Apolipoprotein C-III Isofocusing in the Diagnosis of Genetic Defects in O-Glycan Biosynthesis

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Background: Defects in the biosynthesis of N-glycans may be found by isoelectric focusing (IEF) of plasma transferrin. No test is available to demonstrate O-glycan biosynthesis defects.

Methods: We used isoforms of apolipoprotein C-III (apoC-III) as a marker for the biosynthesis of core 1 mucin type O-glycans. Plasma samples from patients with primary defects and secondary alterations in N-glycan biosynthesis were studied by apoC-III isofocusing.

Results: Age-related reference values for apoC-III were determined. Plasma samples from patients with the primary congenital disorders of glycosylation (CDG) types Ia–Ic, Ie, If, IIA, and IID all showed a normal apoC-III isofocusing profile. Plasma from two patients with CDG type IIx were tested: one showed a normal apoC-III distribution, whereas the other showed a hypoglycosylation profile. In plasma from patients with hemolytic uremic syndrome (HUS), a hypoglycosylation profile was obtained.

Conclusions: IEF of apoC-III is a rapid and simple technique that may be used as a screening assay for abnormalities in core 1 mucin type O-glycans. Evidence that a patient in this study has a primary genetic defect affecting both N- and O-glycosylation provides the first example of an inborn error of metabolism affecting the biosynthesis of core 1 mucin type O-glycans. Our data narrow the options for the position of the primary defect in this patient down to a step in the biosynthesis, activation, or transfer of galactose or N-acetylneuraminic acid to both N- and O-glycans. Circulating neuraminidase excreted by Streptococcus pneumoniae caused the high percentage of asialo apoC-III in two HUS patients.

In 1987, Jaeken et al. (1) were the first to describe an inborn error of metabolism in the biosynthetic pathways of protein N-glycosylation. During the last decade, several defects were found in the biosynthesis of N-glycans. The diseases in this pathway have been collectively referred to as congenital disorders of glycosylation (CDG), formerly called carbohydrate-deficient glycoprotein syndrome (2, 3). The use of transferrin isofocusing on plasma and serum has been pivotal in detecting patients suffering from the various forms of the syndrome. At present, 13 different diseases in N-glycan biosynthesis are known: CDGIa through -Ih and CDGIIa through -IID (4–6). A few inherited diseases have been found in the biosynthesis of O-linked glycans, mainly by genetic approaches. Examples are muscle–eye–brain disease and Walker–Warburg syndrome, in which the defect is situated in the biosynthesis of O-mannose-based glycans (7, 8). The biochemical hallmark in CDGIc patients is hypofucosylation of...
both N- and O-glycosylated proteins. This disease is the first identified in which both N- and O-glycosylation is affected \(^{(9, 10)}\).

The biosynthesis of N-glycans as well as O-glycans can be divided into three stages (see Fig. 1). In the first stage, biosynthesis and activation of monosaccharides occurs. In the second stage, nucleotide sugars enter the endoplasmic reticulum (ER) bound to dolichol phosphate. Nucleotide sugars enter the lumen of the Golgi via specific antiporters. In the third stage, specific transferases attach the glycan in the ER and the Golgi. In mammals, six different types of O-glycans are known, classified on the basis of the first sugar attached to the serine or threonine residue of the protein \(^{(11)}\). The mucin type O-glycan, starting with N-acetylgalactosamine (GalNAc), is most common in humans. In total eight core structures can be distinguished depending on the second sugar(s) and/or sugar binding \(^{(11–13)}\). Further biosynthetic steps finally give rise to at least 50 different O-glycan structures, of which core 1 and 2 are most abundant \(^{(14, 15)}\). In brain and neural tissue, core 1 is most common \(^{(16)}\). The synthesis of core 2 mucin type O-glycans requires the conversion of unsubstituted core 1 mucin type O-glycans by a N-acetylglucosamine (GlcNAc) transferase \(^{(17, 18)}\).

In this study, we developed a test that may be used to demonstrate defects in the biosynthetic pathway of core 1 mucin type O-glycans. The test uses the isoforms of apolipoprotein C-III (apoC-III). This protein carries a single O-glycan at Thr-94 and is not N-glycosylated \(^{(19)}\). The protein is rather stable and is readily detectable in human plasma. Human apoC-III antibodies are commercially available. This study is a step in the development of a biochemical approach to find defects in the biosynthesis of O-glycans. To our knowledge it is the first report describing a patient with an inborn error of metabolism affecting core 1 mucin type O-glycan biosynthesis.

**Patients and Methods**

We analyzed 15 different plasma samples from patients with secondary N-glycosylation alterations [hemolytic uremic syndrome (HUS), \(n = 2\); galactosemia, \(n = 1\); alcohol abuse, \(n = 12\)]. In addition, we studied 21 different plasma samples from patients with primary CDG [CDGIA, \(n = 8\); CDGIB, \(n = 1\); CDGIC, \(n = 8\); CDGID, \(n = 1\); CDGIE, \(n = 1\); CDGIIf, \(n = 1\); CDGIIa, \(n = 1\); CDGIIb, \(n = 1\)]. CDGII includes all defects starting from the assembly of dolichol to the transfer of the oligosaccharide to the protein. CDGI includes all defects that are localized in the processing of glycans on the protein.

We also analyzed plasma samples from two patients with an as yet unidentified primary defect in N-glycan biosynthesis. The patients had been classified as CDGII on the basis of their repeatedly abnormal transferrin isofocusing profile. Patient A is a child of healthy nonrelated German parents. He first presented at the age of 13 years. Until that time, he had been healthy and well and had reached his age-related milestones appropriately. Probably as a result of a thromboembolic complication or aspiration, he suffered from a sudden respiratory arrest, was resuscitated, and needed long-term ventilation. The intensive care period was complicated by a severe derangement of coagulation attributable to very low antithrombin III (<10%; reference interval, 70–130%), protein C (25%; reference interval, 70–130%), and factor XI (11%; reference interval, 70–130%). Recurrent arrhythmia was documented by electrocardiogram. Brain imaging showed slightly dilated ventricles. In the later course, the patient developed seizures with moderate neurologic sequelae and showed recurrently increased transaminases and abnormalities of the clotting factors.

Patient B was born to consanguineous parents with several mentally retarded relatives. The neonatal period
and early infancy were normal. In the course of an infection at 6 months of age she presented with neurologic regression mimicking Leigh disease. The child had infection-triggered episodes with encephalopathy on two other occasions. At 6 years of age she had microcephaly, mental retardation, strabismus, pseudoptosis, and a cerebellar syndrome. She could not walk without support, but she was able to make psychomotor progress. Brain magnetic resonance imaging showed brainstem and cerebellar atrophy. The other clinical data are as follows: failure to thrive, epicanthus, and small hands and feet with hypoplasia of the first phalanx of some fingers and toes. She also has frequent stomatitis, although immunologic studies did not show abnormalities. Increased creatine kinase and transaminase concentrations were observed during the acute phases in the disease. In such periods the coagulation pattern was normal, with protein C and protein S deficiency and decreased prothrombin time and coagulation factors. A detailed clinical description has been published [case 4 in Ref. (20)]. Both children had an abnormal isoelectric focusing (IEF) pattern with an increase of hypoglycosylated transferrin isoforms.

SAMPLES AND SAMPLE PREPARATION
The VLDL pool was isolated by ultracentrifugation, and lipids were extracted by ethanol-acetone (1:1 by volume) and stored in the freezer to sediment the VLDL apoproteins. Identification of the apoC-III isoprotein bands was as published in the literature (21). After centrifugation for cell separation, plasma samples were stored without delay at −80 °C until analysis. Samples used for the apoC-III isoform reference interval for the age group 0–1 year were from healthy babies at an age of 6 months (further use of leftover plasma volumes from a study on calcium homeostasis for which written informed consent was obtained). Samples used for the apoC-III isoform reference interval for the age group 1–18 years were from healthy volunteers (further use of leftover plasma from relatives of patients for routine microsatellite studies after informed consent). Samples used for the apoC-III isoform reference interval for the age group >18 years were obtained from healthy volunteers (Dutch blood bank donors after written informed consent). Their plasma samples showed no abnormalities when tested for N-glycosylation (transferrin IEF). Plasma samples were diluted 10-fold in saline for IEF of apoC-III.

Dry IEF gels for the PhastSystem (cat. no. 17-0677-01; Amersham Pharmacia Biotech) were rehydrated for at least 1.5 h at room temperature in a solution of 8 mol/L urea and 60 mL/L Pharmalyte, which contained Pharmalyte 4.2–4.9 and Pharmalyte 3.5–5.0 (Amersham Pharmacia Biotech) in a ratio of 2:1. The gel was prefocused for 75 V·h at 2000 V. After 0.5 μL of the 10-fold diluted plasma was applied to the gel, IEF was continued for 495 V·h (22).

WESTERN BLOT PROCEDURE
Proteins were blotted onto a nitrocellulose membrane (cat. no. 12806-200-200BLF; Sartorius) by diffusion for 1 h at 60 °C. Nonspecific sites on the nitrocellulose membrane were blocked with 50 g/L nonfat dried milk dissolved in phosphate-buffered saline (PBS) containing 5 mL/L Tween 20. The membrane was washed in PBS containing 0.5 mL/L Tween 20. The primary rabbit anti-human apoC-III antibody (cat. no. 0650-1707; ANAWA Biomedical Services & Products) was diluted 1000-fold in 15 g/L bovine serum albumin dissolved in PBS containing 5 mL/L Tween 20. The secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (cat. no. 1858415; Pierce Perbio Biotechnology) was diluted 5000-fold in the same solution. We then added 0.125 mL of Western blotting detection reagents [electrochemiluminescence (ECL) reagent; cat. no. RPN 2134; Amersham Pharmacia Biotech] per cm² of membrane. A chemiluminescent reaction occurs between horseradish peroxidase and the luminol in the ECL reagents, producing light emission. The blot was placed against an autoradiography film (Hyperform ECL; cat. no. RPN 1681 H; Amersham Pharmacia Biotech) and exposed for different time intervals. The film was developed on a Kodak RP X-Omat Processor (Model M6B) and scanned with the Image master Labscan, Ver. 3.00 (Amersham Pharmacia Biotech). Isoforms of apoC-III were quantified by Image master 1D gel analysis, Ver. 4.10, software (Amersham Pharmacia Biotech).

NEURAMINIDASE TREATMENT
Neuraminidase can remove the negatively charged terminal sialic acid residues from N- and O-linked glycans. After incubation with neuraminidase, all apoC-III isoforms migrate to the position of apoC-III⁰. Polymorphisms in the apoC-III protein can be identified by abnormal mobility of the protein on IEF after neuraminidase treatment. For that purpose neuraminidase (cat. no. 107590; Boehringer Mannheim; 1 g/L in 0.1 mol/L Tris, pH 7.0) and human plasma are incubated in a ratio of 3:2 overnight at room temperature.

Results
HUMAN PLASMA apoC-III ISOFOCUSBING
Isofocusing of human plasma apoC-III shows the three isoforms apoC-III⁰, apoC-III¹, and apoC-III² (see Fig. 2).

![Fig. 2. apoC-III isoforms in human plasma and their structures.](image-url)
Theoretically, nonglycosylated apoC-III, apoC-III with only GalNAc, or apoC-III with GalNAc-Gal could contribute to the apoC-III* fraction. It was not possible to distinguish the nonglycosylated apoC-III or apoC-III with only GalNAc from the asialo form by IEF (Fig. 2). The isoelectric points of the three isoforms were in the pI range 4.5–5.1, which is in accordance with data in the literature (23, 24). The apoC-III isoform profile of the VLDL pool is shown in lane 10 of Fig. 4. apoC-III*, and apoC-III# are the major isoforms occurring in plasma (~55% and 40%, respectively), whereas only trace amounts of apoC-III0 are present. apoC-III is a stable protein; identical apoC-III isoform distribution patterns were found for a fresh plasma sample and the same sample stored frozen at −80 °C for 1 week. No other protein bands were visible in the pI range 4–5.3, illustrating the specificity of the antibody used.

The linearity of the assay was assessed by dilution experiments. Samples had to be diluted to determine the full range at which the assay was linear (Fig. 3A). Exposure time of the blot for a 10-fold diluted plasma sample on the radiography film showed a linear response until at least 60 s of exposure (Fig. 3B).

**REPRODUCIBILITY**

The between-run reproducibility was assessed by performing the test on the same plasma sample on 6 different days. Results for the three isoforms, expressed as mean (SD) as a percentage of total apoC-III, were 4.5 (0.4)%, 59.1 (1.9)%, and 36.4 (1.9)% for apoC-III*, apoC-III#, and apoC-III#, respectively.

**REFERENCE INTERVALS**

The reference values for each age group are shown in Table 1. We found no statistically significant influence of gender (data not shown). apoC-III* was significantly lower in the age group 0–1 year than at older ages. apoC-III* and apoC-III# are equally represented in this youngest age group, whereas at older ages, apoC-III# becomes the most predominant isoform in most individuals.

**apoC-III POLYMORPHISM**

Isofocusing of a plasma sample from one specific blood donor showed four apoC-III isoforms (Fig. 4, lane 9). After treatment with neuraminidase, plasma from other blood donors showed one band in the position of apoC-III* (Fig. 4, lane 7). The plasma sample of this specific blood donor showed bands of equal intensity at the positions of apoC-III*, and apoC-III# (Fig. 4, lane 8). This is evidence for a polymorphism in the apoC-III protein that makes the net charge of the protein more negative.

**PATIENT GROUPS**

*Patients with primary CDG.* Plasma samples from patients with CDGIA, Ib, Ic, Ie, If, IIa, and IIId showed a normal apoC-III isoform distribution (data not shown).

*Patients with secondary N-glycosylation alterations.* Plasma samples from two patients in the acute phase of HUS attributable to *Streptococcus pneumoniae* showed increased apoC-III* (18% and 23%, respectively; reference interval, 0.0–11.6%).

Plasma samples from patients who abused alcohol (n = 12) or had classic galactosemia before dietary treatment (n = 1) showed a normal apoC-III isoform distribution (see Fig. 4).

*Patients with unidentified CDG.* Fig. 4 shows the apoC-III IEF analysis of two patients with unidentified CDG. Patient A has been classified as CDGIIx on the basis of his transferrin isofocusing profile, which showed increased asialo-, monosialo-, and disialotransferrin and decreased tetrasialotransferrin. Thyroxine-binding globulin (TBG), another N-glycosylated plasma protein, also showed an abnormal isofocusing profile, confirming the generalized character of the defect in N-glycan biosynthesis in this patient. Plasma from this patient showed a normal apoC-III isoform distribution (Fig. 4, lane 1). Patient B has also been classified as CDGIIx. Transferrin isofocusing showed that he has increased monosialo- (8.6%; reference interval, 0.0–2.6%), disialo- (22.7%; reference interval, 1.6–6.1%), and trisialotransferrin (28.8%; reference interval, 2.5–15.6%) and decreased tetrasialotransferrin (32.6%; reference interval, 51.2–72.2%). TBG isoforms were also abnormal in this patient. Plasma from this patient showed increased apoC-III* (36.9%; reference interval, 0.0–11.6%) and decreased apoC-III# (5.6%; reference interval, 27.4–60.0%; Fig. 4, lane 2). Apparently the genetic defect in this patient affects N-glycan biosynthesis as well as mucin core 1 type O-glycan biosynthesis.

**Discussion**

Isofocusing of apoC-III is a simple and rapid technique that allows quantitative determination of the three apoC-III isoforms. apoC-III isoforms from plasma may be used as markers for the biosynthesis of core 1 of mucin type O-glycans. This glycan type is widely expressed throughout the human body and is abundant in the nervous
system; it is considered the most common type of O-glycan. Core 2 mucin type O-glycans are synthesized from unsubstituted core 1 mucin type O-glycans. A biosynthesis defect in unsubstituted core 1 O-glycans will also affect the synthesis of core 2 mucin type O-glycans. Alterations in mucin type O-glycan structure have been found in relation to cancer (15). Recently evidence was found for inborn errors of metabolism in the biosynthesis of O-glycans. Defects were found in mannosylated O-glycan biosynthesis in the Walker–Warburg and muscle–eye–brain syndromes (7, 8) and in fucosylation of both N- and O-glycans in CDGIIc (9, 10). As yet there is no evidence for inborn errors in the biosynthesis of mucin type O-glycans.

In this study we tested two patients with a firmly established but as yet unidentified N-glycan biosynthesis defect. We found an abnormal plasma transferrin isofocusing distribution in both patients on several occasions. These patients have a so-called type II pattern with asialo-, monosialo-, and disialotransferrin isoforms overrepresented. This pattern predicts a defect in the Golgi part of N-glycan biosynthesis. Both also have an abnormal TBG isofocusing distribution. Because the primary defect in these patients has not been identified, both were diagnosed as CDG type Ix. In this study patient A showed a normal apoC-III isofocusing profile, whereas patient B showed a hypoglycosylation profile (Fig. 4, lanes 1 and 2). From this we can conclude that the underlying defect causing the disease will be different in the two patients. Because apoC-III is not N-glycosylated, it may be concluded that in patient B the O-glycan biosynthesis of the core 1 mucin type O-glycan is disturbed as well. After CDGIIc this is the second inherited disease affecting the biosynthesis of both N- and O-glycans. It may be the first example of a patient with an inborn error of metabolism affecting the core 1 mucin type O-glycans. The primary defect in this patient remains unidentified, but it must be situated in one of the three biosynthetic stages indicated in Fig. 1. Gal and N-acetyleneuraminic acid (NeuAc) are the only sugars implicated in the biosynthesis of both N-glycans and core 1 mucin type O-glycans; therefore, it is very likely that the genetic defect in patient B is situated in one of the three biosynthetic stages of one Gal or NeuAc.

Many of the transferases involved in O-glycan biosynthesis are potentially N-glycosylated themselves (25). It is therefore an option that O-glycan biosynthesis would be secondarily affected in patients with a primary defect in N-glycan biosynthesis. However, the apoC-III profiles of patients with defined primary defects in N-glycan biosynthesis (CDGIIa−Ib, -Ic, -Ie, -If, -IIa, and -IIId) were all fully normal. It is therefore highly unlikely that the O-glycan abnormality in patient B is secondary to a defect in N-glycan biosynthesis.

Patient A had a normal apoC-III isofocusing profile and an abnormal transferrin isofocusing profile. As a consequence, it is unlikely that the primary defect in this patient is situated in the biosynthesis, activation, or transport to the Golgi of Gal or NeuAc. It seems more likely that the defect in this patient is situated in the pathways for the N-glycan-specific sugars Man, Glc, or GlcNAc. In addition, it may implicate the transferases that transfer Gal or NeuAc to the N-glycan because these enzymes are thought to be N-glycan specific. Our data suggest that apoC-III isofocusing testing may be a helpful step in the elucidation of the primary defect for the relatively large cohort of CDG type Ix and Ix a cases present in the Euroglycan database that serves as a register for CDG patients (26). A preliminary study on this group showed that 12 of 25 cases had an abnormal apoC-III isofocusing profile in addition to their abnormal transferrin isofocusing distribution.

In this study we also tested diseases and conditions leading to secondary N-glycan abnormalities. Among these are galactosemia, alcohol abuse, and HUS. A patient with classic galactosemia attributable to deficiency of galactose-1-phosphate uridyltransferase showed a normal apoC-III isofocusing profile before dietary treatment. The defect leads to the accumulation of galactose-1-phosphate. Transferrin hypoglycosylation in these patients may be attributable to direct inhibition of galactosyltransferase activity by the accumulated galactose-1-phosphate or to an effect on the formation of UDP-galactose, the donor-substrate in the reaction (27). Core 1 mucin type O-glycan
biosynthesis also requires the availability of UDP-galactose in the Golgi. Because the apoC-III O-glycan could be normally synthesized, our data suggest that the abnormal N-glycans of plasma transferrin probably are not attributable to decreased availability of UDP-galactose. The β-1,4-galactosyltransferase (EC 2.4.1.138) involved in N-glycosylation differs from the β-1,3-galactosyltransferase (EC 2.4.1.122) involved in O-glycan biosynthesis of apoC-III (28). Our findings suggest that galactose 1-phosphate inhibits β-1,4-galactosyltransferase but not β-1,3-galactosyltransferase in galactosemic patients.

Secondary transferrin hypoglycosylation has been observed in HUS (29) and in chronic alcohol abuse (30, 31). Plasma apoC-III was hypoglycosylated in the acute phase of S. pneumoniae-associated HUS in two patients. S. pneumoniae excretes neuraminidase, which catalyzes the hydrolysis of α2–3-, α2–6-, and α2–8–linked NeuAc residues from glycoproteins and oligosaccharides. The presence of neuraminidase activity in the plasma of patients with S. pneumoniae-associated HUS explains the abnormal isoform pattern of N-glycosylated proteins in the acute phase of the disease (29). The high percentage of apoC-III, in plasma can be explained as a loss of sialic acid residues from the termini of apoC-III isoforms as a result of the action of circulating neuraminidase.

Plasma samples from 12 patients with chronic alcohol abuse showed a normal apoC-III isofocusing profile. It is widely accepted that chronic ethanol consumption leads to hypoglycosylated plasma transferrin. The mechanism behind this N-glycan abnormality is still not well understood. Flahaut et al. (32) showed that the abnormal transferrin isoforms are caused by the loss of an entire N-linked oligosaccharide and of an alteration in terminal sialylation. It has been suggested that ethanol intake alters the biosynthesis and/or transfer of the dolichol–oligosaccharide intermediates, leading to loss of the entire N-linked oligosaccharide. Alteration of terminal sialylation is suggested to be the result of decreased activity of sialyltransferase in the Golgi. Conversely, it has been suggested that ethanol consumption enhances the activity of sialidase, which can remove the terminal sialic acid residues from glycans. Because sialyltransferase involved in O- and N-glycosylation are specific, our data show that this latter hypothesis is less likely.

It is quite likely that the negative change in pI in the protein found in a healthy volunteer was caused by a heterozygous polymorphism of apoC-III. Three polymorphisms have been described for apoC-III (33, 34). The observed charge heterogeneity could also result from causes such as phosphorylation, acetylation, and sulfation of the protein. The presence of such pI changes may hamper the interpretation of apoC-III isoform profiles. However, incubation with neuraminidase followed by isofocusing is an easy test to show that the abnormal profile in such cases derives from a variation in the protein part of the molecule and is not caused by an O-glycan abnormality.

Further investigations on the biosynthesis of O-glycans are of great importance. O-glycan biosynthesis is very complex, with many different end products. It is very likely that further work in this field will solve the molecular basis of various human diseases or syndromes.

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References


