: one

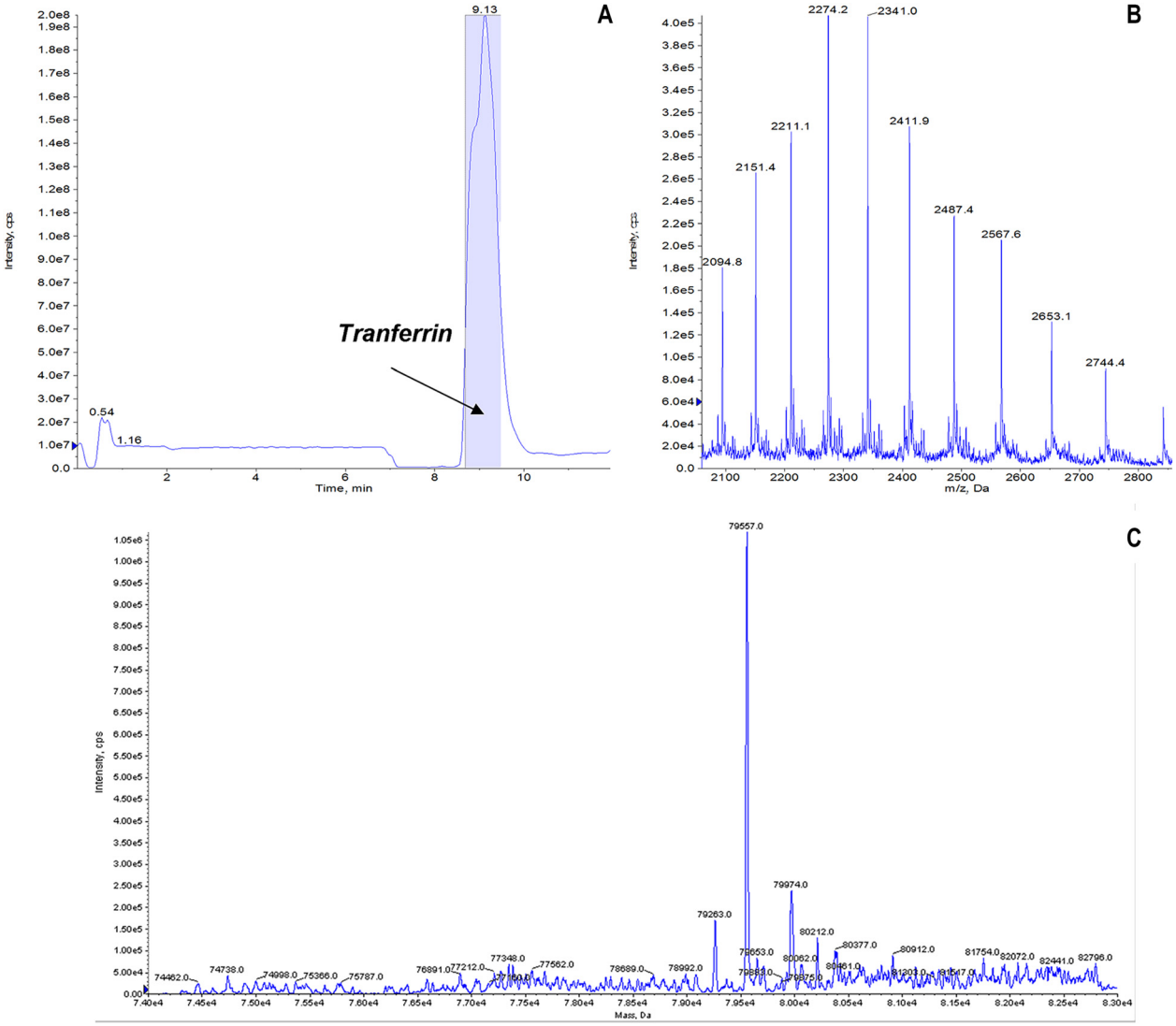

Figure 2: MS tracings of a control sample measurement.
(A) shows the total ion current (TIC) of the chromatographic process where a single peak appears at 9.1 min and corresponds to TRF. (B) displays the related ESI-MS multi-charged spectrum. (C) shows the MW distribution spectrum depicting the various TRF-isoforms after the software deconvolution.

MAN1B1-CDG, one COG5-CDG; mixed CDG-Type I and II: three PGM1-CDG).

In addition, serum samples from five chronic alcohol abusers were collected to test the sensitivity of the method for clinical use in the detection of alcohol addiction.

All samples were stored at –20 °C until analysis.

Experimental

Sample preparation consisted in adding 150 µL of 1 M ammonium formate solution to 50 µL of serum. After vortex-mixing and centrifugation at 12,000 *g* for 5 min, the supernatant was transferred either in a vial or on a titer-plate for a subsequent injection of 25 µL, representing roughly 6.25 µL of the original specimen.

Mass spectrometric measurements were performed by a Sciex API 4000 triple-quadrupole (Sciex, Brugherio, Italy) equipped with ESI probe and operating in single Q1-scan with a range 2,060–2,960 amu covered in 3 s with 0.1 amu steps. Mass calibration was made with a polypropylene-glycols (PPG) mixture (2×10^{-6} M) as suggested by manufacturer. Nebulizer gas and turbo gas (air) were set at 50 psi with the turbo gun temperature at 500 °C.

Collected multiply charged ion spectrum including all the TRF isoform peaks has been processed through the Bayesian Protein Reconstruction option of BioAnalyst (version 1.5.2, Sciex) and by adopting 20 interactions for the range 70–85 kDa with a S/N threshold of 20. These processing parameters associated to the intrinsic quadrupole resolution allowed a precision better than 15 daltons (Da) in the mass assignment over the molecular mass (MW) range 70–85 kDa, precision good enough for an unambiguously characterization of the various forms of glycosylated TRF.

The chromatographic set-up (Online trapping and clean-up) consisted of an Agilent 1200 LC-chain (Agilent, Italy) including a binary pump and an autosampler for implementing an anion exchange chromatographic separation with a Phenomenex AWX Clarity 4.6 × 100 mm column (Phenomenex, Bologna, Italy) flushed at 0.5 mL/min of eluent A as pure H_2O and eluent B as a solution 0.5 M of TRIS-pH 7.5 (Sigma-Aldrich, Milan, Italy) in gradient mode starting at 50% B moving to 90% in 7 min followed by a cleaning step at 95% B for 1 min with the subsequent equilibration of 4 min before the next injection.

The chromatographic set-up is completed by a second Agilent LC-pump with the pumping heads intentionally disjoined. This pump is working in association with an in-house developed manifold centered on two Sciex 10-port switching valves (Figure 1). This assembly is managed in the way that a reverse-phase column Brownlee RP 300 4.6 × 30 mm (PerkinElmer, Milano, Italy) is constantly flushed by an aqueous solution of formic acid 0.5% (solvent A) at 1 mL/min until at 5 min the TRF fraction exiting the anionic exchange chromatography is entrapped for the next minute through valve 1. Once the TRF is trapped, the valves are managed for implementing a cleaning of the collected fraction for the subsequent minute. At 7 min and with valve two activation, solvent A is joined with a solution of acetonitrile containing formic acid 0.1% (solvent B), the latter solvent previously diverted for feeding the mass spectrometer ionization source at a flow rate of 0.4 mL/min. Once joined the two solvents A+B, a fast gradient is operated for a reverse-phase gradient moving from 0 to 80% solvent B in 2 min with a subsequent 100% isocratic step of 4 min. Targeted chromatographic TRF peak appears at 9.1 min and is read by ESI-quadrupole MS as above described.

All the TRF isoforms depicted by the BioAnalyst software on a molecular weight (MW) distribution spectrum were characterized according the scheme proposed by Kleinert [20] (normal TRF isoforms tri-, tetra- and penta-sialo, either fucosylated or not-; and TRF isoforms bi-sialo and asialo). Peaks resulting in the MW distribution spectrum were processed for expressing the ratio of the single isoforms to the total TRF according the following Eqs.:

$$\text{Desialilated isoforms\%} = (\sum \text{bi-sialo isoforms} + \sum \text{asialo isoforms}) \times 100/(\sum \text{all isoforms}) \quad (1)$$

$$\text{Tri-sialo isoforms\%} = (\sum \text{tri-sialo isoforms}) \times 100/(\sum \text{all isoforms}) \quad (2)$$

where the terms "Σ bi-sialo isoforms" and "Σ tri-sialo isoforms" encompass all the isoforms (mono- and bi-glycan, fucosylated or not) hosting respectively two or tri sialic residues.

Adequacy of the whole process (sample preparation and LC-MS) has been proven by testing solutions of 0.3% human TRF as supplied by Sigma.

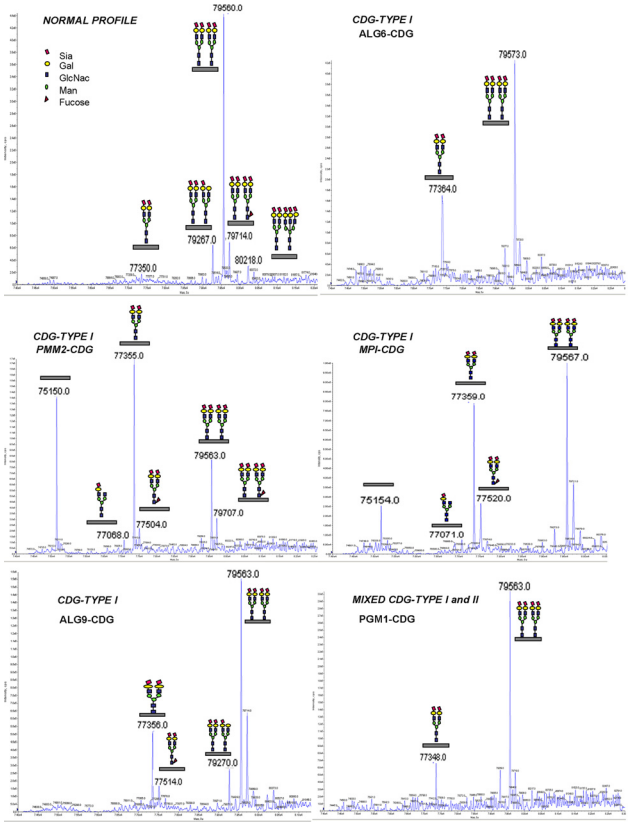

Figure 3: TRF-isoform profiles for different patients.
The upper left panel shows a normal control compared to patients with different sub-types of CDG-I. Peaks are annotated with the glycosylation moiety structure with the following meaning: blue box = N-acetylglucosamine; green circle = mannose; yellow circle = galactose; red triangle = fucose; purple diamond = N-acetylneuraminic acid (sialic acid). Same MW spectra are shown in more detail through Supplementary Figures S3–S8.

Results and discussion

Development of a mass spectrometry assay to analyze undigested glycoproteins is difficult because proteins are both too large for a sensitive mass spectral measurement and usually surrounded by other proteins with concentration range similar or even higher than the one targeted. In order to address this challenge in the TRF application, we performed a first step for trapping the protein on a first ion-exchange LC-column by exploiting the anionic character of TRF due to its sialic residues, which enabled to clean the sample from salts and other interfering compounds. A second step consisted in further trapping and subsequent cleaning the targeted fraction through a second chromatographic column with an optimized solvent management before the final MS measurement.

Although intact proteins often have high molecular weight and should, therefore, not be usually detectable in quadrupole mass spectrometry, their study mainly depends on the m/z range of the instrument. In some commercial instruments, mass range can go up to 3,000 m/z which allows the detection of multi charged proteins of up to a molecular mass of several decades of kDa, including glycoproteins like TRF.

Figure 2 shows the total ion chromatogram (TIC) trace of the TRF of a control serum emerging from the two-step chromatographic run (panel a) and its MS spectrum (panel b). Determination of the mass of the various TRF isoforms is performed by a spectral deconvolution based upon the reconstruction of multiply charged electrospray data by using BioAnalyst software (panel c).

Analysis of intact TRF profiling on 20 confirmed patients and 100 healthy controls has been performed. Figure 3 shows examples of the obtained TRF MW distribution. Note the MW assignments are reported after the deconvolution process: values are within the tolerance as due to the resolving power of the measuring analyser (the "nominal" resolution of quadrupole). Supplementary Figures S3–S8 show the MW distribution spectra in more detail and where to recognize the main tetra-sialo isoform (close to theoretical MW 79,573 Da), the main bi-sialo (theoretical MW 77,365 Da), the asialo (theoretical MW 75,157 Da), the tri-sialo (theoretical MW 79,281 Da), the main penta-sialo (theoretical MW 80,232 Da).

The TRF distribution in a healthy control shows the bi-sialo form to be unambiguously lower than the dominant tetra-sialo TRF peak (Figure 3, upper left panel). The other panels show the MW spectra collected from patients with a CDG-Type I pattern where the desialylated isoforms are clearly increased.

The ratio of desialylated (with 0–2 sialic acid residues) isoforms to total TRF (%) was calculated for all patients and controls with Eq. (1).

Patients with a CDG-Type I pattern showed higher amounts of bi-sialo (range: 6.7–29.6%) compared to controls (<5.5%, mean percentage 3.9%), see, Supplementary Table S1.

Bi-sialo-TRF increment also appears in the chromatogram from patients with PGM1-CDG (mixed CDG Type I–II) as shown in the bottom right panel of Figure 3 (range: 7.2–9.5%).

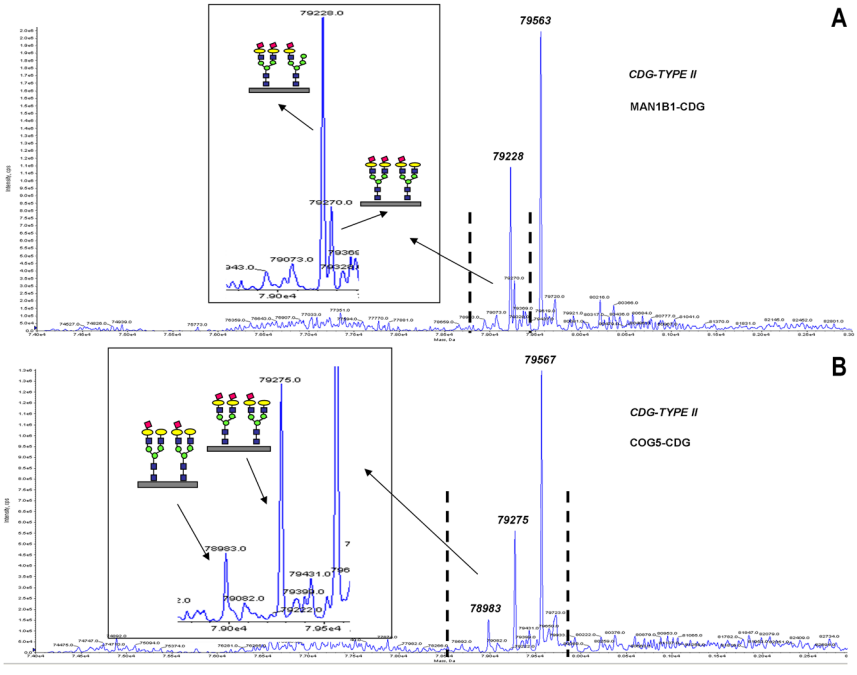

Figure 4: MW distribution of intact TRF from patients affected by CDG-II.
The upper part (A) displays the profile from a MAN1B1-CDG patient while the lower part (B) belongs to a COG5-CDG case. Both profiles display increased amounts of tri-sialo-TRF for both patients.

The defects involved in CDG-Type II occur in the late processing of N-glycans on the glycosylated protein. The MW spectrum pattern showed an increased peak of tri-sialo-TRF glycoforms (Figure 4). The mean percentage of tri-sialo-TRF (Eq. (2)) was 9.3 % (range: 2.9–12.9%) in controls, which was lower than what found in the serum from two patients with COG5-CDG and MAN1B1-CDG (18.5 and 24.5%, respectively) (Supplementary Table S1).

Samples carrying a variant allele TRF polymorphism showed all glycoforms to be split into two peaks on the MS spectrum (see, Supplementary Figure S1). However, the altered spectrum does not impact on the relative abundance of one isoform over the others, as also reported by other MS-based method [21]. Nevertheless, there are rare cases with a TRF mutation in a glycosylation site of the protein. In these cases, the spectrum could reveal equal amounts of tetra-sialo-TRF and bi-sialo-TRF, due to the damaging impacts on gene TRF [22].

We tested the proposed strategy also for detecting chronic alcohol abuse. Analysis of TRF profiles of five samples from heavy daily drinkers showed elevated levels of bi-sialo-TRF (range 6.8–7.5%), which were considerably higher than observed in healthy controls (see, Supplementary Figure S2).

All the above %-results obtained with Eq. (1) imply that every isoform detectable in the MW distribution spectrum must be picked up for expressing the term "Σ all isoforms". Therefore, MW spectrum has been digitalized and processed for considering any feature expressing an isoform and include it in the "Σ all isoforms" term.

In order to overcome this additional processing step and as a further step for facilitating the recognition of a CDG-Type I in a routine large scale running, instead of referring the asialo and bi-sialo forms to total TRF, we have tentatively considered just the ratio "main bi-sialo/main tetra-sialo". In so doing, we have tried to verify whether the latter ratio is suitable for making recognition of abnormalities easier and faster.

To evaluate how the performance of this proposed strategy is consistent with other approaches used for CDG-Type I diagnosis, we retrieved the main bi-sialo form/tetra-sialo form ratios from the MW distribution spectrum and plotted the results as shown in Figure 5. Panel (a) clearly shows as this simplified ratio unambiguously enables the recognition of the CDG-Type I. Panel (b) shows the same data but with a log-scale where the grouping between CDG-patients and healthy subjects is even more evident.

Figures of merit of the method were determined by calculating intra-day and inter-day precision of bi-sialo and tri-sialo TRF on one normal sample run in six replicates

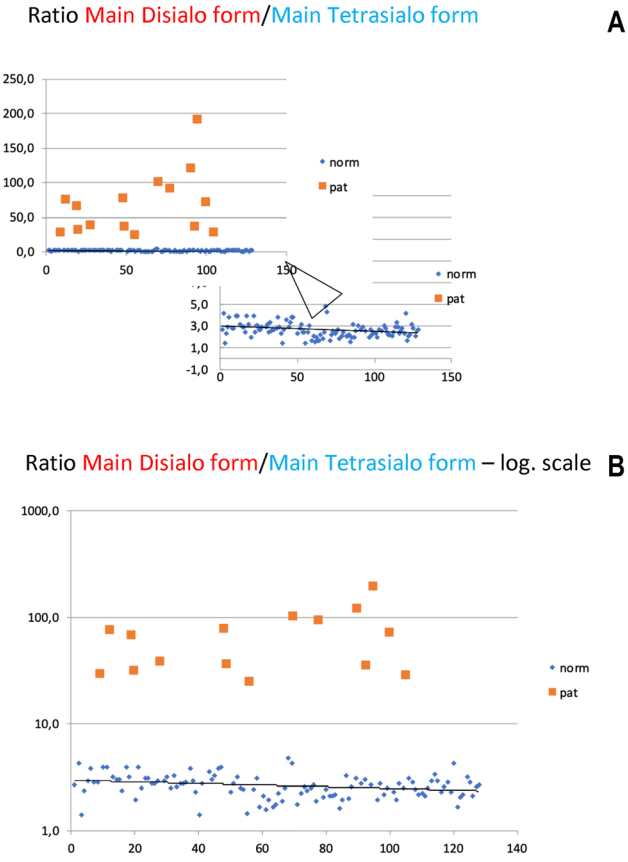

Figure 5: Plot of relationship between normal population and CDG-I patients with the simplified calculation of main bi-sialo-TRF/main tetra-sialo-TRF ratios.
The upper panel (A) shows the distribution of the ratio values for controls and the dispersed CDG-Type I patients. Blue-filled squares represent the patients meanwhile the light-blue dots represent the controls. In lower panel (B) same data are reported on a log-scale.

for three consecutive days. Intra-day and inter-day precisions were less than 9 and 16%, respectively, for bi-sialo- and less than 3 and 6% for tri-sialo-TRF. These RSD% values support the adequacy of the hereby described strategy for a large routine clinical biochemistry laboratory (see, Supplementary Table S2)

Conclusions

The hereby-proposed method looks robust, simple, low-cost and rapid (taking less than 20 min). Sample preparation requires a simple dilution with no need for further sample pre-treatment. Since the workflow estimates the various isoforms as intact proteins, their lengthy digestion and its associated burden (preventive protein class segregation, reduction and alkylation) are omitted. The required isolation of the serum TRF has been implemented in the

chromatographic set-up leaving the user to make just a dilution of the specimen. Investment and running costs are limited to a quadrupole technology and normal LC-solvents and chemicals (no immunoaffinity stuff).

Compared with others, this method provides several benefits, including high sensitivity and specificity and it has demonstrated a great potential for use in clinical biochemistry laboratories, where LC-MS plays an increasingly important diagnostic role.

The simplified calculation of main bi-sialo/main tetra-sialo ratio looks as an interesting option in a large-scale screening scenario.

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Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: This study was approved by the Institutional Ethics Board of Meyer Hospital of Florence (No. 137/2020).

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