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RAPID COMMUNICATION

Two dimensional gel electrophoresis of apolipoprotein C-III and MALDI-TOF MS are complementary techniques for the study of combined defects in *N*- and mucin type *O*-glycan biosynthesis

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In the field of diseases related to glycosylation disorders, congenital defects associated with abnormalities in both *O*- and *N*-glycosylation of proteins constitute arising novel entities. Defects in subunits of the conserved oligomeric Golgi protein complex have been shown to be involved in an important part of previously unsolved CDG type II combining abnormalities in both mucin type core1 *O*- and *N*-glycans; furthermore, recent studies revealed that autosomal recessive cutis laxa type II could also be associated with such combined glycosylation defects. Based on the studies of serum samples from three patients including a case of cutis laxa, we present here evidence that 2-DE of apolipoprotein C-III in combination with MALDI-TOF-MS analysis of serum *O*- and *N*-glycans allow the detection and the biochemical characterization of these newly recognized glycosylation disorders.

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Together with IgA1 [1], apolipoprotein C-III (apoC-III) is one of the better characterized mucin type core1 *O*-glycosylated circulating proteins in humans. As it carries the most common form of *O*-linked glycans [2], IEF of apoC-III has been proposed as a biological test for the screening of *O*-glycosylation disorders [3] such as those retrieved in conserved oligomeric Golgi (COG) complex subunits congenital defects [4–6], in sialuria [7] and in autosomal recessive cutis laxa type II (ARCL-II) [8]. It has been shown that IEF could separate three isoforms of apoC-III corresponding to the disialyl (apoC-III_2), the monosialyl (apoC-III_1), and the asialyl (apoC-III_0) glycoforms of this protein. Although allowing unambiguous separation of apoC-III_2 and apoC-III_1, IEF did not permit to distinguish between the three theoretical components of apoC-III_0, *i.e.*, nonglycosylated, with only *N*-acetylgalactosamine (GalNAc) (also called "Tn antigen") and with galactose (Gal)-GalNAc. Using IEF, some defects of *O*-sialylation were characterized in cases of ARCL-II or sialuria [7,

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Abbreviations: apoC-III, apolipoprotein C-III; **ARCL-II,** autosomal recessive cutis laxa type II; **COG,** conserved oligomeric Golgi; **Gal,** galactose; **GalNAc,** *N*-acetylgalactosamine

8], but those described as affecting the initial steps of *O*-glycosylation in COG subunits defects remained to be or were partially characterized using additional techniques such as MS and/or lectin binding of patient's cells [4–6]. Concerning MS techniques, it has been shown that MALDI-TOF MS could be efficiently applied not only to the accurate separation but also to the relative quantification of serum *N*-glycans and of major usual forms of core1 mucin type serum *O*-glycans [9, 10]. We recently described a 2-DE method allowing us to separate the two sialylated apoC-III major isoforms and up to four minor asialylated ones [11]; nevertheless, the potential of 2-DE for the screening and characterization of disorders combining *O*- and *N*-glycans abnormalities has not been evaluated. In this purpose, we present here the 2-DE and MALDI-TOF MS-based studies of three human sera revealing abnormal undersialylation and undergalactosylation of *N*- and of mucin type core1 *O*-glycans.

Patient 1 is a French girl from nonconsanguineous healthy parents presenting at birth with marked developmental delay, cardiac intraventricular communication, axial hypotonia, and large anterior fontanel. Today, she is 14 years-old and shares mild mental retardation and marked developmental delay associated with typical symptoms of ARCL-II such as localized skin wrinkles, hypermobile joints, and nasal voice. Syndactylism, short toes, and convergent strabismus could also be noted. Standard and high resolution caryotypes were normal as well as haemostasis, hepatic, and iron biological investigations.

Patients 2 and 3 were Lebanese male siblings from consanguineous healthy parents; they both developed severe hypotonia and severe developmental/mental retardations with a start of symptoms during neonatal period and a rapid lethal issue before 1 year-old.

For these three patients, secondary causes of glycans abnormalities, *i.e.*, galactosaemia, fructose intolerance, and septicaemia were excluded based on clinical and biological findings.

2-DE and Western blotting of apoC-III isoforms from 1 to 5 μL of serum was carried out as previously described [10] using IPG ranging from 4.0 to 7.0 and 15% acrylamide SDS-PAGE mini-gels. MALDI-TOF MS analysis of permethylated *O*- and *N*-glycans released from circulating glycoproteins were conducted also as previously described using up to 30 μL of serum [9].

When compared with controls (Fig. 1A, left), the 2-DE pattern of apoC-III from patient 1 (Fig. 1B, left) showed an increased percentage of the monosialyl isoform (apoC-III_1 = 89%, reference interval 40–75%, n = 36) associated with decreased percentage of the disialyl isoform (apoC-III_2 = 10%, reference interval 25–60%) and normal percentage of asialyl isoforms (apoC-III_0 = 1%, reference interval <5%); the study of one additional independent sample from the same patient confirmed the abnormality with, in this case, undetectable asialyl isoform (not shown). MALDI-TOF MS analysis of whole circulating core1 *O*-linked glycans from patient 1 (Figs. 1A and B, right; Table 1) corroborated these results at the overall level showing a decreased relative percentage of the disialyl residue (m/z = 1256). Lastly, MALDI-TOF MS analysis of serum N-linked glycans from patient 1 (Figs. 2A and B) showed discrete abnormally increased levels of various undergalactosylated (m/z = 1836 and 2040) and undersialylated (m/z = 2431 and 2605) structures.

The 2-DE patterns of apoC-III from patients 2 and 3 showed dramatically increased percentages of the protein spot corresponding to apoC-III linked to GalNAc and/or to nonglycosylated apoC-III (*i.e.*, localized clearly below the virtual straight-line joining apoC-III_1 and apoC-III_2) in association with decreased percentage of the mono- and disialyl isoforms for patient 2 and of the disialylated one for patient 3 (Figs. 1C and D, left). For patient 2, a similar abnormal pattern (apoC-III_0 = 75%) was retrieved in other independent serum sample (not available for patient 3). For these two siblings, MALDI-TOF MS applied to *O*-glycans (Figs. 1C and D, right) appeared to be unable to detect any structure corresponding to *O*-GalNAc at theoretical m/z = 330. Because of the high volatility of the methylated derivative, this compound was probably lost during the purification step [8]. When considering this analytical limitation, measured relative levels of usual core1 *O*-glycans (at m/z = 534, 895, and 1256) from patients 2 and 3 appeared to us difficultly interpretable (data not shown). Furthermore, since we also failed in detecting the apoC-III isoform linked to GalNAc (as well as others "normal" isoforms) on 2-DE gels using periodate-based glycoprotein detection technique, MS study of the entire glycoprotein and/or of glycopeptides [12] will be undertaken in order to formally check for its presence in the two investigated patients. By contrast with the case of *O*-glycans, MALDI-TOF MS spectra of serum *N*-glycans from patients 2 and 3 (Figs. 2C and D) were more easily interpretable showing markedly (notably for patient 2) increased relative levels of various undergalactosylated (m/z = 1662, 1836, 2040, and 2228) and undersialylated structures (m/z = 2244, 2431, and 2605).

IEF of serum apoC-III has been shown to allow the detection of core1 mucin type *O*-glycosylation disorders [3, 13]. In one hand, IEF of apoC-III from consanguineous patients with congenital ARCL-II revealed an "apoC-III_1" profile sharing increased percentages of the monosialylated isoform [8, 14]; in the other hand, all identified COG subunits congenital defects were accompanied by a similar "apoC-III_0" profile with increased levels of the protein band corresponding to the asialylated isoforms [4–6]. In all cases, *N*-glycosylation was also impaired with congenital disorders of glycosylation (CDG) type II transferrin IEF patterns. Clinically, the patients with ARCL-II and apoC-III_1 profile notably shared localized skin folds, large fontanels, hypermobile joints, eye anomalies, motor developmental delay, and mild mental retardation [14]. In the field of COG deficiencies, those involving COG1 and COG8 led to a mild clinical phenotype, *e.g.*, with hypotonia, mild mental retardation, and cerebellar atrophy [5, 6], in radical contrast with

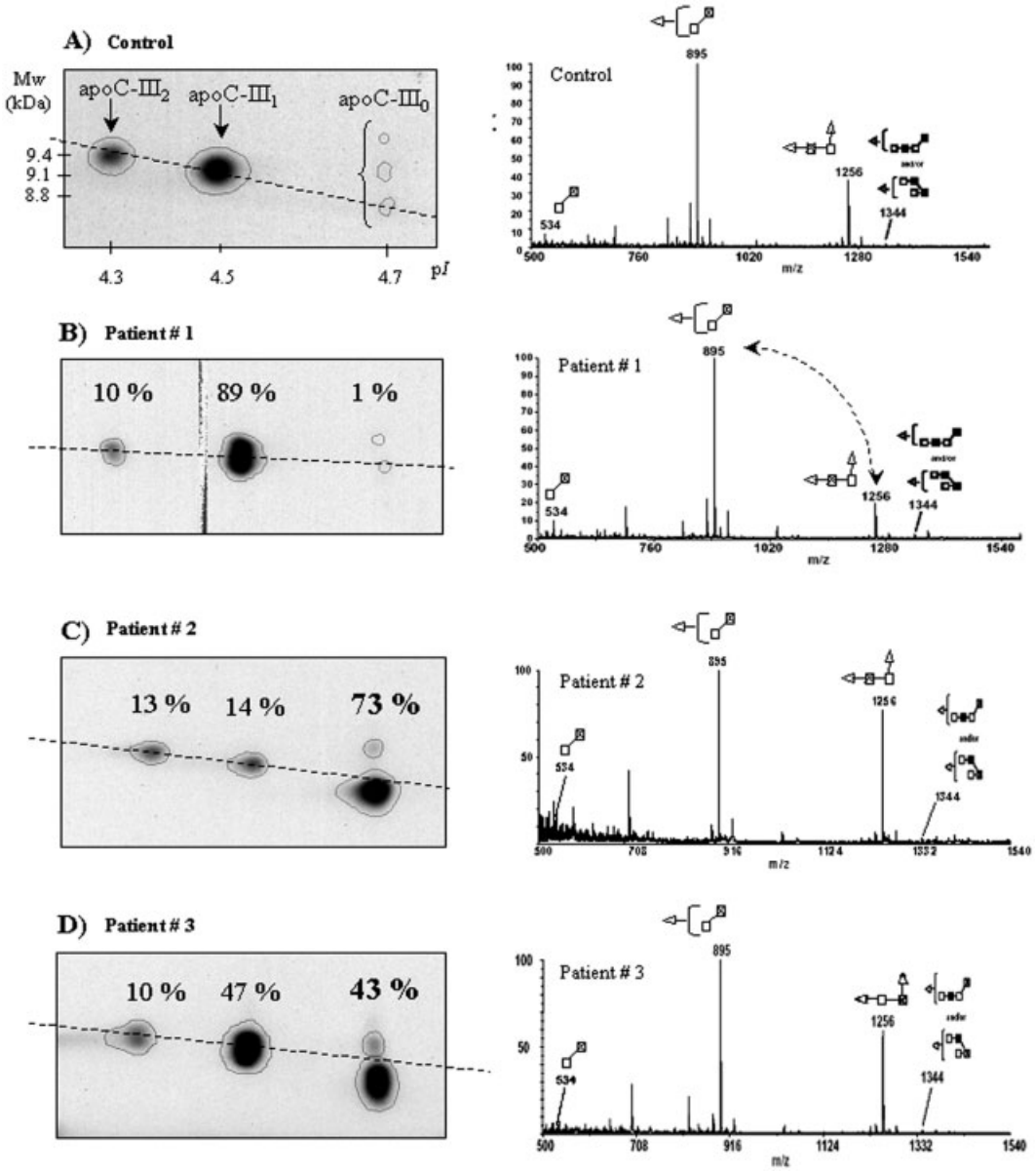

Figure 1. The 2-DE patterns of serum apoC-III (left) and corresponding serum *O*-glycans MALDI-TOF mass spectra (right) from control (A), patient 1 (B), patient 2 (C), and patient 3 (D). "Double-arrow" in Fig. 1B (right) indicate significant relative decrease in the bi-sialylated *O*-glycan structure at m/z = 1256. Symbols: □, Gal; ■, *N*-acetylglucosamine; ⊠, GalNAc; △, *N*-acetyl neuraminic (*i.e.*, sialic) acid.

those related to COG7 notably leading to severe hypotonia, severe liver failure, episodes of hyperthermia, and rapid lethal issue [4, 15].

In this study, 2-DE of apoC-III allowed low background separation of glycoforms into well-resolved and easy-to-quantify protein spots. For patient 1, it clearly showed an apoC-III_1 profile which was corroborated at the whole serum *O*-glycan level by MALDI-TOF MS. Concerning N-linked glycans from patient 1, MALDI-TOF showed discrete under-sialylation and undergalactosylation. When combined with clinical findings, these glycans deficiencies altogether strongly suggest major similarities with described patients with ARCL-II and *O*- and *N*-glycans abnormalities. Of interest is to note that the causative mutations in these patients have been recently found as affecting the gene encoding the α2 subunit of the V_0-H^+ ATPase protein [16].

For patients 2 and 3, 2-DE patterns of apoC-III revealed for the first time dramatically increased levels of the protein spot corresponding to the nonglycosylated isoform and/or to the isoform substituted with only GalNAc. Since MALDI-TOF MS clearly showed that *N*-glycosylation was also impaired with undersialylation and undergalactosylation,

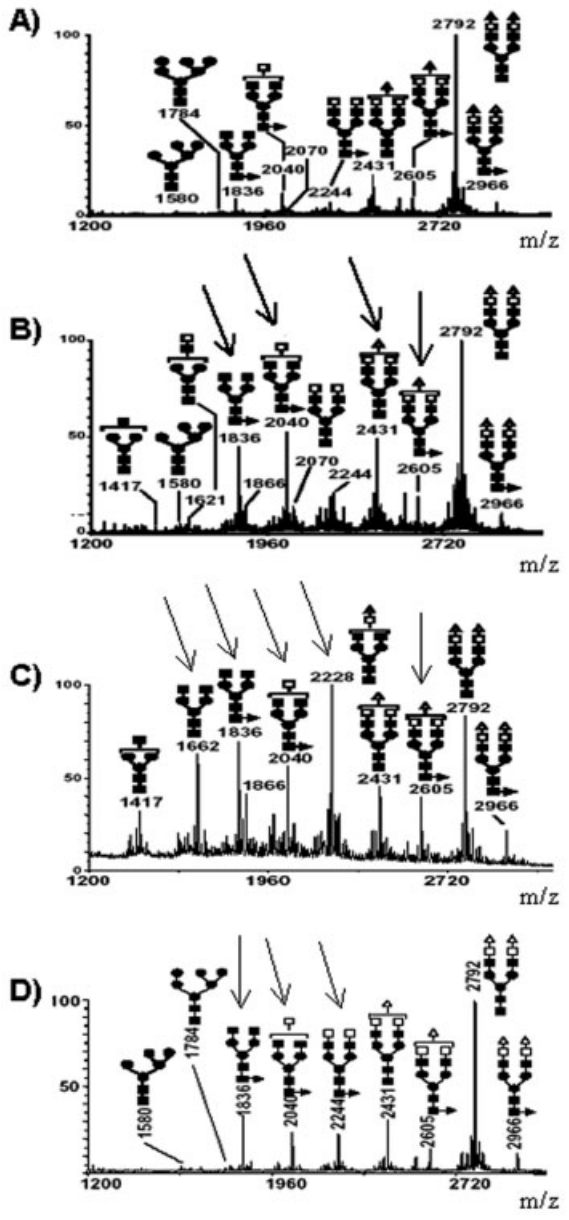

Figure 2. Serum *N*-glycans MALDI-TOF mass spectra from control (A), patient 1 (B), patient 2 (C), and patient 3 (D). Arrows indicate abnormally increased *N*-glycan structures. Symbols: ●, mannose; □, Gal; ■, *N*-acetylglucosamine; ▲, fucose; △, *N*-acetyl neuraminic (*i.e.*, sialic) acid.

these original biological data suggested a global defect in the glycosylation machinery similar to those retrieved in COG subunits deficiencies. More precisely, very severe and typical associated clinical findings in these siblings from consanguineous parents were highly suggestive of a congenital defect in COG7 subunit.

The 2-DE of apoC-III and MALDI-TOF-MS analysis of permethylated *O*- and *N*-glycans released from whole serum glycoproteins should be considered as complementary techniques for the biochemical study of both *O*- and *N*-glycosylation biosynthesis disorders. The 2-DE of apoC-III can be used to rapidly determine the different apoC-III isoforms in quantitative terms to get an overview of core1 *O*-glycans abnormalities. Most importantly, by contrast with IEF only, this technique allows to differentiate between various asialylated apoC-III isoforms. Furthermore, although still "immature" for the detection of *O*-GalNAc, MALDI-TOF MS of permethylated *O*-glycans can be proposed as a global confirmation method (at least in cases with apoC-III_1 profile) since *O*-glycans are released from whole serum glycoproteins of different cellular origin, *e.g.*, highly *O*-glycosylated IgA1 from lymphocytes and apoC-III from hepatocytes. More evidently in the field of *N*-glycosylation, MALDI-TOF MS can successfully be dedicated to the detection and the structural characterization of abnormally accumulated N-linked glycans. Thus, combination of both techniques led us to describe alterations in mucin type core1 *O*-glycans and in *N*-glycans from three patients opening the way toward future identification of new cases among these emerging combined glycosylation disorders.

Table 1. Relative percentage values of the major ions observed in MALDI-TOF MS spectra of permethylated mucin type core1 *O*-glycans from patient 1 by comparison with 16 controls (0–1 year, $n = 8$; 1–18 years, $n = 8$)

		Ion at *m/z* 534 (%)	Ion at *m/z* 895 (%)	Ion at *m/z* 1256 (%)
Reference range	0–1/1–18 years	0–6/0–12	100/100	30–39/23–41
Patient 1	14 years	9	100	19

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References

[1] Tarelli, E., Smith, A. C., Hendry, B. M., Challacombe, S. J. *et al.*, Human serum IgA1 is substituted with up to six O-glycans as shown by matrix assisted laser desorption ionisation time-of-flight mass spectrometry. *Carbohydr. Res.* 2004, *339*, 2329–2335.

[2] Wopereis, S., Lefeber, D. J., Morava, E., Wevers, R. A., Mechanisms in protein O-glycan biosynthesis and clinical and molecular aspects of protein O-glycan biosynthesis defects: A review. *Clin. Chem.* 2006, *52*, 574–600.

[3] Wopereis, S., Grunewald, S., Morava, E., Penzien, J. M. *et al.*, Apolipoprotein C-III isofocusing in the diagnosis of genetic defects in O-glycan biosynthesis. *Clin. Chem.* 2003, *49*, 1839–1845.

[4] Wu, X., Steet, R. A., Bohorov, O., Bakker, J. *et al.*, Mutation of the COG complex subunit gene COG7 causes a lethal congenital disorder. *Nat. Med.* 2004, *10*, 518–523.

[5] Foulquier, F., Vasile, E., Schollen, E., Callewaert, N. *et al.*, Conserved oligomeric Golgi complex subunit 1 deficiency reveals a previously uncharacterized congenital disorder of glycosylation type II. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 3764–3769.

[6] Foulquier, F., Ungar, D., Reynders, E., Zeevaert, R. *et al.*, A new inborn error of glycosylation due to a Cog8 deficiency reveals a critical role for the Cog1-Cog8 interaction in COG complex formation. *Hum. Mol. Genet.* 2007, *16*, 717–730.

[7] Wopereis, S., Abd Hamid, U. M., Critchley, A., Royle, L. *et al.*, Abnormal glycosylation with hypersialylated O-glycans in patients with Sialuria. *Biochim. Biophys. Acta* 2006, *1762*, 598–607.

[8] Wopereis, S., Morava, E., Grunewald, S., Mills, P. B. *et al.*, A combined defect in the biosynthesis of N- and O-glycans in patients with cutis laxa and neurological involvement: The biochemical characteristics. *Biochim. Biophys. Acta* 2005, *1741*, 156–164.

[9] Faid, V., Chirat, F., Seta, N., Foulquier, F. *et al.*, A rapid mass spectrometric strategy for the characterization of N- and O-glycan chains in the diagnosis of defects in glycan biosynthesis. *Proteomics* 2007, *7*, 1800–1813.

[10] Wada, Y., Azadi, P., Costello, C. E., Dell, A. *et al.*, Comparison of the methods for profiling glycoprotein glycans – HUPO human disease glycomics/proteome initiative multi-institutional study. *Glycobiology* 2007, *17*, 411–422.

[11] Bruneel, A., Robert, T., Lefeber, D. J., Benard, G. *et al.*, Two-dimensional gel electrophoresis of apolipoprotein C-III and other serum glycoproteins for the combined screening of human congenital disorders of *O*- and N-glycosylation. *Proteomics Clin. Appl.* 2007, *1*, 321–324.

[12] Wada, Y., Mass spectrometry for congenital disorders of glycosylation, CDG. *J. Chromatogr., B Anal. Technol. Biomed. Life Sci.* 2006, *838*, 3–8.

[13] Noll, B., Hackler, R., Pelzer, M., Pelzer, S. *et al.*, Semi-automated rapid isoelectric focusing of apolipoproteins C from human plasma using Phastsystem and immunofixation. *Clin. Chem. Lab. Med.* 1999, *37*, 643–648.

[14] Morava, E., Wopereis, S., Coucke, P., Gillessen-Kaesbach, G. *et al.*, Defective protein glycosylation in patients with cutis laxa syndrome. *Eur. J. Hum. Genet.* 2005, *13*, 414–421.

[15] Morava, E., Zeevaert, R., Korsch, E., Huijben, K. *et al.*, A common mutation in the COG7 gene with a consistent phenotype including microcephaly, adducted thumbs, growth retardation, VSD and episodes of hyperthermia. *Eur. J. Hum. Genet.* 2007, *15*, 638–645.

[16] Kornak, U., Reynders, E., Dimopoulou, A., van Reeuwijk, J. *et al.*, Impaired glycosylation and cutis laxa caused by mutations in the vesicular H+-ATPase subunit ATP6V0A2. *Nat. Genet.* 2008, *40*, 32–34.

