RESEARCH ARTICLE

MALDI-TOF MS applied to apoC-III glycoforms of patients with congenital disorders affecting O-glycosylation. Comparison with two-dimensional electrophoresis

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Purpose: The O-glycan abnormalities accompanying some congenital disorders of glycosylation, namely conserved oligomeric Golgi-congenital disorders of glycosylation (COG-CDGs) and ATP6V0A2-CDGs, are mainly detected using electrophoresis methods applied to circulating apolipoprotein C-III. The objective of this study was to evaluate the reliability of MALDI-TOF MS of apoC-III for the detection and characterization of CDG-associated O-glycan defects.
Experimental design: plasmas from CDG-negative, COG-CDG, and ATP6V0A2-CDG patients were analyzed and results were compared to those obtained using 2DE followed by Western blot.
Results: MALDI-TOF of apoC-III allowed to detect various significant O-glycan abnormalities in CDG-patients with emphasis to COG-CDG. Furthermore, in CDG samples, comparison study between 2DE and MALDI-TOF showed a particular behavior of monosialylated apoC-III in the mass spectrometer that could be related to an abnormal O-glycan structure.
Conclusions and clinical relevance: MALDI-TOF MS appears as a powerful technique for the analysis of apoC-III glycoforms for potential routine screening of COG- and ATP6V0A2-CDGs.

Keywords:
Apolipoprotein C-III / ATP6V0A2 / CDG / COG complex / MALDI-TOF / O-glycosylation

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1 Introduction

Glycosylation is a PTM involving a huge variety of molecular partners (lipid anchor, activated nucleotide sugars, monosaccharides, enzymes, transporters . . .) within the secretory pathway in ER and Golgi apparatus. Depending on the site it occurs, glycosylation is mainly subdivided into N-glycosylation (amide linkage to Asn) and O-glycosylation (glycosidic linkage to Ser/Thr) [1]. Congenital disorders of glycosylation (CDG) typically share N-glycosylation defects and are defined as type I or type II according to the defective biosynthesis step [2]. Mutations in structural or functional Golgi proteins can disturb overall glycosylation leading to N- and O-glycan abnormalities [3]. This group of CDGs essentially corresponds to mutations in genes encoding for conserved oligomeric Golgi complex subunits

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Abbreviations: **ApoC-III**, apolipoprotein C-III; **CDG**, congenital disorder of glycosylation; **COG**, conserved oligomeric Golgi

Clinical Relevance

As all congenital disorders of glycosylation (CDG), those affecting O-glycosylation (COG-CDG and ATP6V0A2-CDG) share very heterogeneous clinical symptoms. Consequently, although rare, these inherited diseases are frequently suspected in specialized genetic consultations. In this context, we show in this work that MALDI-TOF MS of apoC-III, by allowing efficient, simple, and fast screening of O-glycosylation defects, could be a very interesting analytical tool for reducing very frequent and highly painful "wandering" toward accurate CDG diagnosis and potential prenatal diagnosis.

(COG-CDG) and for the α2 subunit of the vesicular ATPase (ATP6V0A2-CDG). The COG complex consists in eight protein subunits (COG-1 to COG-8) organized into two lobes and importantly involved as a tethering factor in the trans- to cis-Golgi retrograde membrane trafficking [4]. The α2 subunit of the vesicular ATPase H^+ pump (ATP6V0A2) plays an important role in medial- and trans-Golgi pH acidification and in retrograde membrane trafficking [5]. Clinically, COG-CDGs and ATP6V0A2-CDGs present heterogeneous symptoms from mild to very severe neurological defects associated with other multiorgan features such as dysmorphia, skin abnormalities, microcephaly, and liver disease [5–7]. Biologically, deficiencies in COG subunits and in ATP6V0A2 have been shown to indirectly disturb N-glycosylation as well as mucin type O-glycosylation. Regarding N-glycosylation, COG- and ATP6V0A2-CDGs share similar IEF transferrin patterns, showing that terminal maturation steps are affected (CDG type II). Regarding O-glycosylation, these CDGs are associated with abnormal IEF profiles of apolipoprotein C-III (apoC-III) a mucin core 1 O-glycosylated protein whose glycoforms are detailed Fig. 1 [8–11]. Indeed, COG mutations share an increase of asialylated glycoforms (apoC-III_0 patterns) while ATP6V0A2 genetic defects are associated to a decrease of bisialylated apoC-III_2 and a relative increase of the monosialylated glycoform (apoC-III_1 patterns) [7, 12, 13]. We use 2DE followed by Western blot for the screening of inherited defects of mucin core 1 O-glycosylation. By comparison with IEF that separates apoC-III glycoforms according to the number of negatively charged sialic acids (SA), 2DE, by adding a molecular weight separation step, has been shown to be more resolving for the asialylated species, i.e.: unglycosylated apoC-III (apoC-III_{0a}), GalNAc-apoC-III (apoC-III_{0b}), and Gal-GalNAc-apoC-III (apoC-III_{0c}) [14]. Since IEF and 2DE are relatively laborious techniques, progresses in MS analysis of apoC-III suggest interesting potentialities for the screening of COG- and ATP6V0A2-CDGs. Indeed, after minimal pretreatment of few microliters of serum/plasma, MALDI-TOF MS allows accurate *m/z*-based separation of apoC-III glycoforms, including asialylated ones [9]. Investigating a set of clinical conditions such as obesity, liver fibrosis, or sepsis, Harvey and col. showed altered ratios of the two major full sequence apoC-III glycoforms, i.e. apoC-III_1 and apoC-III_2 [11]. In the field of CDG with O-glycans abnormalities, Wada and col. applied MALDI-TOF to apoC-III of an individual with ATP6V0A2 mutation and showed an unusual decrease in O-glycans site occupancy [15].

In order to evaluate the reliability and robustness of MALDI-TOF for the screening and the study of CDGs affecting O-glycosylation, plasmas from CDG-negative and from COG- and ATP6V0A2-CDGs patients were analyzed, and results were compared to those obtained using 2DE Western blotting.

2 Materials and methods

2.1 Samples

With exception of one COG1-, one COG5-, and two ATP6V0A2-CDG samples (provided by D.J. Lefeber, Nijmegen, Netherlands), all plasmas used in this study were obtained at Bichat Hospital (Groupe d'étude des glycopathies congénitales, AP-HP, Paris, France). For MALDI-TOF determination of CDG-negative reference values of apoC-III glycoforms, samples were obtained from 15 CDG-negative patients (previously screened by normal transferrin and apoC-III electrophoresis patterns) encompassing a large clinical spectrum. Concerning the testing of CDG-patients, eight samples were analyzed: one COG1-, two COG5-, one COG7-, and four ATP6V0A2-CDGs. Samples from COG-CDG and ATP6V0A2-CDG share rather similar N-glycosylation abnormalities (CDG type II profiles) as determined using IEF or 2DE of transferrin. For MALDI-TOF/2DE comparison study, 23 plasmas were analyzed, including samples from 12 CDG-negative patients, eight CDG patients, and three patients with isolated abnormal apoC-III patterns but without identified causative genes.

2.2 MALDI-TOF analysis

MALDI-TOF analysis of plasma samples was performed as previously described [9]. Briefly, 1.0 μL of plasma was diluted in 15 μL of water/ACN/TFA (95:5:0.1), extracted using C4 Zip-Tip (Millipore) and eluted in 0.75 μL of water/ACN/TFA (25:75:0.1). Eluates were applied on MALDI target and mixed with equal volume of sinapinic acid as the matrix (saturated solution in water/ACN/TFA, 50:50:0.1).

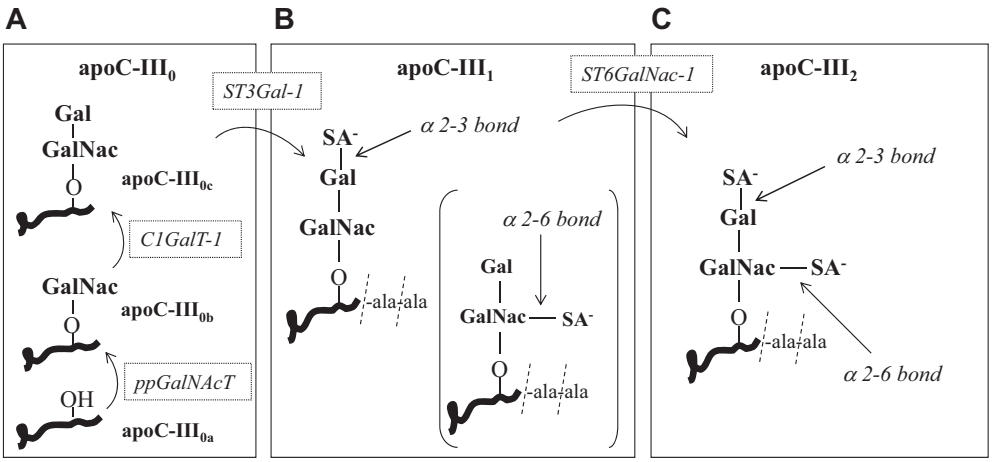

Figure 1. Mucin Core1 O-glycan structures of apoC-III. (A) Asialylated glycoforms (apoC-III_0): unglycosylated apoC-III (apoC-III_{0a}), GalNAc-apoC-III (apoC-III_{0b}), and Galβ1–3GalNAc-apoC-III (apoC-III_{0c}). ApoC-III_{0b} and apoC-III_{0c} respectively result from the sequential and concerted action of polypeptide GalNAc transferase (ppGalNAcT) and core1 galactosyl transferase-1 (C1GalT-1), two enzymes located in cis/median Golgi and organized in heteromeric complexes [18, 21]. (B) Monosialylated glycoforms (apoC-III_1): "normal" apoC-III_1 glycoform NeuAcα2,3Galβ1–3GalNAc-apoC-III as produced by beta-galactoside alpha-2,3-sialyl transferase-1 (ST3Gal-1). ST3Gal-1 is located in median/trans Golgi [18, 19]. In brackets: putative "abnormal" apoC-III_1 glycoform Galβ1–3(NeuAcα2,6)GalNAc-apoC-III as aberrantly produced by alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase-1 (ST6GalNac-1) acting prior to ST3Gal-1. These two apoC-III_1 glycoisomers can also result from the loss of one sialic acid from bisialylated apoC-III_2. Minor apoC-III_1 truncated isoforms lacking one or two C-terminal alanine have also been described [8, 11]. (C) Bisialylated glycoform (apoC-III_2): NeuAcα2,3Galβ1–3(NeuAcα2,6)GalNAc-apoC-III as produced by ST6GalNac-1. ST6GalNac-1 is located in the median/trans Golgi [18]. Minor apoC-III_2 isoforms lacking one or two C-terminal alanine have also been described [8, 11].

After crystallization, samples were analyzed with a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems) operating in positive linear mode with 200 laser shots per sample (accelerating voltage: 25.000 V, grid voltage: 93%, extraction delay time: 350 nsec, acquisition mass range: 5000–15 000 Da). Optimization on laser intensity was carried out to determine a power leading to high S/N with minimal terminal sialic acid (SA) removal. Compromise was reached for a laser power of 2211. Background substraction, internal calibration (m/z 9423, apoC-III_1), and smoothing of spectra (Gaussian smooth: 31 points) allowed to label peaks corresponding to apoC-III glycoforms and to generate the respective peaks intensity list. Comparisons were done according to peak intensity percentages.

2.3 2DE Western blotting analysis

2DE of proteins from 1 μL of plasma was conducted using ZOOM strip pH 4–7 for the first dimension and ready-made 4–12% NuPAGE Bis-Tris gel for the second dimension as recommended by the manufacturer (Life technologies). After 2DE, proteins were transferred (100 volts for 1 h) to nitrocellulose sheets and apoC-III glycoforms were revealed using rabbit anti-apoC-III primary antibody (1/5000 v/v; BioDesign International), HRP-linked anti-rabbit IgG secondary antibody (1/5000 v/v; GE Healthcare) and Chemidoc XRS system from Bio-Rad. Relative quantification of apoC-III glycoforms was performed using ImageLab software (Bio-Rad, Marnes la Coquette).

2.4 Statistical analysis

Pearson's correlation coefficients were determined using Microsoft Excel program. Significance of comparisons between CDG-patients (COG and ATP6V0A2) and CDG-negatives was determined using nonparametric Mann–Whitney test (GraphPad Prism 6 software).

3 Results

3.1 ApoC-III MALDI-TOF profiles of CDG-negative samples

Typical apoC-III glycoforms profile (m/z range: 8600–10 000 Da) of plasma from a CDG-negative patient is shown Fig. 2. In agreement with prior studies [9, 11, 16], in all samples, up to seven major peaks can be respectively labeled as nonglycosylated apoC-$III_{0a,}$ apoC-II, Gal-GalNAc-apoC-III (apoC-III_{0c}), SA-Gal-GalNAc-apoC-III minus C-term Ala (apoC-III_1 minus ala), full sequence apoC-$III_{1,}$ SA_2-Gal-GalNAc-apoC-III minus C-term ala (apoC-III_2 minus ala), and full sequence apoC-III_2. Peak corresponding to apoC-III_1 ($m/z = 9422$) appears to be the base peak among apoC-III glycoforms while peaks corresponding to apoC-III_{0c} (m/z

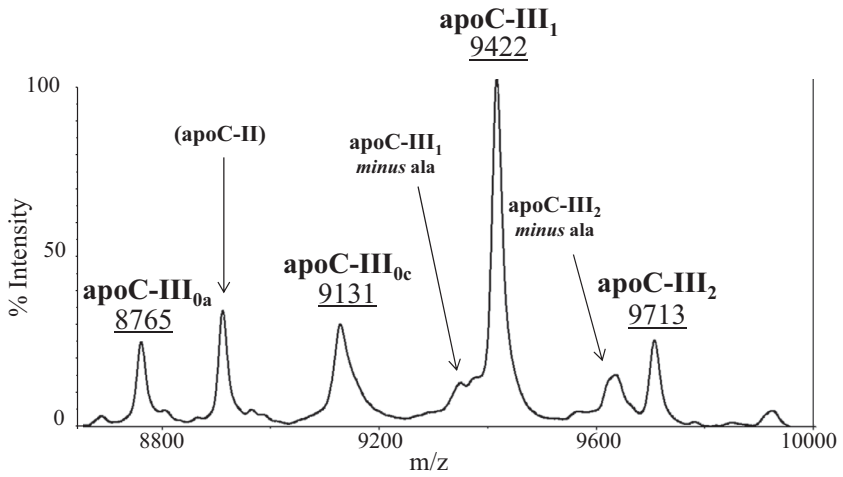

Figure 2. MALDI-TOF spectrum of apoC-III glycoforms from a CDG-negative patient. Average masses of protonated molecular ions were detected in the region of 8.600–10 000 Da, covering all apoC-III glycoforms. Underlined *m/z* values correspond to full length major apoC-III glycoforms.

= 9131) and apoC-III$_2$ (m/z = 9713) share similar relative abundance. Furthermore, no peak can be detected as GalNAc-apoC-III (apoC-III$_{0b}$, theoretical m/z = 8963) in any samples. MALDI-TOF analysis of the CDG-negative samples allowed to establish reference range values (mean ± 2SD) for each full sequence glycoform percentage as well as for corresponding apoC-III$_1$/apoC-III$_2$ and apoC-III$_{0a}$/apoC-III$_2$ percentage ratios (Table 1).

3.2 MALDI-TOF analysis and 2DE profiles of COG-CDGs and ATP6V0A2-CDGs

MALDI-TOF mass spectra of CDG-patients show at least one out of reference % value in apoC-III glycoforms distribution (Table 1). COG-CDGs share increased median % value for apoC-III$_{0a}$ while ATP6V0A2-CDGs show decreased median % value for apoC-III$_2$ ($p < 0.001$; Supplementary file 1). ApoC-III$_{0a}$/apoC-III$_2$ ratio is discriminating for COG-CDGs while apoC-III$_1$/apoC-III$_2$ is discriminating for ATP6V0A2-CDGs. Taken together, at least one of the two ratios is out of reference range for each CDG-patient (Fig. 3).

In agreement with MALDI results, 2DE Western blot patterns of COG-CDG samples share increase of the spot corresponding to apoC-III$_{0a}$ (apoC-III$_0$ patterns), while 2DE patterns of ATP6V0A2-CDGs share decrease of apoC-III$_2$ (Fig. 4).

3.3 MALDI-TOF MS/2DE comparison

Given that we use 2DE Western blotting of apoC-III for the screening of CDGs affecting O-glycosylation [14, 17], a comparison study with MALDI-TOF was undertaken. Concerning the apoC-III$_{0c}$ glycoform (Gal-GalNAc-apoC-III), it is nearly never detected using 2DE in all samples (in agreement with our screening experience) but reaches percentages around 20% using MALDI-TOF. Since antibodies we used have been

Table 1. MALDI-TOF-determined relative abundances of apoC-III glycoforms and related % apoC-III$_1$/% apoC-III$_2$ and % apoC-III$_{0a}$/% apoC-III$_2$ ratios

	% apoC-III$_2$	% apoC-III$_1$	% apoC-III$_{0c}$	% apoC-III$_{0a}$	% apoC-III$_1$ /% apoC-III$_2$	% apoC-III$_{0a}$ /% apoC-III$_2$
CDG-negatives (n = 15) Mean (SD)	21.4 (5.5)	54.2 (3.3)	18.1 (1.7)	6.2 (2.9)	2.7 (0.9)	0.35 (0.22)
Reference intervals	10.4–32.4	47.6–60.8	14.7–21.5	0.4–12.0	0.9–4.5	0.0–0.79
COG-1	**8.3**	48.8	18.2	**24.8**	**5.9**	**3.0**
COG-5[a]	16.0	**35.0**	**13.0**	**36.0**	2.2	**2.25**
COG-5[b]	**7.2**	47.8	26.1	**18.8**	**6.6**	**2.60**
COG-7	**6.5**	**45.7**	19.6	**28.3**	**7.0**	**4.33**
ATP6VOA2[a]	11.0	**64.5**	19.4	5.2	5.9	0.47
ATP6VOA2[b]	**8.5**	**65.4**	18.3	7.8	**7.7**	**0.92**
ATP6VOA2[c]	**10.4**	**61.0**	15.2	**13.4**	**5.9**	**1.29**
ATP6VOA2[d]	**6.1**	60.0	**22.6**	11.3	**9.8**	**1.86**

Bold values are out of reference intervals.

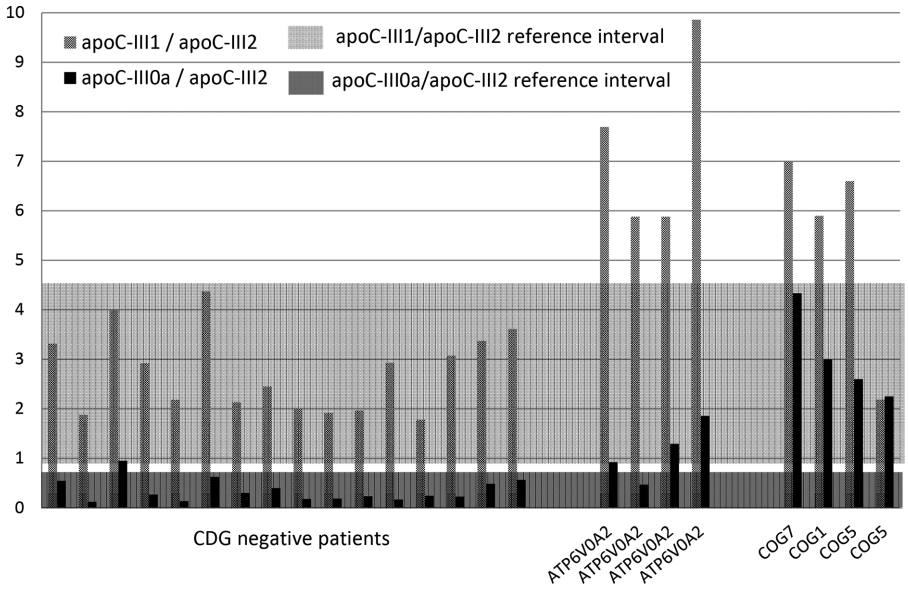

Figure 3. ApoC-III_1/apoC-III_2 and apoC-III_{0a}/apoC-III_2 percentage ratios. Histogram representation of apoC-III_1/apoC-III_2 (dotted black) and apoC-III_{0a}/apoC-III_2 (black) percentage ratios. Positioning of CDG-negative patients, ATP6V0A2-CDG patients and COG-CDG patients compared to reference intervals.

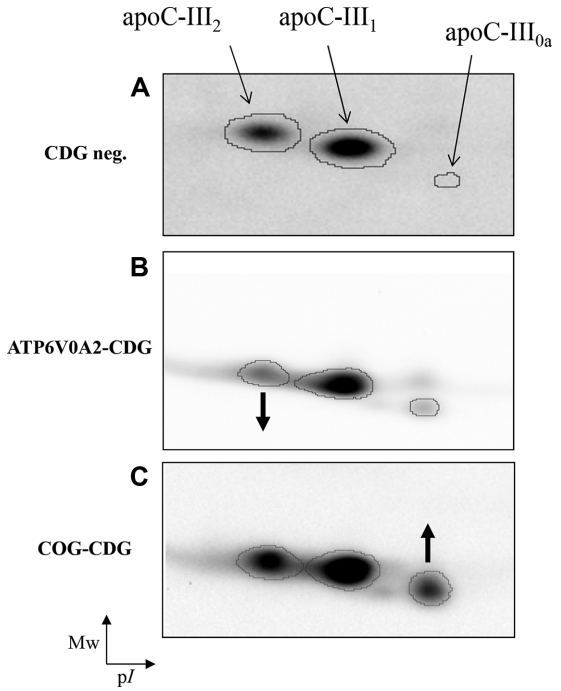

Figure 4. 2DE Western-blot of apoC-III. Zoom of 2DE Western blotting of apoC-III glycoforms from 1 μL of plasma (pH 4–7; 4–12% NuPAGE Bis-Tris gel followed by specific imunostaining of apoC-III). (A) CDG-negative patient (B) ATP6V0A2-CDG patient, and (C) COG-CDG patient. In ATP6V0A2-CDG patients, a decrease of apoC-III_2 (left spot) is consistently observed, while COG-CDG patients share an increase of apoCIII_{0a} (right spot). Arrows embodies noticeable variation by comparison with CDG-negative.

shown to fully recognize apoC-III_{0c}, this suggests that apoC-III_{0c} probably exists at very low-level in vivo and mainly results, as previously described, from artefactual laser-induced SA loss [11]. 2DE Western blotting is performed with a polyclonal anti-apoC-III antibody that recognizes apoC-III glycoforms lacking C-terminal alanine (Fig. 1). In this comparison study, since 2DE spots corresponding to apoC-III_1 minus ala and apoC-III_2 minus ala are combined with full length apoC-III_1 and apoC-III_2, respectively, % of detectable MALDI-TOF MS peaks related to truncated glycoforms were added to their full-length counterparts. Under these conditions, when applied to both CDG-negative and CDG-positive samples, the methods are well correlated for apoC-III_2 ($r = 0.907$; $p < 0.01$) but less correlated for apoC-III_1 ($r = 0.793$) (Fig. 5). For apoC-III_1, correlation becomes satisfactory if considering both COG- and ATP6V0A2-CDGs only ($r = 0.946$) or non-CDG only ($r = 0.896$) (Supplementary file 2).

4 Discussion

MALDI-TOF was applied to plasmas from fifteen CDG-negative patients and from eight patients with COG-CDG ($n = 4$) and ATP6V0A2-CDG ($n = 4$). In all samples, all already characterized apoC-III glycoforms could be detected with reproducible profiles emphasizing that MALDI-TOF is a reliable and robust technique for the separation and relative quantification of apoC-III glycoforms. For CDG-negative samples, reference values obtained under our experimental conditions are rather different that those determined by others in other MS conditions [9, 11, 15, 16]. This observation illustrates the lack of ionization standardization from one MALDI instrument to another and highlights the necessity to establish its own reference ranges before considering analysis of apoC-III glycoforms in pathological conditions.

Indeed, MALDI-TOF of apoC-III is a convenient tool for the study of mucin core1 O-glycan biosynthesis pathway that consists in the sequential actions of

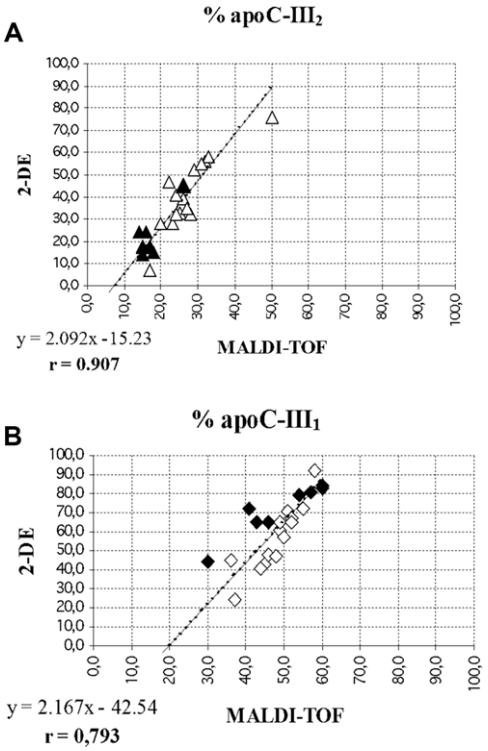

Figure 5. MALDI-TOF/2DE comparison for both CDG-negative and positive patients. For (A) bisialylated apoC-III$_2$ and (B) monosialylated apoC-III$_1$. Plain symbols correspond to CDG-patients.

GalNAc transferase (GalNAcT), core1 galactosyl transferase (C1GalT-1), beta-galactoside alpha-2,3-sialyl transferase-1 (ST3Gal-1), and alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase-1 (ST6GalNac-1) organized in a highly regulated spatial interplay along the Golgi apparatus (see legend of Fig. 1 for details). In particular, we assume in the following discussion that, in normal conditions (i.e. non-CDG patients), ST3Gal-1 acts prior to ST6GalNac-1 leading to homogeneous O-glycan monosialylated apoC-III$_1$ structure mainly bearing α2–3 SA linkage [13, 18, 19].

MALDI-TOF analysis of COG- and ATP6V0A2-CDG samples systematically shows various apoC-III glycoforms abnormalities when compared to reference ranges. For COG-CDG patients, we show for the first time that the increase of asialylated apoC-III glycoforms can be specifically related to non-glycosylated apoC-III$_{0a}$ abundance. This abnormality shows that a defect of the COG complex is accompanied to a decrease of O-glycans site occupancy, probably related to abnormal retrograde trafficking between trans- and cis-Golgi leading to partial mislocalization of GalNAcT and heterodimer with C1GalT-1 [4, 20, 21]. The lower abundance of apoC-III$_2$ over all COG-CDGs, while apoC-III$_1$ seems to be less impacted, also suggests that COG mutations are probably associated with altered positioning and/or activity of ST3Gal-1 and/or ST6GalNac-1 in the late compartments of the Golgi. Indeed, the increase of the ratio apoC-III$_{0a}$/apoC-III$_2$, combining the differences with CDG negative patients, is highly discriminating for COG-CDG patients (Fig. 3).

MALDI-TOF profiles of ATP6V0A2-CDGs show a systematic increase of the percentage ratio apoC-III$_1$/apoC-III$_2$ as described in numerous IEF or 2DE studies [12, 15, 17]. In the context of CDG, this abnormality illustrates the importance of the Golgi proton pump in sialylation steps catalyzed by ST3Gal-1 and ST6GalNac-1 within the medial and trans-Golgi [22]. However, the respective role of pH misregulation and/or impaired retrograde trafficking in this abnormality remains to be clarified. Additionally, when considering that this apoC-III$_1$/apoC-III$_2$ ratio has been shown to be aberrantly elevated in obesity [11], its specificity for ATP6V0A2-CDG could be questioned. A discrete increase of the unglycosylated apoC-III$_{0a}$ isoform has been found for one ATP6V0A2-CDG patient. Such an increase, previously described as "unusual" in one individual by Wada and col. [15], also suggests a possible influence of the α2 subunit of Golgi ATPase in the initiation of O-glycosylation by GalNAcT.

The comparison study between MALDI-TOF and 2DE shows satisfactory correlation for bisialylated apoC-III$_2$ (r = 0.897), with percentages obtained by MALDI approximately 1.6-fold lower than 2DE values. If considering that 2DE Western blotting correctly reflects the in vivo situation, this observation suggests, as evoked by others [11, 15], the loss of one SA linkage in a substantial part of apoC-III$_2$ due to fragmentation during ionization. In agreement with this hypothesis, the MS analysis of the same spot under increasing laser intensity showed a progressive decrease of apoC-III$_2$ relative abundance (data not shown). Lower ionization efficiency of this doubly negative glycoform can also be suspected [23]. Nevertheless, apoC-III$_2$ correlation shows that it homogeneously behaves within the different stages of MALDI-TOF analysis (laser shot, ionization, TOF . . .) in agreement with its structural homogeneity (Fig. 1). For the monosialylated glycoform apoC-III$_1$, when considering both CDG-negative and positive samples, the correlation study shows variable and sometimes inconsistent percentage differences (r = 0.793). Interestingly, correlation becomes very satisfactory if individualizing CDG positive patients (r = 0.944) as well as CDG negative ones (r = 0.896). These findings show different apoC-III$_1$ behaviors from one group to another and homogeneous behavior inside each group. This observation could be explained by unequal ionization response related to irregular structural composition of apoC-III$_1$ in CDG patients. More precisely, while apoC-III$_1$ bearing α2–3 SA linkage is mainly expected in non-CDG patients, our findings suggest the presence (or increased abundance) of abnormal α2–6 SA linkage in apoC-III$_1$ from COG- and ATP6V0A2-CDG patients reinforcing the already evoked probable dysregulation of ST3Gal-1/ST6GalNac-1 interplay in these two inherited pathologies.

In conclusion, MALDI-TOF allows fast, sensitive, and reproducible analysis of circulating apoC-III glycoforms and appears as a promising technique for the screening of COG-

CDGs and ATP6V0A2-CDGs. Furthermore, when coupled to 2DE, it could be a useful tool for helping in the molecular characterization of mucin core1 O-glycan abnormalities.

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