Electrophoretic screening for genetic variation in apolipoprotein C-III: identification of a novel apoC-III variant, apoC-III(Asp45→Asn), in a Turkish patient

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Abstract Screening of 6,840 plasma samples by isoelectric focusing (IEF) led to the identification of a novel apolipoprotein C-III variant. The underlying molecular defect was established by sequencing of exons 3 and 4 of the apoC-III gene subsequent to their amplification by the polymerase chain reaction (PCR). A G→A transition in the first nucleotide of codon 45 results in a replacement of aspartic acid by asparagine. ApoC-III(Asp45→Asn) was detected in a Turkish patient who previously had undergone coronary bypass surgery. Family studies identified two of the three children of the index patient as heterozygous variant carriers. The family was too small to demonstrate a significant effect of the variant on lipid metabolism. However, as judged by two-dimensional immunoelectrophoresis as well as IEF and subsequent scanning densitometry, the concentrations of the variant allele products were increased twofold in very low density lipoproteins (VLDL) and slightly decreased both in low density lipoproteins (LDL) and in high density lipoproteins (HDL) relative to the concentrations of the normal allele products. The disproportional distribution of the variant apoC-III isoproteins may indicate differences in the metabolism of variant and normal apoC-III. We conclude that genetically determined structural variants of apoC-III with changes in complete net charges are very rare and, hence, do not significantly contribute to the formation of dyslipidemia in the German population. Although heterozygosity for apoC-III(Asp45→Asn) is not associated with severe dyslipidemia, the disproportional distribution of the allele products among plasma lipoproteins indirectly indicates some impact on lipoprotein metabolism.—**Lüttmann, S., A. von Eckardstein, W. Wei, H. Funke, E. Köhler, R. W. Mahley, and G. Assmann.** Electrophoretic screening for genetic variation in apolipoprotein C-III: identification of a novel apoC-III variant, apoC-III(Asp45→Asn), in a Turkish patient. *J. Lipid Res.* 1994. **35:** 1431–1440.

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Apolipoprotein C-III (apoC-III), a glycoprotein consisting of 79 amino acids of known primary structure, is a major protein constituent of chylomicrons and of very low density lipoproteins (VLDL) and a minor constituent of high density lipoproteins (HDL). The human apoC-III gene has been mapped to chromosome 11,q23, where it is flanked by the genes for apoA-I and apoA-IV (reviewed in ref. 1). In plasma, apoC-III occurs in three isoforms differing by their content of O-linked sialic acids: apoC-III-0 (no sialic acid), apoC-III-1 (1 mol sialic acid), and apoC-III-2 (2 mol sialic acid) (2–4). The in vivo role of apoC-III is not well understood. In vitro, apoC-III inhibits lipoprotein lipase (LPL) (5–7) and hepatic triglyceride lipase (HTGL) (8), two enzymes involved in the catabolism of triglyceride-rich lipoproteins. Previous studies have shown that apoC-III also may represent an important LPL inhibitor in vivo (9) and that the aminoterminus is likely responsible for the modulation of LPL activity by apoC-III (7). Furthermore, apoC-III seems to compete with the apoE-mediated hepatic uptake of triglyceride-rich lipoproteins and their remnants

Abbreviations: IEF, isoelectric focusing; LPL, lipoprotein lipase; PCR, polymerase chain reaction; PROCAM, Prospective Cardiovascular Munster Study; RFLP, restriction fragment length polymorphism; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

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(10–13). Overexpression of the human apoC-III gene causes severe hypertriglyceridemia in transgenic mice, owing to decreased removal of triglyceride-rich particles from the circulation (14, 15).

To date, the role of genetic variation in apoC-III for the regulation of lipid metabolism is not well understood. ApoC-III deficiency has been found only in association with deficiency of apoA-I (16–19). Hence, the contribution of the lack of apoC-III to HDL deficiency and atherosclerosis in these patients is not known. Moreover, three structural variants of apoC-III have been described. One variant, designated apoC-III-3, is caused by oversialylation (3). In another variant, apoC-III(Thr74→Ala), the attachment site threonine was lost (20). These two apoC-III variants were not associated with dyslipidemia. The third variant, apoC-III(Lys58→Glu), was identified in two hyperalphalipoproteinemic women with low plasma concentrations of apoC-III and atypically large HDL (21).

The present study was performed to determine the frequency of structural variants of apoC-III in the Caucasian population and to unravel their possible impact on the formation of dyslipidemia.

MATERIALS AND METHODS

Subjects

Plasma samples from 6,840 individuals were screened for the presence of apoC-III variants; 4,228 samples were selected from probands of the Prospective Cardiovascular Münster (PROCAM) study (22), and 2,612 samples were from patients of a rehabilitation center for cardiovascular diseases.

In the course of the screening, a 50-year-old Turkish patient was identified as a heterozygous carrier of an apoC-III variant. Subsequent family studies were restricted to family members who live in Germany, namely his wife and three children.

The index patient's family originated from Capadokia (Central Anatolia, Turkey). To verify that the identified variant is not a frequent polymorphism in this Turkish population, 750 plasma samples from the Turkish Heart Study, collected in Capadokia, were also screened for the presence of apoC-III variants.

Quantitative analyses

Serum concentrations of triglycerides and cholesterol were determined with an autoanalyzer (Hitachi, Boehringer Mannheim GmbH, Mannheim, Germany). The concentration of HDL cholesterol was measured subsequent to precipitation of apoB-containing lipoproteins with phosphotungstic acid/$MgCl_2$ (Boehringer). LDL cholesterol was calculated using the Friedewald algorithm (23).

Concentrations of apoA-I, apoA-II, and apoB were quantified with a modified, commercially available turbidimetric assay (24). Concentrations of apoC-III both in total plasma and in apoB-free plasma (apoC-III Lp non-B) were quantified by electroimmunodiffusion in commercially available agarose gels as recommended by the supplier (Sebia, Paris, France). The difference between these two values gave the concentration of apoC-III in apoB-containing lipoproteins (apoC-III Lp B).

Preparation of lipoproteins, lipoprotein fractions, and apolipoproteins

VLDL ($d < 1.006$ g/ml), IDL (d 1.006 $<$ 1.019 g/ml), LDL (d 1.019 $<$ 1.063 g/ml), and HDL (d 1.063 $<$ 1.21 g/ml) were isolated by sequential isopyknic ultracentrifugation (25). Subsequently, the fractions were thoroughly dialyzed against 5 mM ammonium bicarbonate or 150 mM NaCl.

Density gradient ultracentrifugation of 1 ml plasma was performed using an SW 40 rotor in an ultracentrifuge from Beckman Instruments (Palo Alto, CA) by a procedure principally described by Redgrave, Roberts, and West (26) and Terpstra, Woodward, and Sanchez-Muniz (27). A fraction collector and a peristaltic pump were used to fractionate a density gradient ranging from 1.022 to 1.205 g/ml. Concentrations of cholesterol, triglycerides, apoA-I, and apoB in these fractions were determined as described above.

Prior to IEF, the lipoprotein emulsions were delipidated with ethanol–ether 4:1 (v/v) (28). The precipitated apolipoproteins were solubilized in a buffer containing 1% decylsulfate (wt/wt) (Eastman Kodak Co., Rochester, NY) and 0.01 M Tris-HCl (pH 8.2).

Desialylation of apoC-III

To desialylate apoC-III, 1 ml plasma or lipoprotein emulsion was incubated in 5 ml 0.01 M sodium acetate (pH 5.5) with 0.15 U neuraminidase from *Clostridium perfringens* (EC 3.2.1.18, Sigma Chemical Co., St. Louis, MO) at 37°C for 12 h. Subsequent to digestion with neuraminidase, the samples were delipidated and solubilized as described above.

Electrophoretic techniques

Isoelectric focusing of 5 μl plasma, apoVLDL, apoLDL, or apoHDL was performed in polyacrylamide gels (T=5%, C=3%) containing 2% (w/v) carrier ampholytes (pH 3.5–5.5) and 8 M urea (29). Gel strips containing the separated proteins either were directly stained with Coomassie blue R 250 (Serva) or were used for further analyses, i.e., immunoblotting, two-dimensional SDS-electrophoresis, or two-dimensional immunoelectrophoresis. After electroblotting to Immobilon Polyvinyliden Difluorid (Millipore Corp., Bedford, MA) (30), apoC-III isoproteins were detected with a sheep anti-human apoC-III-antiserum and a rabbit peroxidase-conjugated anti-sheep IgG antiserum (Dakopatts, Glos-

trup, Denmark). Two-dimensional SDS-electrophoresis of VLDL apolipoproteins was performed in a polyacrylamide gradient gel ranging from 10 to 25% according to Neville (31). Two-dimensional immunoelectrophoresis was carried out in agarose gels, containing 1% agarose (wt/vol), 2.5% polyethylenglycol 6,000 (wt/vol), and 1% sheep anti-human apoC-III-antiserum.

The size distribution of HDL particles, preseparated by ultracentrifugation, was analyzed by nondenaturing gradient gel electrophoresis principally as described by Nichols, Krauss, and Musliner (32). Fifty μl undelipidated HDL emulsion (d 1.069–1.21 g/ml) was applied to a polyacrylamide gradient gel ranging from 4 to 30% containing a buffer system as described by Altland, Hackler, and Knoche (33). Electrophoresis was interrupted when the indicator bromphenol blue left the gel.

Densitometry

Subsequent to IEF of desialylated apoC-III isoproteins from total plasma and from isolated lipoproteins, the relative concentration of normal and variant allele products was determined by scanning densitometry (Ultrascan, LKB Instruments, Bromma, Sweden) of immunoblotted proteins (total plasma and HDL apolipoproteins) or of Coomassie blue-stained proteins (VLDL and LDL apolipoproteins).

The Stokes diameters of HDL particles were determined as described by Nichols et al. (32), using nondenaturing gradient gel electrophoresis and scanning densitometry.

Analysis of the structural defect in the apoC-III variant

Genomic DNA was isolated from leukocytes of the five family members. Exons 3 and 4 of the propositus' apoC-III gene were selectively amplified by the polymerase chain reaction (PCR) (34) using oligonucleotides 1–4 described in **Table 1.** Thirty cycles of PCR were carried out as recommended by the supplier of the Taq polymerase (Cetus Corp., Emeryville, CA) with denaturation at 96°C for 90 s, annealing at 62°C for 60 s, and extension at 70°C for 60 s. After purification by electrophoresis in a 2.5% NuSieve agarose/1% agarose gel (wt/vol) (FMC Corp., Rockland, ME), the DNA was electroeluted into 0.5 × TAE (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0). Asymmetric PCR (35) was used to obtain single-strand DNA as described previously (36). For sequencing, primers 3 and 5 (Table 1) were used. Electrophoresis was carried out in a 6% polyacrylamide gel ([wt/vol] 29:1 acrylamide–bisacrylamide), 135 mM Tris, 45 mM boric acid, and 2.5 mM EDTA. Films were exposed overnight.

Vertical transmission of a G→A transition found in codon 45 was analyzed by an artificially created allele-specific *Cla*I-restriction fragment length polymorphism (RFLP) (37) after PCR amplification of exon 4 of the apoC-III gene primers 5 and 6. The latter is distinct from the proband's apoC-III sequence by the presence of a C instead of a T in the penultimate position (Table 1) and thereby introduces a cutting site for ClaI into the PCR product (AT↓CGAT). A heterozygous C→T transition in the third nucleotide of codon 14 was analyzed by digestion with *Bst*EII whose cutting site (G↓GTNACC) was specifically inserted into mutant DNA by the mutagenic primer 7 (Table 1). PCR was performed with primers 1 and 7. Finally, a T→G transversion of base pair 3206 (38) in the 3' -untranslated nucleotide sequence of exon 4 was analysed as a *Fnu*4HI-RFLP after PCR amplification of exon 4 with oligonucleotides 3 and 4.

RESULTS

Identification of an apoC-III variant

To determine the frequency of genetically determined structural variants of apoC-III in the Caucasian population, we performed a systematic screening of 6,840 plasma samples by the use of IEF and subsequent immunoblotting. In the course of the screening, one sample

TABLE 1. Sequence of primers used for PCR amplification, sequencing, and RFLP analysis of the apoC-III gene

No.	Sequence	Base Pairs[a]
Primer 1:	5'-CCCTACTCCTTCTGGCAGACCCAGC-3'	733–757
Primer 2:	5'-TTCCATTGTTGGGATCTCACCAGGG-3'	1241–1265
Primer 3:	5'-TCGTCCAGTGGGGACATGGGTGTGG-3'	2926–2950
Primer 4:	5'-ACCTGGAGTCTGTCCAGTGCCCACC-3'	3386–3410
Primer 5:	5'-GGAGCTGGCAGGATGGATAGG-3'	3161–3181
Primer 6:	5'-TGACTGATTTAGGGGCTGGGTGATC-3'	3003–3027
Primer 7:	5'-GCGGTCTTGGTGGCGTGCTTCAGGT-3'	1102–1126

Nucleotides underlined in primers 6 and 7 indicate deviations from the sequence of the apoC-III gene. PCR amplification with these primers introduced artificial cutting sites for restriction endonucleases *Cla*I and *Bst*EII in the presence of the G→A transition in codon 45 and a C→T transition in codon 14, respectively. Primers 1 and 2 were used for PCR amplification of exon 3, primers 3 and 4 for the amplification of exon 4.

[a] The enumeration of base is taken from reference 38.

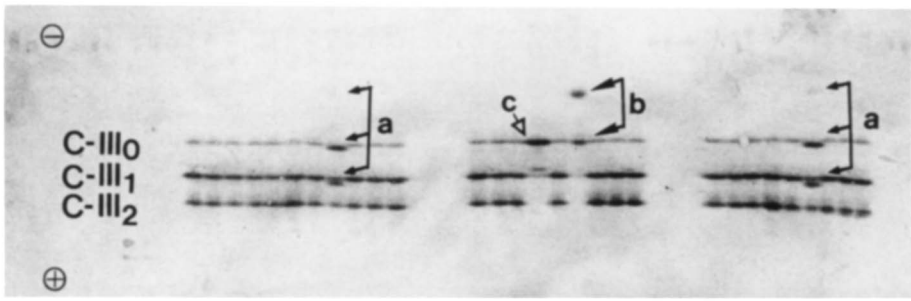

Fig. 1. Demonstration of apoC-III isoproteins in serum by IEF and subsequent immunoblotting; a) native serum of the propositus; b) neuraminidase-treated serum of the propositus; c) neuraminidase-treated serum of a normal subject.

was found to exhibit an atypical banding pattern with additional anti-apoC-III immunoreactive bands focusing cathodic from normal apoC-III isoproteins (**Fig. 1**). IEF performed after incubation of the proband's plasma with neuraminidase led to the detection of two anti-apoC-III immunoreactive bands, differing by one relative charge unit. As analyzed by two-dimensional SDS-polyacrylamide gel electrophoresis of the index patient's VLDL apolipoproteins, the additional bands exhibited a molecular weight identical to that of normal apoC-III isoproteins (**Fig. 2**). A family study revealed that two of three children of the propositus also presented additional anti-apoC-III immunoreactive proteins with atypical isoelectric points in their plasma, whereas these abnormal apoC-III isoproteins were not detectable in the plasma samples of the proband's wife or his younger son. In summary, the results indicated that the Turkish patient was heterozygous for a heritable apoC-III variant.

No other sample contained anti-apoC-III immunoreactive proteins with atypical isoelectric points. Therefore, the frequency of structural apoC-III variants with differences in complete net charges appears to be less than 0.02% in the German population. The index patient's family originated from Capadokia (Central Anatolia, Tur-

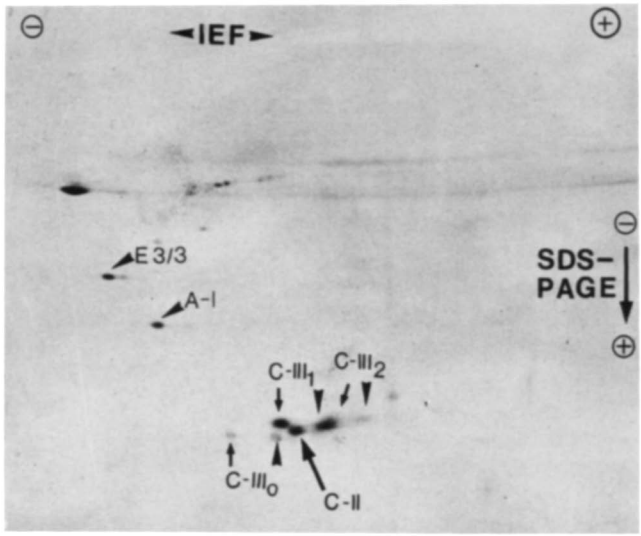

Fig. 2. Demonstration of apoC-III isoproteins by two-dimensional SDS electrophoresis of the index patient's VLDL apolipoproteins. Variant isoforms of apoC-III are marked by arrows; normal isoforms of apoC-III as well as apoA-I, apoE, and apoC-II are marked by triangles.

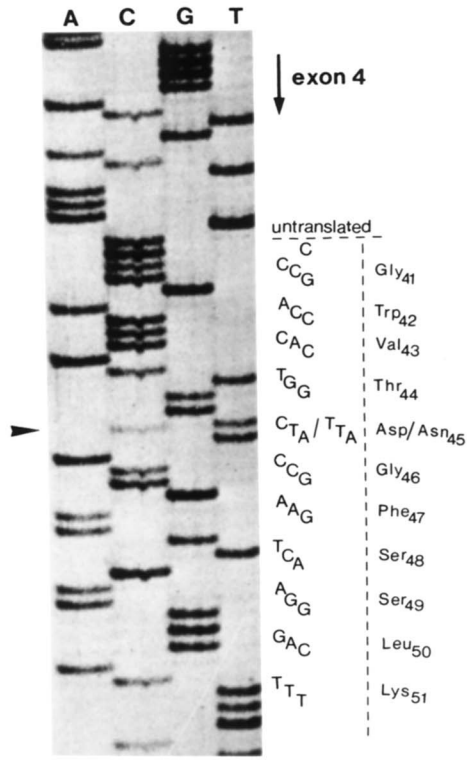

Fig. 3. Demonstration of heterozygosity for the G→A transition in the first nucleotide of codon 45, resulting in a substitution of aspartic acid by asparagine. Exon 4 of the proband's apoC-III gene was sequenced subsequent to its amplification by the PCR so that sequence information was obtained for both alleles simultaneously. The figure depicts the DNA strand complementary to template DNA. The base pair, affected by the nucleotide substitution, is marked by an arrow.

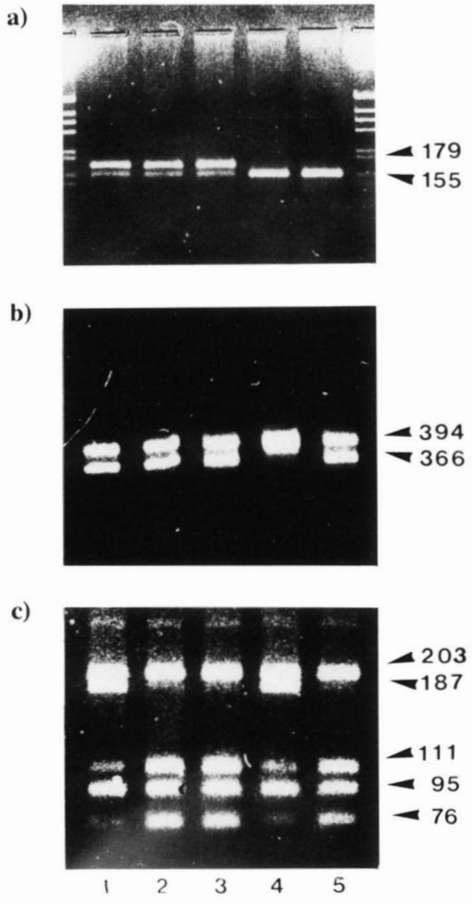

Fig. 4. a) Demonstration of vertical transmission of apoC-III(Asp45→Asn) by genotyping the family members' DNA by an allele-specific *Cla*I-RFLP. The G→A transition in the first nucleotide of codon 45 does not allow the formation of the *Cla*I cutting site so that heterozygosity for apoC-III(Asp45→Asn) is characterized by an additional 179-bp-long DNA fragment supplementary to the 24-bp- and 155-bp-long wild-type-specific DNA fragments; b) Demonstration of a C→T transition in the third nucleotide of codon 11 by analysis of an artificial *Bst*EII-RFLP. The presence of the rare nucleotide leads to the formation of 28-bp- and 366-bp-long DNA fragments instead of the 394-bp-long wild-type fragment (see Methods) c) Demonstration of a T→G transversion in the 3'-untranslated region of exon 4 of the apoC-III gene by analysis of an *Fnu*4HI-RFLP. The presence of the rare nucleotide leads to the formation of 76-bp- and 111-bp-long DNA fragments instead of the 187-bp-long wild-type fragment. Additional fragments of 95 bp and 203 bp in length are present in all cases; 1, propositus; 2, elder son; 3, daughter; 4, younger son; 5, wife.

key). To verify that the identified mutation is not a frequent polymorphism in this population, we subsequently screened 750 plasma samples from a Capadokian population for the presence of genetically determined structural variants of apoC-III. The above-described apoC-III variant was not identified in any other sample.

Determination of the molecular defect in the apoC-III variant

To establish the underlying molecular defect, exons 3 and 4 of the index patient's apoC-III gene were sequenced subsequent to their amplification by PCR. We found heterozygosity for a G→A transition in the first nucleotide of codon 45 (**Fig. 3**), which causes a replacement of aspartic acid by asparagine in the encoded protein and, hence, explains the altered isoelectric point of the variant apoC-III isoproteins.

The DNA from other family members was analyzed for the presence of the G→A transition in codon 45 by the use of an allele-specific *Cla*I-RFLP. The G→A exchange removes the *Cla*I cutting site and leads to the formation of a 179-bp-long DNA fragment instead of 24-bp- and 155-bp-long fragments in wild-type DNA. This genotype analysis verified the heterozygous presence of the mutation in the propositus, his elder son, and his daughter and excluded its presence in his wife and his younger son (**Fig. 4**).

Determination of haplotypes

Besides the nonsynonymous mutation, two additional mutations were identified in the proband's apoC-III gene. A heterozygous C→T transition in the third nucleotide of codon 14 was verified upon cleavage with *Bst*EII by the presence of 366-bp- and 28-bp-long DNA fragments in addition to the 394-bp-long wild-type fragment (see Methods). Heterozygosity for this polymorphic site was also observed in his elder son, his daughter, and his wife, whereas his younger son was homozygous for the frequent nucleotide (Fig. 4). Furthermore, a heterozygous T→G transversion of base pair 3,206 (37) in the 3'-untranslated nucleotide sequence of exon 4 of the proband's apoC-III gene was confirmed by digestion with *Fnu*4HI. This nucleotide exchange was characterized by the

TABLE 2. Genotypes and deduced haplotypes determined by RFLP analysis of one nonsynonymous mutation and two additional mutations in the apoC-III gene of a family with an apoC-III variant; haplotype A identifies the mutant allele

	*Cla*I	*Bst*EII	*Fnu*4HI	
		Genotypes		
Propositus	+ / −	+ / −	+ / −	
Wife	− / −	+ / −	+ / +	
Elder son	+ / −	+ / −	+ / +	
Daughter	+ / −	+ / −	+ / +	
Younger son	− / −	− / −	+ / −	
		Deduced Haplotypes		Allele
Propositus	+	+	+	A
	−	−	−	B
Wife	−	+	+	C
	−	−	+	D
Elder son	+	+	+	A
	−	−	+	D
Daughter	+	+	+	A
	−	−	+	D
Younger son	−	−	−	B
	−	−	+	D

(−) Indicates the presence of the frequent nucleotide; (+) the presence of the rare nucleotide.

TABLE 3. Lipid and apolipoprotein concentrations of members from a Turkish family with an apoC-III variant and reference values for Turkish and German participants in the PROCAM study (38)

Subjects	Triglycerides	Total Cholesterol	HDL Cholesterol	LDL Cholesterol	ApoA-I	ApoA-II	ApoB
age, mutant				*mg/dl*			
Propositus (50, +)	150	230 (> 80)	31 (p<30)	169 (p>90)	104	35	101
Wife (46, −)	154	209 (>70)	33 (p<30)	145 (p>70)	98	37	93
Elder son (25, +)	109	227 (>80)	33 (p<30)	172 (p>90)	110	43	97
Daughter (23, +)	58	161 (>50)	36 (p<40)	113 (p>60)	106	39	68
Younger son (21, −)	81	176 (>50)	41 (p<60)	119 (p>50)	98	37	93
Turkish men (n = 396)	74.7<135.3<244.9	193.2 ± 41.7[b]	37.7 ± 9.5[b]	123.9 ± 36.2[b]	n.d.	n.d.	n.d.
German men (n = 23,115)[a]	67.8<119.4<210.3	217.8 ± 43.6	46.6 ± 12.0	143.6 ± 38.2	141.8 ± 21.1	42.9 ± 7.4	106.5 ± 33.4
Turkish women (n = 155)	51.3<87.5<149.2	174.2 ± 35.6[b]	45.9 ± 11.8[b]	108.2 ± 30.7[b]	n.d	n.d	n.d
German women (n = 10,496)[a]	56.8<88.4<137.4	210.8 ± 42.7	59.5 ± 15.0	131.7 ± 39.7	157.7 ± 26.8	42.9 ± 8.0	96.0 ± 30.6

Values in parentheses give percentiles for total cholesterol, HDL cholesterol, and LDL cholesterol from sex-matched Turkish PROCAM controls. Reference values are expressed as means ± SD, and as $-s<x<+s$ confidence intervals as determined by lge transformation (triglycerides); n.d., not determined.

[a] Control populations for plasma concentration comparisons of apolipoproteins were recruited from a smaller number of German participants in the PROCAM study (n>435).

[b] Significant difference between Turkish and German sex-matched mean values ($P < 0.001$, two-tailed t-test).

presence of both the 111-bp- and 76-bp-long DNA fragments and the 187-bp-long wild-type fragment. The proband's younger son also was heterozygous for this polymorphic site, whereas his elder son, his daughter, and his wife were homozygous for the rare nucleotide (Fig. 4). Haplotypes are given in **Table 2.**

The nonsynonymous nucleotide substitution in codon 45, resulting in the replacement of aspartic acid by asparagine, was identified to be *cis*-localized both with the nucleotide substitution of codon 11 and the nucleotide substitution in the 3'-untranslated region of exon 4 of the apoC-III gene.

Analysis of the effect of apoC-III(Asp45→Asn) on lipid metabolism

ApoC-III(Asp45→Asn) was identified in a Turkish patient who previously had undergone coronary bypass surgery. At the time of the studies, the propositus was treated with gemfibrozil. His relatives appeared healthy and were not treated with drugs that influence lipoprotein metabolism. Lipid values and apolipoprotein concentrations of the three apoC-III(Asp45→Asn) heterozygotes and of the two unaffected family members are given in **Table 3.** Compared to Germans, Turks generally exhibit significantly lower plasma concentrations of total cholesterol, LDL cholesterol, and especially HDL cholesterol ($P < 0.001$, two-tailed t-test, Table 3). Therefore, control populations for plasma concentration comparisons of lipids were recruited from Turkish participants in the PROCAM study. Compared to the unaffected younger son and to sex-matched Turkish PROCAM controls, the propositus and his heterozygous elder son exhibited relatively high plasma concentrations of total cholesterol (above the 80th percentile of sex-matched Turkish PROCAM controls) and of LDL cholesterol (above the 90th percentile). Similarly, plasma concentrations of HDL cholesterol were relatively low in the two male variant carriers when compared to Turkish controls (below the 30th percentile). Lipid values of the fe-

TABLE 4. Plasma concentrations of apoC-III in total plasma, apoB-containing, and apoB-free lipoproteins

Subjects	Total ApoC-III	ApoC-III Lp B	ApoC-III Lp non B	ApoC-III Lp non B/ Total ApoC-III
(mutant)		*mg/dl*		
Propositus (+)	1.6	0.9	0.7	0.44
Wife (−)	2.1	0.9	1.2	0.57
Elder son (+)	1.5	0.8	0.7	0.47
Daughter (+)	1.2	0.7	0.5	0.42
Younger son (−)	1.3	0.4	0.9	0.69
Variant carriers (n = 3)	1.43 ± 0.21	0.80 ± 0.10	0.63 ± 0.12	0.44 ± 0.03[a]
Unaffected family members (n = 2)	1.70 ± 0.57	0.65 ± 0.35	1.05 ± 0.21	0.63 ± 0.08
Reference values	1.6–4.5	0.1–2.2	0.4–4.8	n.d.

[a] Significant difference between apoC-III(Asp45→Asn) heterozygotes and unaffected family members ($P < 0.05$, two-tailed t-test); n.d., not determined.

male apoC-III(Asp45→Asn) heterozygote were normal. While plasma concentrations of HDL cholesterol and of apoC-III were low in all investigated subjects, the ratio of apoC-III in HDL to apoC-III in total plasma was significantly decreased in the three variant carriers compared to the two unaffected family members ($P < 0.05$, two-tailed t-test, **Table 4).**

The distribution of cholesterol, triglycerides, apoA-I, and apoB throughout lipoprotein classes separated by density gradient ultracentrifugation did not significantly differ among apoC-III(Asp45→Asn) heterozygotes and unaffected family members. Scanning densitometry of HDL particles separated by nondenaturing gradient gel electrophoresis revealed low concentrations of HDL_{gge2a} and HDL_{gge2b} in both affected and unaffected family members.

Relative concentrations of normal and variant allele products

The relative concentrations of normal and variant allele products in total plasma and in isolated lipoproteins from the variant carriers were investigated by two-dimensional immunoelectrophoresis as well as by IEF and subsequent scanning densitometry. In serum, the concentration of the variant asialo-apoC-III was slightly increased relative to the concentration of the normal asialo-apoC-III (1.12 ± 0.06; n = 3) (**Fig. 5**). In desialylated VLDL apolipoproteins, the ratio of variant apoC-III to normal apoC-III was significantly increased, approximately by a factor of 1.75, compared to the ratio in serum (**Fig. 6**). In both LDL and HDL, the concentration of the variant allele products was slightly decreased relative to the concentration of the normal allele products (**Fig. 7, Table 5**).

DISCUSSION

Genetically determined variation in plasma apolipoproteins contributes to the formation of dyslipidemia and has provided unique insights into the structure–function relationships of many apolipoproteins. In the present study, we investigated the frequency and the importance for lipoprotein metabolism of genetically determined structural variants of apoC-III. Only one of 6,840 plasma samples contained a structural apoC-III variant detectable by IEF. This resulted from the replacement of an aspartic acid in position 45 by an asparagine residue. Thus, structural variants of apoC-III are very rare and, hence, do not contribute considerably to the formation of dyslipidemia in the German population. To our knowledge, no further data on the frequency of apoC-III variants are available so far. Comparable electrophoretic screening programs have been performed for the identification of structural variants of apoA-I, whose gene closely neighbors that for apoC-III and which has descended from the same ancestral gene (reviewed in ref. 1). The frequency of apoA-I variants ranges from 1:1000 to 1:2000 (39). Eight hundred thirteen of 2,187 possible single-base-pair substitutions in the cDNA sequence of the apoA-I gene cause complete net charges changes in the encoded protein and thus are detectable by IEF as compared to 206 of 711 possible single-base-pair substitutions in the coding part of the apoC-III gene. The low frequency of apoC-III variants observed in this screening, therefore, appears to be within the statistical expectation.

ApoC-III(Asp45→Asn) was identified in a Turkish patient who previously had undergone coronary bypass surgery and in two of his three children. Although subsequent family studies did not reveal any severe effect of apoC-III(Asp45→Asn) on lipoprotein metabolism, we cannot yet exclude minor effects of this mutant allele on lipid metabolism as the investigated family is very small and as the a priori low concentration of HDL cholesterol in Turks may have masked any association of the apoC-III variant with reduced HDL cholesterol levels. Further-

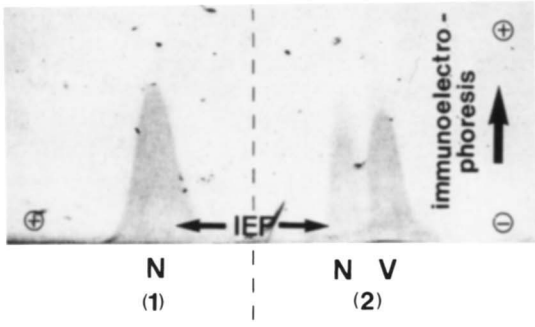

Fig. 5. Two-dimensional immunoelectrophoresis of desialylated apoC-III isoproteins in serum; 1, from a normal subject; 2, from an apoC-III(Asp45→Asn) heterozygote. Gel strips, containing desialylated apoC-III isoproteins, preseparated by IEF, were transferred to a gel containing 1% agarose, 2.5% PEG 6000, and 1% sheep anti-human apoC-III-antiserum; N denotes normal $apoC\text{-}III_{neuraminidase}$; V denotes variant $apoC\text{-}III_{neuraminidase}$.

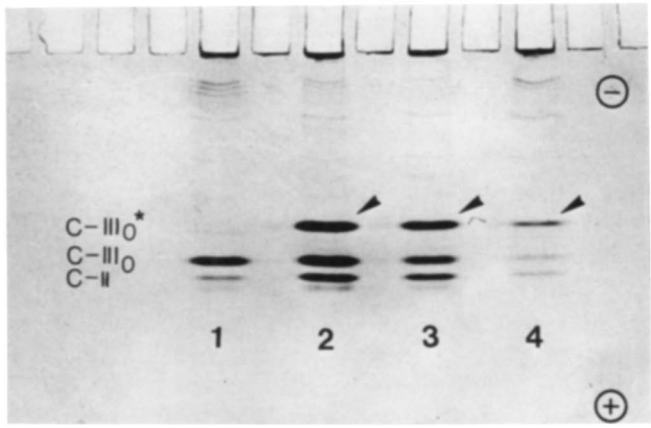

Fig. 6. Demonstration of desialylated apoC-III isoproteins in VLDL by IEF; 1, normal subject; 2, propositus; 3, elder son; 4, daughter.

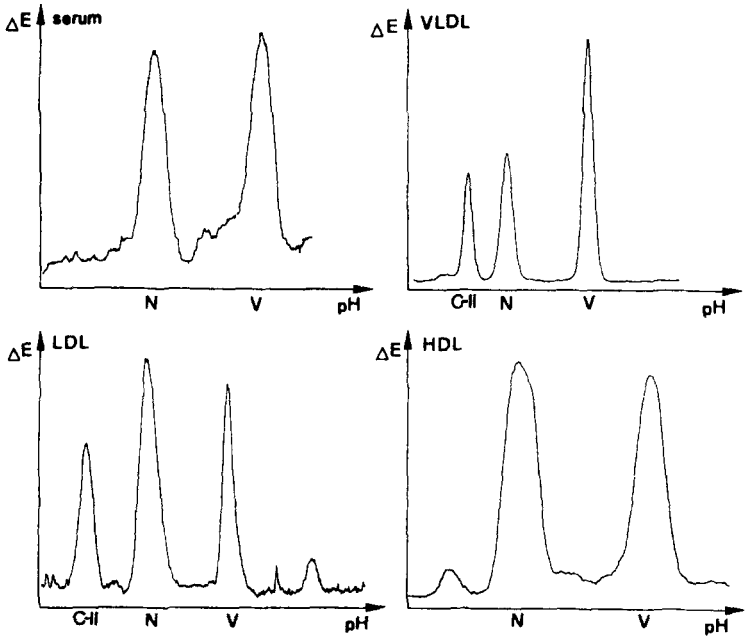

Fig. 7. Densitometry of desialylated apoC-III isoproteins subsequent to IEF of total plasma and of isolated lipoproteins from the propositus. ApoC-III isoproteins were either detected by anti apoC-III immunoblotting (serum, HDL) or by staining with Coomassie blue (VLDL, HDL); N denotes normal apoC-III$_{neuraminidase}$, V denotes variant apoC-III$_{neuraminidase}$; C-II denotes apoC-II, which was detectable only after protein staining.

more, although unlikely, we cannot yet exclude the possibility that the additionally identified polymorphic sites may be of functional importance. Some of our findings, however, indirectly indicate that the allele carrying apoC-III(Asp45→Asn) does affect lipoprotein metabolism. The ratio of apoC-III in HDL to apoC-III in total plasma was significantly decreased in the three variant carriers compared to the two unaffected family members. In this context it is also noteworthy that, in the Cholesterol Lowering Atherosclerosis Study, the relative distribution of apoC-III between HDL and apoB-containing lipoproteins was the most powerful predictor for the progression of coronary heart disease (40).

The enrichment of apoC-III in apoB-containing lipoproteins of heterozygotes for apoC-III(Asp45→Asn) was paralleled by a disproportional distribution of normal and variant allele products with a twofold increased ratio of variant to normal apoC-III in VLDL and a slightly decreased ratio in both LDL and HDL. Taking into consideration that the C-apolipoproteins are readily exchanged between triglyceride-rich lipoproteins and HDL (41), this disproportional distribution of normal apoC-III and apoC-III(Asp45→Asn) among VLDL, LDL, and HDL could be explained by changes in the lipid-binding properties of the mutant protein. Actually, circular dichroism studies of apoC-III and its peptide analogues demonstrated that the lipid-binding domain of apoC-III probably resides in its carboxyterminal thrombin cleavage fragment, which encompasses residues 41–79 (42). The Asp→Asn replacement in position 45 occurs in a region with predicted amphipathic α-helical conformation (43). Computer-assisted protein sequence analysis (44–46) did not reveal considerable differences between the predicted secondary structure of normal apoC-III and apoC-III(Asp45→Asn). Furthermore, as both aspartic acid and asparagine contain a polar side chain, the amino acid substitution probably does not alter the amphipathic character of the presumed α-helix. The Edmundson wheel presentation of apoC-III$_{40-67}$, however, demonstrates that Asp45 may be located within a cluster of negatively charged amino acid residues (43). Although apoC-III has been little conserved during the evolution of mammals, the apoC-III sequences from humans (1), cynomolgus monkey (47), dog (48), and cow (49) all contain a negatively charged amino acid residue at position 45, suggesting that Asp45 contributes to the regular function of apoC-III. As the aminoterminus of apoC-III is responsible for its inhibitory role on lipoprotein lipase (7) it is unlikely that the replacement of aspartic acid by asparagine

TABLE 5. Relative concentrations of variant and normal desialylated apoC-III isoforms in total serum and in isolated lipoproteins of heterozygotes for apoC-III(Asp45→Asn)

	Variant ApoC-III/Normal apoC-III
Serum	1.12 ± 0.06
VLDL	1.98 ± 0.08 ($P < 0.001$)
LDL	0.86 ± 0.03 ($P < 0.01$)
HDL	0.91 ± 0.06 ($P < 0.02$)

Subsequent to IEF, the relative concentrations of variant and normal desialylated apoC-III isoforms were determined by scanning densitometry of immunoblotted proteins (serum, HDL) or of Coomassie blue-stained proteins (VLDL, LDL). Means ± SD; n = 3. Using the two-tailed *t*-test, significant differences were demonstrated for the ratio variant/normal apoC-III in VLDL, LDL, and HDL as compared to the ratio in serum.

at residue 45 affects the modulation of LPL activity by apoC-III.

In summary, although various association studies have implicated genetic variation in the apoC-III gene in dyslipidemia and premature coronary heart disease (reviewed in ref. 50), our data indicate that genetically determined structural variants of apoC-III with differences in complete net charges are very rare in the German population. Like two other previously identified apoC-III variants, apoC-III-3 (10) and apoC-III(Thr74→Ala) (20), apoC-III(Asp45 → Asn) has no tremendous effect on lipoprotein metabolism. Unlike apoC-III(Lys58→Glu) (21), apoC-III(Asp45→Asn) is certainly not associated with increased concentrations of HDL cholesterol. By contrast, we cannot yet exclude the possibility that apoC-III(Asp45→Asn) decreases HDL cholesterol.

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