Clinical Chemistry 53:2
180–187 (2007)

Transferrin and Apolipoprotein C-III Isofocusing Are Complementary in the Diagnosis of N- and O-Glycan Biosynthesis Defects

Suzan Wopereis,[1] Stephanie Grünewald,[2] Karin M.L.C. Huijben,[1] Éva Morava,[3] Rosella Mollicone,[4] Baziel G.M. van Engelen,[5] Dirk J. Lefeber,[1] and Ron A. Wevers[1*]

Background: **Apolipoprotein C-III (apoC-III) isoelectric focusing (IEF) can be used to detect abnormalities in the biosynthesis of core 1 mucin-type O-glycans.**
Methods: **We studied plasma samples from 55 patients with various primary defects in N- and/or O-glycosylation, 21 patients with secondary N-glycosylation defects, and 6 patients with possible glycosylation abnormalities. Furthermore, we analyzed 500 plasma samples that were sent to our laboratory for selective screening for inborn errors of metabolism.**
Results: **Plasma samples from patients with congenital disorders of glycosylation (CDG) types –IIe and –IIf showed a hypoglycosylated apoC-III isoform profile, as did plasma samples from 75% of the patients with an unspecified CDG type II. Hyposialylated O-glycan profiles were also seen in plasma from 2 patients with hemolytic-uremic syndrome. In the 500 plasma samples from the selective screening, 3 patients were identified with a possible isolated defect in the biosynthesis of core 1 mucin-type O-glycans.**
Conclusions: **To our knowledge this is the first study in which use of a plasma marker protein has identified patients in whom only O-glycan biosynthesis might be affected. The primary defect(s) remain as yet unknown. Plasma apoC-III IEF is complementary to transferrin isofocusing. In conjunction both tests identify biosynthesis defects in N-glycan and mucin-type core 1 O-glycan biosynthesis. The apoC-III IEF assay is likely to help metabolic laboratories to identify and unravel further subtypes of inborn errors of glycan biosynthesis.**

Hypoglycosylation of glycoproteins is characteristic for congenital disorders of glycosylation[6] (CDG). Transferrin isoelectric focusing (TIEF) is generally applied in the screening for inborn errors in the biosynthesis of N-glycans, whereas apolipoprotein C-III (apoC-III) isoelectric focusing (IEF) can be used in the screening for inborn errors in the biosynthesis of mucin-type core 1 O-glycans *(1)*. Plasma transferrin contains 2 complex type N-glycans with terminal sialic acid residues. The complex type N-glycan has a common core structure containing 2 *N*-acetylgalactosamine (GlcNAc) and 3 mannose residues with heterogeneous antennae consisting of GlcNAc, galactose and sialic acid (NeuAc) residues. Because the terminal residues and the branching of N-glycans are variable, several isoforms of transferrin can be distinguished. ApoC-III is a plasma protein containing 1 core 1 mucin-type O-glycan. Three isoforms of apoC-III can be distinguished, apoC-III_0, apoC-III_1 and apoC-III_2, for which the isoform number, as in transferrin, is the num-

Departments of [1] Laboratory of Pediatrics and Neurology, [3] Pediatrics, and [5] Neurology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.
[2] Department of Pediatric Metabolic Medicine and Institute for Child Health, Great Ormond Street Hospital, London, United Kingdom.
[4] Institut National de la Santé et de la Recherche Médicale (INSERM U602), Groupement de Recherche, Paul Brousse Hospital, University of Paris Villejuif, France.
* Address correspondence to this author at: Laboratory of Pediatrics and Neurology (830), Radboud University Nijmegen Medical Center, Geert Grooteplein 10, 6525 GA Nijmegen, The Netherlands. Fax 31-24-3668754; e-mail r.wevers@cukz.umcn.nl.
Received May 27, 2006; accepted November 14, 2006.
Previously published online at DOI: 10.1373/clinchem.2006.073940

[6] Nonstandard abbreviations: CDG, congenital disorders of glycosylation; TIEF, transferrin isoelectric focusing; ApoC-III, apolipoprotein C-III; IEF, isoelectric focusing; GalNAc, *N*-acetylgalactosamine; NeuAc, sialic acid; FTC, familial tumoral calcinosis (MIM 211900); HIBM, hereditary inclusion body myopathy (MIM 600737); MEB, muscle-eye-brain disease (MIM 253280); HUS, hemolytic uremic syndrome; HGPS, Hutchinson Gilford progeria syndrome (MIM 176670), F5F8D, a combined deficiency of factor V and factor VIII (MIM 227300); ER, endoplasmic reticulum; COG7, conserved oligomeric Golgi complex; CMP-NeuAc: cytidine 5′monophospho-*N*-acetylneuraminic acid; GNE/MNK, uridine-5′-diphosphate-*N*-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase; GALNT3, UDP-GalNAc transferase 3.

ber of sialic acid residues attached to the core 1 mucin-type O-glycan *(2)*. Because sialic acid has a negative charge, the IEF patterns of hyposialylated transferrin and apoC-III show a cathodal shift that can be caused either by structural glycan changes or by a different number of glycans *(3)*, whereas hypersialylated glycoproteins show an anodal shift. TIEF can detect abnormal glycosylation in primary defects that include enzyme deficiencies in the N-glycosylation pathway and secondary defects that include genetic diseases and conditions that influence the N-glycan biosynthesis.

In mammals, the most common form of O-glycosylation is mucin-type O-glycosylation in which glycans are attached to the protein via GalNAc. This mucin-type O-glycosylation can be subdivided into 8 core structures. The core 1 O-glycan, with Galβ1–3GalNAc-(Ser/Thr) as the core structure, is the most common subtype and occurs on many membrane and secreted proteins *(4, 5)*. Until now, only 1 disorder with a genetic defect in the biosynthesis of mucin-type O-glycans has been described: familial tumoral calcinosis (FTC) associated with mutations in the UDP-*N*-acetyl-α-D-galactosamine:polypeptide-*N*-acetylgalactosaminyltransferase 3 gene (*GALNT3*)[7] *(6)*. O-glycosylation biosynthesis routing and different O-glycan biosynthesis defects have recently been reviewed by Wopereis et al. *(7)*.

Three years ago, our group developed apoC-III IEF as a means to study biosynthesis defects in mucin-type core 1 O-glycans and addressed technical aspects such as linearity, reproducibility, and reference intervals *(1)*. This study is an exploratory retrospective study into the diagnostic significance of apoC-III isoform analysis. We investigated abnormalities in apoC-III isoform profiles in samples from 4 patient groups. Among these are samples from patients with (genetically) confirmed primary and secondary glycosylation defects.

Materials and Methods

PATIENT GROUP 1; PRIMARY GLYCOSYLATION DEFECTS

Group 1 patients had a specified CDG subtype, another genetically confirmed glycosylation disorder, or repeatedly abnormal TIEF profiles obtained and known within Euroglycanet. We obtained 54 plasma samples from patients with primary CDG, 21 from patients with specified CDG type I (CDG-Ia, n = 8; CDG-Ib, n = 3; CDG-Ic, n = 8; CDG-Ie, n = 1; CDG-If, n = 1), 7 from patients with unspecified CDG type I [= CDG-Ix *(8)*; the patients are in this group because of the characteristically abnormal TIEF profile with asialo- and disialotransferrin markedly increased], 6 from patients with specified CDG type II [CDG-IIa, n = 1 *(9)*; CDG-IId, n = 1 *(10)*; CDG-IIe, n = 3—P2 in *(11)* and 2 as yet unpublished cases—CDG-IIf, n = 1, the case from *(12)*], 12 from patients with unspecified CDG type II [= CDG-IIx *(8)*; the patients are in this group because of the characteristically hypoglycosylated TIEF profile with variably increased asialo-, monosialo-, disialo- and trisialotransferrin fractions] and 8 from other genetically confirmed types of glycan biosynthesis disorders [hereditary inclusion body myopathy (HIBM), n = 1 *(13)*; FTC, n = 6 *(6)*; and muscle-eye-brain disease (MEB), n = 1].

The 2 unreported CDG-IIe cases (1 female patient, 1 month old; 1 male, 7 months old) presented with growth retardation; progressive, severe microcephaly; hypotonia; adducted thumbs; feeding problems from gastrointestinal pseudoobstruction; failure to thrive; cardiac anomalies, wrinkled skin; and episodes of extreme hyperthermia. Both patients were found to have the same homozygous, intronic splice site mutation (c.169 + 4A>C) of the *COG7* gene identified in the patients described by Wu et al. *(11)*. Clinical signs and symptoms of these 2 CDG-IIe patients will be described in more detail separately by Morava et al.

PATIENT GROUP 2; SECONDARY DEFECTS IN THE N-GLYCOSYLATION

Group 2 patients had secondary defects in N-glycosylation. These patients had genetically confirmed secondary diseases or confirmed syndromes with abnormal TIEF profiles. We obtained 21 plasma samples from patients with secondary N-glycosylation alterations [fructosemia due to aldolase B deficiency, n = 2; galactosemia due to galactose-1-phosphate uridyltransferase deficiency, n = 5; chronic alcohol abuse, n = 12; and hemolytic-uremic syndrome (HUS), n = 2]. The plasma samples from the patients with fructosemia and galactosemia were obtained before dietary treatment. The plasma samples from the HUS cases were obtained in the acute phase of the disease before or soon after the start of the appropriate treatment.

PATIENT GROUP 3; POSSIBLE GLYCOSYLATION ABNORMALITIES

Group 3 patients had confirmed genetic defects suspected to influence the biosynthesis of glycans or a syndrome with abnormal glycosylation patterns described in the literature. We studied 6 plasma samples from patients with disorders suspected to cause abnormal glycosylation [Hutchinson Gilford progeria syndrome (HGPS), n = 2; and a combined deficiency of factor V and factor VIII (F5F8D), n = 4]. In HGPS, abnormal N-glycosylation has been reported *(14)*. Patients with F5F8D have a defect in component 53 of the endoplasmic reticulum (ER)-Golgi

[7] Human genes: *GALNT3,* UDP-*N*-acetyl-α-D-galactosamine:polypeptide-*N*-acetylgalactosaminyltransferase 3; *COG7,* component of oligomeric golgi complex 7; *LMNA*, lamin A/C (previous symbols: LMN1, CMD1A); *MGAT*2, mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase; *GCS*1, glucosidase; *SLC35C*1, solute carrier family 35, member C1; *B4GALT*1, UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1; *SLC35A*1, solute carrier family 35 (CMP-sialic acid transporter), member A1; *POMGNT*1, protein O-linked mannose beta1,2-*N*-acetylglucosaminyltransferase; *GNE*, glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase; *GALT*, galactose-1-phosphate uridylyltransferase; *ALDOB*, aldolase B, fructose-bisphosphate.

intermediate compartment, which could influence the biosynthesis and trafficking of glycoproteins.

PATIENT GROUP 4; SELECTIVE SCREENING FOR INBORN ERRORS OF METABOLISM

Group 4 patients had plasma samples sent to our laboratory for selective metabolic screening. In a prospective study, we analyzed 500 different plasma samples collected during the last 3 years from patients presenting with psychomotor retardation that was isolated or with additional symptoms, muscle diseases, encephalopathy, and other symptoms compatible with metabolic disorders.

SAMPLES AND SAMPLE PREPARATION

Blood samples were obtained from patients with informed parental consent. Plasma was prepared by centrifugation and stored immediately at −80 °C until required for analysis.

IEF OF APOC-III

IEF of apoC-III was carried out as described by Wopereis et al. *(7)*. The apoC-III IEF profile was defined as abnormal when the ratio of the 3 apoC-III isoforms was outside the described reference intervals in at least 2 different plasma samples from 1 patient sample in duplicate *(1)*. In general, 2 abnormal apoC-III IEF profile types can be differentiated; the apoC-III_0 IEF profile characterized by increased concentrations of apoC-III_0, and the apoC-III_1 profile characterized by increased concentrations of the monosialo apoC-III form *(8)*. The day-to-day (total) imprecision (CV), assessed by performing the test on the same plasma sample on 6 different days, was <2% for each of the 3 isoforms *(1)*. The apoC-III IEF assays were performed and interpreted by 2 technicians with 3 years of expertise.

IEF OF TRANSFERRIN

TIEF was carried out as described by Wopereis et al. *(7)*.

NEURAMINIDASE TREATMENT

Human plasma was incubated with neuraminidase (5 kU/L) from *Clostridium perfringens* (cat. no. 1585886; Roche; 5U in 0.5 mL 0.1 mol/L Tris, pH 7.0) overnight at room temperature. Samples were analyzed for transferrin and apoC-III IEF as described above.

Results

To identify the causes of abnormal apoC-III IEF profiles we defined 4 groups of patients.

PATIENT GROUP 1; PRIMARY CDG

All plasma samples from 28 patients with CDG type I (CDG-Ia, -1b, -Ic, -Ie, -If, and CDG-Ix) showed a normal apoC-III isoform distribution. The plasma sample from 3 CDG-IIe patients showed an apoC-III_0 IEF profile with increased amounts of the apoC-III_0 isoform (35%, 27%, and 23% respectively; reference interval 0%–8%) and decreased amounts of the apoC-III_2 isoform (15%, 20%, and 22% respectively; reference interval 40%–62%; Fig. 1, lane 2). The plasma sample from the CDG-IIf patient showed a different isoform profile, an apoC-III_1 IEF profile with increased amounts of the apoC-III_1 isoform (78%, reference interval 33%–67%) and decreased amounts of the apoC-III_2 isoform (16%, reference interval 27%–60%; Fig. 1, lane 3). Of the 12 CDG-IIx patients, 3 had a normal apoC-III IEF profile, whereas 9 had an abnormal apoC-III profile. Of the 9 patients with abnormal apoC-III IEF results, 5 had an apoC-III_0 IEF profile and 4 had the apoC-III_1 profile. The relative amounts of the apoC-III isoforms in the patients with abnormal apoC-III IEF profiles are summarized in

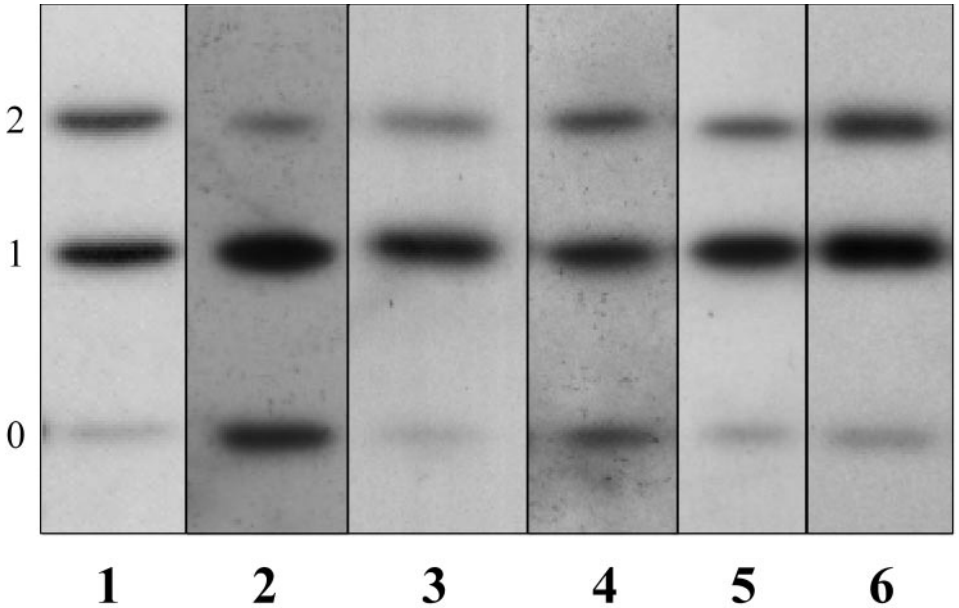

Fig. 1. Plasma apoC-III isofocusing profiles from patients with primary and secondary mucin-type core 1 O-glycan abnormalities.

Lane 1, control; *lane 2*, CDG-IIe; *lane 3*, CDG-IIf; *lane 4*, HUS; *lane 5*, Hutchinson Gilford progeria; *lane 6*, control (age >18 years) with apoC-III_2 fraction around the upper reference interval limit.

Table 1. Summary of the relative amounts of apoC-III isoforms in patients with abnormal apoC-III profiles.

		ApoC-III$_0$, %	ApoC-III$_1$, %	ApoC-III$_2$, %	
Reference interval 0–1/1–18/>18 years[a]		0–8/0–12/3–19	34–59/33–67/43–69	40–62/27–60/23–50	ApoC-III
Mean (SD) 0–1/1–18/>18 years		4 (2)/5 (3)/9 (4)	47 (6)/53 (9)/55 (6)	9 (6)/42 (8)/36 (7)	profile type
CDG-IIe (*11*)	1 month	35	50	15	ApoC-III$_0$
CDG-IIe	7 month	27	53	20	ApoC-III$_0$
CDG-IIe	1 month	23	55	22	ApoC-III$_0$
CDG-IIf	4 month	6	78	16	ApoC-III$_1$
CDG-IIx[b]	7 years	14	70	16	ApoC-III$_0$
CDG-IIx[c]	3 years	37	58	5	ApoC-III$_0$
CDG-IIx[d]	2 years	11	73	16	ApoC-III$_1$
CDG-IIx[e]	3 years	6	86	8	ApoC-III$_1$
HUS	1 year	18	56	26	ApoC-III$_0$
HUS	1 year	23	53	24	ApoC-III$_0$
HGPS + *LMNA*	10 years	4	76	20	ApoC-III$_1$
HGPS − *LMNA*	Child	1	70	29	ApoC-III$_1$
Patient 1	9 years	7	80	13	ApoC-III$_1$
Patient 2	18 years	2	17	81	Hyper
Patient 3	5 years	18	70	12	ApoC-III$_0$
Patient 4	16 years	2	79	19	ApoC-III$_1$
Mother of patient 4	45 years	3	75	22	ApoC-III$_1$

[a] As published in (*1*).
[b] Patient 11 in (*8*). This patient has the mildest apoC-III$_0$ IEF profile of the patients investigated.
[c] Patient 15 in (*8*). This patient has the most severe apoC-III$_0$ IEF profile of the patients investigated.
[d] Patient 17 in (*8*). This patients has the mildest apoC-III$_1$ IEF profile of the patients investigated.
[e] Patient 19 in (*8*). This patient has the most severe apoC-III$_1$ IEF profile of the patients investigated.

Table 1. Clinical signs and biochemical results of the 12 CDG-IIx patients have been described in more detail (*8*). The plasma samples from a CDG-IIa, CDG-IId, HIBM, MEB, and 6 FTC patients showed a normal apoC-III isoform distribution. The apoC-III IEF and TIEF results from patients with primary CDG are summarized in Table 2.

PATIENT GROUP 2; SECONDARY N-GLYCOSYLATION ALTERATIONS

Several disorders and/or conditions are known to cause N-glycan abnormalities, such as galactosemia (*16*), fructosemia (*17*), chronic alcohol abuse (*18*), and HUS due to a *Streptococcus pneumoniae* infection (*19*). Plasma samples

Table 2. Overview of apoC-III IEF and TIEF results on samples from patients with primary and secondary disorders in the glycosylation.

CDG type	MIM	Gene	Protein	TIEF	ApoC-III IEF
Primary CDG					
CDG-I (a,b,c,e,f,x)				+	−
IIa	212 066	*MGAT2*	GlcNAc transferase II	+	−
IIb	606 056	*GCS1*	Glucosidase I	− (*28*)	ND
IIc	266 265	*SLC35C1*	GDP-fucose transporter	− (*29*)	ND
IId	607 091	*B4GALT1*	Galactosyltransferase	+	−
IIe	608 779	*COG7*	COG7	+	+
IIf		*SLC35A1*	CMP-NeuAc transporter	−	+
IIx				+	+ or − (*8*)
FTC	211 900	*GALNT3*	Protein GalNActransferase	−	−
MEB	253 280	*POMGNT1*	*O*-mannosyl-β-1,2-GlcNAc transferase	−	−
HIBM	600 737	*GNE*	UDP-GlcNAc 2-epimerase/ManNAc kinase	−	−
Secondary CDG					
Galactosemia	230 400	*GALT*	Galactose-1-phosphate uridyltransferase	+	−
Fructosemia	229 600	*ALDOB*	Aldolase B	+	−
HUS				+	+
Alcohol abuse				+	−
HGPS	176 670	*LMNA*	Lamin A/C	−	+

+, IEF profile is abnormal; −, IEF profile is normal; ND, not determined.

from 2 patients in the acute phase of HUS resulting from a *S. pneumoniae* infection showed an apoC-III_0 profile with increased relative amounts of apoC-III_0 (18% and 23%, respectively, reference interval 0%–12%) and slightly decreased amounts of apoC-III_2 (26% and 24% respectively, reference interval 27%–60%; Fig. 1, lane 4). Relative amounts of the apoC-III isoforms in the HUS patients are summarized in Table 1. The profiles normalized soon after the start of appropriate treatment. The plasma samples from 12 chronic alcohol abuse, 2 fructosemia, and 5 galactosemia patients all had normal plasma apoC-III isoform profiles (Table 2).

PATIENT GROUP 3; POSSIBLE GLYCOSYLATION ABNORMALITIES

In patient group 3, we investigated 2 different disorders that were suspected to have abnormal glycan biosynthesis, namely HGPS and F5F8D. Robinson et al. *(14)* reported that proteome analysis in HGPS showed abnormal glycosylation in patients with and without mutations in the lamin A/C gene. Our HGPS patient with a mutation in the *LMNA* gene had an apoC-III_1 profile with increased amounts of apoC-III_1 (76%, reference interval 33%–67%) and decreased amounts of apoC-III_2 (20%, reference interval 27%–60%; Fig. 1, lane 5). The apoC-III isoform distribution in the HGPS patient without a mutation in the *LMNA* gene (the underlying defect is still unknown) was slightly but not convincingly abnormal. The ApoC-III_1 isoform was just above the reference interval (70%, reference interval 33%–67%). The results for TIEF were normal in both HGPS patients. The relative amounts of the apoC-III isoforms in the HGPS patients are summarized in Table 1.

F5F8D also might lead to glycosylation abnormalities. As is seen in patients with a defect in subunit 7 of the conserved oligomeric Golgi complex (COG7) or CDG-IIe, defects in Golgi trafficking can influence the biosynthesis of glycoproteins. Patients with F5F8D have a defect in component 53 of the ER–Golgi intermediate compartment, thought to function as a molecular chaperone for transport of a specific subset of secreted proteins, including factor V and VIII, from the ER to the Golgi *(20)*. Therefore, this defect might lead to a disturbance of glycosylation. The plasma samples from 4 patients with F5F8D, however, gave normal results for both apoC-III IEF and TIEF.

PATIENT GROUP 4; SELECTIVE SCREENING FOR INBORN ERRORS OF METABOLISM

ApoC-III IEF was performed in 500 plasma samples sent to our laboratory for selective screening for inborn errors of metabolism. Four samples were found to have an abnormal apoC-III IEF profile (Table 1). All patients had normal results for TIEF.

Patient 1. In this 9-year-old boy with pronounced developmental regression and deterioration of verbal communication at age 2 years, ApoC-III IEF repeatedly showed an apoC-III_1 profile with increased amounts of apoC-III_1 (80%, reference interval 33%–67%) and decreased amounts of apoC-III_2 (13%, reference interval 27%–60%).

Patient 2. In this 18-year-old woman with psychomotor retardation, rhabdomyolysis, and kidney insufficiency, ApoC-III IEF of a plasma sample obtained in the acute phase of rhabdomyolysis showed a hypersialylated apoC-III profile with decreased amounts of apoC-III_1 (17%, reference interval 43%–69%) and increased amounts of apoC-III_2 (81%, reference interval 23%–50%).

Patient 3. This 5-year-old boy with psychomotor retardation and dysmorphic features had a repeatedly abnormal apoC-III IEF profile; an apoC-III_0 profile with increased amounts of apoC-III_0 (18%, reference interval 0%–12%), slightly increased amounts of apoC-III_1 (70%, reference interval 33%–67%), and decreased amounts of apoC-III_2 (11%, reference interval 27%–60%).

Patient 4. In this 16-year-old woman with muscle cramps of unknown cause and excessive sweating, ApoC-III IEF repeatedly showed an apoC-III_1 profile with increased amounts of apoC-III_1 (79%, reference interval 33%–67%), and decreased amounts of apoC-III_2 (19%, reference interval 27%–60%). Interestingly, her mother presented similar clinical symptoms and also an apoC-III_1 profile with increased amounts of apoC-III_1 (75%, reference interval 43%–69%) and decreased amounts of apoC-III_2 (22%, reference interval 23%–50%). Serum creatine kinase was normal and a quadriceps muscle biopsy showed no morphological or enzyme histochemical abnormalities. Enzyme histochemistry showed no abnormal mitochondrial staining and also Periodic Acid Schiff-staining was normal. Biochemical investigations of the muscle biopsy showed normal overall oxidation rates and normal respiratory chain complex activity.

APOC-III PATTERN IN PREMATURE NEONATES AND APOC-III POLYMORPHISMS

We found that apoC-III isoforms in premature neonates were hyperglycosylated in 6 of 13 cases, suggesting abnormal O-glycan biosynthesis possibly due to liver immaturity. Furthermore, in the 500 plasma samples we found 2 from individuals who had a polymorphism in the protein backbone of apoC-III. These results have been described in the Supplemental data [see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue2].

Discussion

In the group with primary glycan biosynthesis defects, samples from patients with CDG-IIe and -IIf showed an abnormal apoC-III IEF profile. CDG-IIe is caused by mutations in subunit 7 of the COG complex *(11)*. The

COG complex is involved in retrograde trafficking processes through the Golgi, a defect that affects the regulation, compartmentalization, transport, and activity of several Golgi enzymes *(11, 21)*. Wu et al. *(11)* reported defective N-glycosylation and subsequently showed altered biosynthesis of mucin-type core 1 O-glycans in lectin-stained fibroblasts. ApoC-III IEF resulted in an apoC-III_0 IEF profile *(8)* with increased amounts of apoC-III_0 and decreased amounts of apoC-III_2 in the plasma of a CDG-IIe patient. The altered mucin-type core 1 O-glycosylation in CDG-IIe can thus be picked up with apoC-III IEF.

The patient with CDG-IIf had a deficient cytidine 5′monophospho-N-acetylneuraminic acid (CMP-NeuAc) transporter *(12)*. Martinez-Duncker et al. *(12)* found that the sialylation pattern of several N-glycosylated plasma proteins was normal, but lectin staining revealed defective sialylation of core 1 mucin-type O-glycans. ApoC-III IEF showed an apoC-III_1 profile *(8)* with increased amounts of the apoC-III_1 isoform and decreased amounts of apoC-III_2. The patient with CDG-IIf was identified by apoC-III IEF but had normal TIEF results. A comparison with other sialylation defects shows that in all cases mainly mucin-type O-glycosylation is affected, rather than N-glycosylation. Three defects affecting sialylation are known to date: CDG-IIf, HIBM, and sialuria. Sialuria patients have a defect in uridine-5′-diphosphate-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE/MNK) leading to an overproduction of CMP-NeuAc. The patients have a clear hypersialylation of plasma core 1 O-glycans as shown in the apoC-III IEF assay but have only minor changes in the sialylation pattern of the N-glycans *(22)*. Patients with HIBM also have a defect in the gene coding for the enzyme GNE/MNK, which is a bifunctional enzyme that catalyzes the first 2 steps in the biosynthesis of CMP-NeuAc. The theoretical decrease in CMP-NeuAc biosynthesis leads to hyposialylated muscle core 1 O-glycans shown by lectin staining *(23)* and to a decrease of muscle α-dystroglycan staining suggestive for hyposialylation of O-mannosylglycans *(13)*. The sialylation of N-glycans, however, seems to be unaffected *(13, 23)*. ApoC-III IEF resulted in a normal sialylation pattern in plasma of a HIBM patient and did not confirm the hyposialylation of core 1 O-glycans found in muscle tissue. The disturbance in mucin-type core 1 O-glycan biosynthesis may be tissue specific, leading to abnormal O-glycans in muscle and possibly not affecting the hepatic synthesis of apoC-III.

The primary defect in 12 CDG-IIx patients, most probably presenting a genetic heterogeneous group, is probably localized in the processing of N-glycans, which mainly occurs in the Golgi compartment. The 3 CDG-IIx patients with normal apoC-III IEF likely had the primary defect in an N-glycan specific processing step, whereas the 9 CDG-IIx patients with abnormal apoC-III IEF likely had the defect in an enzyme or protein that plays a role in the biosynthesis of both N- and core 1 O-glycans.

In patients with other glycosylation disorders, patients with FTC have the only genetically defined defect in the biosynthesis of mucin-type O-glycosylation identified so far. The defective *GALNT3* gene encodes UDP-GalNAc transferase 3 (GALNT3), responsible for the transfer of UDP-GalNAc to Thr/Ser to the protein backbone. *GALNT3* is highly expressed in human pancreas, skin, kidney, and testis and weakly expressed in prostate, ovary, intestine, and colon *(24)*. The results of a normal apoC-III IEF profile reflected this expression pattern because GALNT3 is not expressed in liver tissue where apoC-III is synthesized *(6)*. The normal results in the CDG-I, CDG-IIa, -IId, and MEB patients are explained by the fact that the defective enzymes in these disorders are not involved in the biosynthesis of apoC-III O-glycan.

In patient group 2, where we tested for abnormalities in the biosynthesis of mucin-type core 1 O-glycans, we found diseases/conditions leading to secondary N-glycan abnormalities. Among these are galactosemia, fructosemia, alcohol abuse, and HUS. Plasma apoC-III was hyposialylated in the acute phase of *S. pneumoniae*–associated HUS of 2 patients. *S. pneumoniae* excrete neuraminidase, which catalyzes the hydrolysis of NeuAc residues from glycoproteins, explaining the apoC-III hyposialylation profile. Plasma from patients with galactosemia, fructosemia (all before dietary treatment), and alcohol abuse showed normal results for apoC-III IEF. The 3 conditions result in a transferrin type 1 profile *(17, 18, 25)*, which suggests that these conditions have an influence on the early N-glycan biosynthesis pathway, localized in the ER or cytoplasm. Because the biosynthesis of mucin-type core 1 O-glycans is situated in the Golgi, these results were as expected.

Finally, we analyzed 500 plasma samples that were sent to our laboratory for the selective screening of inborn errors of metabolism. We found 4 patients with an abnormal apoC-III IEF profile. Three of the 4 patients had a hypoglycosylated apoC-III IEF profile, whereas 1 patient had a hypersialylated apoC-III IEF profile. The sample from patient 2 was taken in the acute phase of rhabdomyolysis, and apoC-III isoforms showed a hypersialylated profile. The rhabdomyolysis in our patient caused secondary renal failure. Six additional patients with kidney failure had normal apoC-III IEF profiles (data not shown), so it is unlikely that the kidney failure in the rhabdomyolysis patient caused the hypersialylation. Unfortunately, the patient was lost for follow-up so it remains unclear whether this patient also had an abnormal apoC-III IEF profile after the rhabdomyelytic phase. The mechanism behind the hypersialylation remains unknown.

The other 3 patients displayed a hypoglycosylated apoC-III isoform distribution on several occasions (patients 1, 3 and 4). Patients 1 and 4 had an apoC-III_1 profile with increased amounts of apoC-III_1 and decreased amounts of apoC-III_2. The mother of patient 4 had symptoms similar to those of her daughter and turned out to have an abnormal apoC-III_1 IEF pattern as well, suggest-

ing dominant or X-linked inheritance. Patient 3 had an apoC-III_0 profile with increased apoC-III_0 and apoC-III_1 and decreased apoC-III_2. The results for TIEF were normal in all 3 patients. We were unable to reach a classifying diagnosis in any of the 3 patients. They are likely to have the primary defect situated in one of the steps involved in the biosynthesis of mucin-type core 1 type O-glycans. To our knowledge this is the first time that patients have been identified in which only the O-glycan biosynthesis might be affected while N-glycan biosynthesis if unaffected.

In conclusion, the apoC-III IEF test is helpful, especially in patients with CDG type II, and should be considered complementary to selective screening with TIEF. The apoC-III isoforms have a broad reference interval in all age cohorts. In some patients only slightly abnormal values are found for apoC-III isoforms (as in case CDG-IIx[*1] in Table 1). When such slight abnormalities are confirmed in a 2nd sample they may be meaningful, but as long as the primary defect in these patients has not been determined the significance of slight abnormalities remains uncertain. No alternative techniques for the screening of O-glycan biosynthesis disorders are available, although newly developed mass spectrometric approaches are promising *(27, 28)*. The apoC-III IEF test is pivotal to pick up inborn errors in mucin-type core 1 O-glycan biosynthesis. The assay is likely to help metabolic screening laboratories to identify and unravel a further series of inborn errors of glycan biosynthesis.

This work was supported by the European commission, Euroglycan (contract no. QLG-CT2000-0047), and Euroglycanet (contract no. 512131). We thank M. Huizing, L. Spaapen, E. Sprecher, M. Adamowicz, A. Evangeliou, G. Damen, P. Clayton, and U. Seligsohn for allowing us to investigate their patients' plasma samples.

References

1. Wopereis S, Grünewald S, Morava E, Penzien JM, Briones P, Garcia Silva MT, et al. Apolipoprotein C-III isofocusing in the diagnosis of genetic defects in O-glycan biosynthesis. Clin Chem 2003;49:1839–45.
2. Vaith P, Assmann G, Uhlenbruck G. Characterization of the oligosaccharide side chain of apolipoprotein C-III from human plasma very low density proteins. Biochim Biophys Acta 1978;541:234–40.
3. Bergen HR, Lacey JM, O'Brien JF, Naylor S. Online single-step analysis of blood proteins: the transferrin story. Anal Biochem 2001;296:122–9.
4. van den Steen P, Rudd P, Wormald M, Dwek R, Opdenakker G. O-linked glycosylation in focus [Review]. Trends Glycosci Glycotechnol 2000;63:35–49.
5. Hounsell EF, Davies MJ, Renouf DV. O-linked protein glycosylation structure and function. Glycoconj J 1996;13:19–26.
6. Topaz O, Shurman DL, Bergman R, Indelman M, Ratajczak P, Mizrachi M, et al. Mutations in GALNT3, encoding a protein involved in O-linked glycosylation, cause familial tumoral calcinosis. Nat Genet 2004;36:579–81.
7. Wopereis S, Lefeber DJ, Morava E, Wevers RA. 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.
8. Wopereis S, Morava E, Grünewald S, Adamowicz M, Huijben K, Lefeber DJ, et al. Patients with unsolved congenital disorders of glycosylation type II can be subdivided in six distinct biochemical groups. Glycobiology 2005;15:1312–9.
9. Jaeken J, Schachter H, Carchon H, De Cock P, Coddeville B, Spik G. Carbohydrate deficient glycoprotein syndrome type II: a deficiency in Golgi localised N-acetyl-glucosaminyltransferase II. Arch Dis Child 1994;71:123–7.
10. Peters V, Penzien JM, Reiter G, Körner C, Hackler R, Assmann B, et al. Congenital disorder of glycosylation IId (CDG-IId) – a new entity: clinical presentation with Dandy-Walker malformation and myopathy. Neuropediatrics 2002;33:27–32.
11. Wu X, Steet RA, Bohorov O, Bakker J, Newell J, Krieger M, et al. Mutation of the COG complex subunit gene COG7 causes a lethal congenital disorder. Nat Med 2004;10:518–23.
12. Martinez Duncker I, Dupre T, Piller V, Piller F, Candelier JJ, Trichet C, et al. Genetic complementation reveals a novel human congenital disorder of glycosylation of type II, due to inactivation of the Golgi CMP-sialic acid transporter. Blood 2005;105:2671–6.
13. Huizing M, Rakocevic G, Sparks SE, Mamali I, Shatunov A, Goldfarb L, et al. Hypoglycosylation of α-dystroglycan in patients with hereditary IBM due to GNE mutations. Mol Genet Metab 2004;81:196–202.
14. Robinson LJ, Karlsson NG, Weiss AS, Packer NH. Proteomic analysis of the genetic premature aging disease Hutchinson Gilford progeria syndrome reveals differential protein expression and glycosylation. J Proteome Res 2003;2:556–7.
15. van Eijk HG, van Noort WL. The analysis of human serum transferrins with the PhastSystem: quantitation of microheterogeneity. Electrophoresis 1992;13:354–8.
16. Charlwood J, Clayton P, Keir G, Mian N, Winchester B. Defective galactosylation of serum transferrin in galactosemia. Glycobiology 1998;8:351–7.
17. Jaeken J, Pirard M, Adamowicz M, Pronicka E, Van Schaftingen E. Inhibition of phosphomannose isomerase by fructose 1-phosphate: an explanation for defective N-glycosylation in hereditary fructose intolerance. Pediatr Res 1996;40:764–6.
18. Stibler H. Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed. Clin Chem 1991;37:2029–37.
19. de Loos F, Huijben KM, van der Kar NC, Monnens LA, van den Heuvel LP, Groener JE, et al. Hemolytic uremic syndrome attributable to *Streptococcus pneumoniae* infection: a novel cause for secondary protein N-glycan abnormalities. Clin Chem 2002;48:781–4.
20. Nichols WC, Seligsohn U, Zivelin A, Terry VH, Hertel CE, Wheatley MA, et al. Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. Cell 1998;93:61–70.
21. Oka T, Ungar D, Hughson FM, Krieger M. The COG and COPI complexes interact to control the abundance of GEARs, a subset of Golgi integral membrane proteins. Mol Biol Cell 2004;15:2423–35.
22. Wopereis S, Abd Hamid UM, Critchley A, Royle L, Dwek RA, Morava E, et al. Abnormal glycosylation with hypersialylated O-glycans in

patients with Sialuria. Biochim Biophys Acta 2006;1762:598–607.

23. Tajima Y, Uyama E, Go S, Sato C, Tao N, Kotani M, et al. Distal myopathy with rimmed vacuoles: impaired O-glycan formation in muscular glycoproteins. Am J Pathol 2005;166:1121–30.

24. Bennett EP, Hassan H, Clausen H. cDNA cloning and expression of a novel human UDP-N-acetyl-α-D-galactosamine. Polypeptide N-acetylgalactosaminyltransferase, GalNAc-t3. J Biol Chem 1996; 271:17006–12.

25. Adamowicz M, Pronicka E. Carbohydrate deficient glycoprotein syndrome–like transferrin isoelectric focusing pattern in untreated fructosaemia. Eur J Pediatr 1996;155:347–8.

26. Hortin GL. The MALDI-TOF Mass Spectrometric View of the plasma proteome and peptidome. Clin Chem 2006;52:1223–37.

27. Mills K, Mills P, Jackson M, Worthington V, Beesly C, Mann A, et al. Diagnosis of congenital disorders of glycosylation type-I using protein chip technology. Proteomics 2006;6:2295–304.

28. Volker C, De Praeter CM, Hardt B, Breuer W, Kalz Fuller B, Van Coster RN, et al. Processing of N-linked carbohydrate chains in a patient with glucosidase I deficiency (CDG type IIb). Glycobiology 2002;12:473–83.

29. Grünewald S, Matthijs G, Jaeken J. Congenital disorders of glycosylation: a review. Pediatr Res 2002;52:618–24.