Apolipoprotein C-III(Lys_{58} → Glu)

Identification of an Apolipoprotein C-III Variant in a Family with Hyperalphalipoproteinemia

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Abstract

Apolipoprotein C-III is a major protein constituent of triglyceride rich lipoproteins and HDL. It occurs in plasma in three isoforms differing by their sialic acid content. Apo C-III putatively inhibits lipolysis and the apo E mediated hepatic uptake of remnants from triglyceride rich particles. We identified a heterozygous carrier of an apolipoprotein C-III variant by the presence of additional bands after isoelectric focusing (IEF) of VLDL. Structural analysis of the variant protein by HPLC, time-of-flight secondary ion mass spectrometry, and automated gas phase sequencing revealed a lysine to glutamic acid replacement in position 58. The underlying A to G exchange was verified by direct sequencing subsequent to amplification by polymerase chain reaction of exon 4 of the apo C-III gene. Family studies revealed vertical transmission of this defect.

The two variant carriers exhibited plasma concentrations of HDL cholesterol and apo A-I above the 95th percentiles of sex matched controls whereas the unaffected father and sister showed normal values. The plasma concentrations of apo C-III in the two variant carriers were decreased by 30–40% compared with those of the two unaffected family members and to random controls. Using two-dimensional immunoelectrophoresis as well as IEF and subsequent scanning densitometry, we found that the low serum concentration of apo C-III was a consequence of diminished concentrations of the variant apo C-III isoproteins in both VLDL (15% of normal) and HDL (25% of normal). Apo C-III(Lys_{58} → Glu) heterozygotes possessed unusual HDL as demonstrated by nondenaturing gradient gel electrophoresis. They consisted mainly of HDL_{2b} and contained a proportion of atypically large particles, enriched in apo E, with a Stokes diameter of 13–18 nm and resembling HDL_c.

In conclusion, heterozygosity for a structural apo C-III variant – apo C-III(Lys_{58} → Glu)—was identified in two hyperalphalipoproteinemic subjects characterized by the presence of low plasma apo C-III concentrations and atypically large HDL. (*J. Clin. Invest.* 1991. 87:1724–1731.) Key words: triglyceride rich particles • HDL subclasses • lipolysis • time-of-flight secondary ion mass spectrometry • gradient gel electrophoresis

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Introduction

Several prospective epidemiological studies revealed an inverse correlation between the plasma concentration of HDL cholesterol and the risk of coronary heart disease (reviewed in 1). The variation of HDL cholesterol levels in the population is determined by environmental as well as by genetic factors. Family and twin studies have estimated the impact of genes to be ~ 50% (2). Efforts undertaken to unravel the gene loci influencing the plasma concentration of HDL cholesterol have focused on APOLP1, as this locus codes for the apolipoproteins A-I, C-III, and A-IV, all of which are involved in the formation and metabolism of HDL (3–5). Whereas some mutations in the apo A-I gene have been shown to be associated with familial HDL deficiency (6), little is known about the effects of mutations in the genes for apo C-III and apo A-IV on HDL cholesterol plasma concentration.

Apo C-III, a protein consisting of 79 amino acids of known sequence (7–10), is found predominantly in VLDL and in HDL (11). Isoelectric focusing (IEF)[1] separates apo C-III into three isoforms that differ in their degree of O-linked sialyation at the threonine residue in position 74: apo $C\text{-}III_0$ (no sialic acid), apo $C\text{-}III_1$ (1 mol sialic acid) and apo $C\text{-}III_2$ (2 mol sialic acid) (12–15). The physiological function of apo C-III is not yet fully understood. In vitro, high concentrations of apo C-III have been shown to inhibit lipoprotein lipase (LPL) (16, 17) and hepatic triglyceride lipase (18), enzymes responsible for the clearance of triglyceride rich particles from the plasma. In contrast to normal plasma, apo A-I/apo C-III deficient plasma is not able to inhibit LPL activity, thus supporting the hypothesis that apo C-III also inhibits LPL in vivo (19). Furthermore, apo C-III was shown to inhibit the hepatic uptake of triglyceride rich particles (20–22). Apo C-III also appears to interfere with HDL receptor mediated uptake of lipoproteins; apo C-III containing proteoliposomes were shown to compete for the specific binding of HDL and of apo A-I proteoliposomes to cell surface receptors of various rat cells (23–25).

Previously, two genetic variants of apo C-III have been detected on the basis of additional bands after IEF: One variant was characterized by the presence of a fourth anodic band, which was shown to be due to oversialyation of the protein (14). In the other apo C-III variant, the substitution of threonine in position 74 by alanine prevented O-glycosylation (26–28). Both apo C-III variants were not found associated with any dyslipoproteinemic phenotype. Two cases of familial apo C-III deficiency that were characterized by increased prevalence of

1. *Abbreviations used in this paper:* IEF, isoelectric focusing; LPL, lipoprotein lipase; PCR, polymerase chain reaction; PROCAM, Prospective Cardiovascular Munster study; TOF-SIMS, time-of-flight secondary ion mass spectrometry.

precocious coronary heart disease were also deficient in apo A-I. Therefore, the potential role of apo C-III in the pathogenesis of premature atherosclerosis in these families remained undefined (29–32).

Here, we report on a mutant apo C-III that we found in two family members with relatively low plasma concentrations of apo C-III and high plasma concentrations of HDL cholesterol and apo A-I.

Methods

Subjects. In a screening program, apo E polymorphism was analyzed in 200 students by IEF of VLDL apolipoproteins. In the course of the study, the C apolipoproteins of a 27-yr old woman (P.Sch.) were found to yield six bands instead of the normal four. Further studies on family members revealed that the two additional anodic bands were vertically transmitted.

For the family study blood was collected from the proband's parents and sister. For analyses of lipoproteins by density gradient ultracentrifugation and polyacrylamide gradient gel electrophoresis the blood was collected on 50 mM EDTA and stored on ice at once. Blood for leucocyte isolation was also collected on 50 mM EDTA. For the structural analysis of the mutant protein, 1 l plasma was obtained from the proposita by plasmapheresis. Control populations for plasma concentration comparisons of lipids and apolipoproteins were recruited from the Prospective Cardiovascular Münster (PROCAM) study (33).

Quantitative analyses. Serum concentrations of triglycerides and cholesterol were quantified with an autoanalyzer (Hitachi/Boehringer Mannheim GmbH, Mannheim, FRG). Phosphatidylcholine and sphingomyeline were determined as described previously (34, 35). Concentrations of HDL lipids were measured after precipitation with phosphotungstic acid (Boehringer). LDL cholesterol was calculated using the Friedewald formula (36).

Concentrations of apo A-I, apo A-II, B, and E were determined with a modified commercially available turbidimetric assay (Boehringer) (37). Apo C-III was quantified by electroimmunodiffusion in 1% agarose containing 2.5% polyethylenglycol and 8% (vol/vol) rabbit anti-human apo C-III antiserum (38). Calibration was done with a purified apo C-III of known protein concentration (39) dissolved in a solution of 0.9% sodium chloride and 3% BSA (wt/vol) at final apo C-III concentrations of 20, 10, 5, and 2.5 mg/dl. For a comparative evaluation of apo C-III serum concentrations in members of the Sch. Family and in the normal population, we also analyzed sera of 58 women and 61 men participating in the PROCAM study.

Preparation of lipoproteins and apolipoproteins. VLDL, LDL, and HDL were prepared by sequential ultracentrifugation (40). The fractions were exhaustively dialyzed against 50 mM EDTA. Lipoprotein emulsions were delipidated with ethanol:ether (3:1, [vol/vol]) (41). The precipitated apolipoproteins were solubilized in 50 μl of a buffer containing 1% decylsulfate (wt/wt) (Eastman Kodak Co., Rochester, NY), 20% glycerol (wt/wt), 0.01 M Tris HCl (pH 8.2), and 2% carrier ampholytes (wt/wt).

Desialyation of apo C-III. For desialyation of apo C-III, 3 ml VLDL and HDL emulsions adjusted to 0.01 M sodium acetate (pH 5) were incubated with 0.1 U neuraminidase (Sigma Chemical Co., St. Louis, MO) at 37°C for 16 h and then delipidated and solubilized as described above.

Demonstration of apo C-III by isoelectric focusing, two-dimensional SDS-electrophoresis, two-dimensional immunoelectrophoresis and immunoblotting. Apo C-III isoproteins from 5 μl plasma, 50 μl apo VLDL or apo LDL, 2 μl apo HDL or 50 μl of fractions obtained by density gradient ultracentrifugation and subsequent dialysis against EDTA (see below) were separated by isoelectric focusing in a one step procedure (42). Gel strips containing the separated proteins were either directly stained with Coomassie blue R 250 or were used for further analyses by two-dimensional SDS electrophoresis, two-dimensional immunoelectrophoresis, and immunoblotting. SDS electrophoresis was performed in a gel with 20% polyacrylamide according to Neville (43). Immunoelectrophoresis was performed in a 1% agarose gel containing 8% antiserum against apo C-III (42). After electroblotting to nitrocellulose (44), apo C-III isoproteins were detected with a rabbit anti-apo C-III antiserum, a biotinylated goat anti-rabbit IgG antiserum, and streptavidin-horseradish peroxidase.

Density gradient ultracentrifugation. Density gradient ultracentrifugation was performed with an SW 40 rotor in a centrifuge (L8-70; Beckman Instruments, Inc., Palo Alto, CA) by a procedure principally described by Redgrave et al. and Terpstra et al. (45, 46). After a 24-h run at 40,000 rpm, the sample was portioned into fractions of 500 μl using a fraction collector and a peristaltic pump (Pharmacia-LKB Instruments Inc., Bromma, Sweden). Concentrations of total and free cholesterol, triglycerides, phosphatidylcholine, sphingomyeline, and apo A-I were determined as described above. 50 μl of each fraction was analyzed by nondenaturing polyacrylamide gradient electrophoresis (see below) as well as by IEF and subsequent immunoblotting (see above).

Gradient gel electrophoresis. Nondenaturing polyacrylamide gradient gel electrophoresis of undelipidated HDL ($1.069 < 1.21$ g/ml) or of fractions obtained by density gradient ultracentrifugation was performed principally as described by Nichols et al. (47) but using lab-made gels of 1.5-mm thickness and a buffer system described by Altland et al. (48). Either 40 μl HDL emulsion or 50 μl of the fractions obtained by density gradient ultracentrifugation were applied to polyacrylamide gradient gels ranging from 5 to 30% or from 5 to 21% (wt/vol), respectively. Electrophoresis was interrupted when the bromphenol blue front had left the gel.

Densitometry. The relative distribution of the apo C-III isoproteins and the size distribution of HDL particles were analyzed by densitometry of Coomassie blue stained gels subsequent to isoelectric focusing of apo VLDL and apo HDL or subsequent to nondenaturing gradient gel electrophoresis, respectively. Scanning densitometry was performed with Ultrascan (LKB Instruments Inc., Bromma, Sweden). Peak areas reflecting the intensity of the apo C-III bands and thus the concentration of specific isoproteins were integrated automatically with SP 4270 (Spectra-Physics Inc., Mountain View, CA). The Stokes diameters of the HDL particles were determined as described by Nichols et al. (47).

Isolation and structural analysis of apo C-III isoproteins. Isolation and structural analysis of the apo C-III variant was done by a procedure that has been described by our laboratory previously (14, 49, 50). Briefly, VLDL apolipoproteins isolated from 1 l plasma by ultracentrifugation and subsequent delipidation were separated by isoelectric focusing in pH 3.75 to pH 5.25. The gels were rehydrated with 6 M urea and 15% glycerol (wt/vol). Separation was performed overnight at 2,000 V, 15 mA, and 5 W, and for 4 further h at 3,000 V. For band localization, a 1-cm wide gel strip was removed from one side of the gel and stained with Coomassie blue. By referring to the bands in the stained gels, gel strips containing the normal and the variant apo C-III isoforms were cut out of the remaining unstained gel. After electroelution the proteins were dialyzed against 0.01 M ammonium hydrogen carbonate (pH 7.8) for 24 h and subsequently lyophilized. After purification by reversed phase HPLC, 500 μg of the lyophilized apo C-III isoproteins were solubilized in 0.01 M ammonium hydrogen carbonate (pH 7.8) and digested with trypsin (Cooper Biomedical, Wiesbaden, FRG, activity: 212 U/mg protein) at a ratio of 40:1 (wt/wt). The solution was incubated for 24 h at 37°C. The proteolytic digest was separated by reversed phase HPLC. A C18 column with a length of 250 mm and a diameter of 4 mm (E. Merck, Darmstadt, FRG) was run with a gradient from 0% acetonitrile in 0.1% trifluoro acetic acid (eluent A) to 100% acetonitrile in 0.1% trifluoro acetic acid (eluent B). The flow rate was 1 ml/min. After isocratic elution at 0% acetonitrile for the first 10 min, the acetonitrile content of the gradient was increased to 50% within 50 min and to 100% during the following 2 min. A peak detector monitoring at 215 nm and a fraction collector (Pharmacia-LKB) were used to collect the peptides separately. HPLC fractions were analyzed by time-of-flight secondary ion mass spectrometry (TOF-SIMS) (49). Mass differences between peptides of normal apo C-III that were lost from the chromatogram, and peptides of variant apo C-III that were

newly generated, were used to calculate possible amino acid substitutions. The predicted amino acid substitution was confirmed by sequence analysis of the affected tryptic peptide. Sequence analysis was performed by automated gas phase protein sequencing (Applied Biosystems, Inc., Foster City, CA).

Verification of the mutation by direct sequencing of the apo C-III gene. Genomic DNA was isolated from leucocytes of four family members. Exon 4 of the apo C-III gene with its flanking regions was selectively amplified by the polymerase chain reaction (PCR) (51) using 5′-TCGTCCAGTGGGGACATGGGTGTGG-3′ as the 5′-primer and 5′-ACCTGGAGTCTGTCCAGTGCCCACC-3′ as the 3′-primer. 30 cycles of PCR were done as described by the supplier of the Taq polymerase (Cetus Corp., Emeryville, CA) except that (*a*) initial denaturation was done at 96°C for 90 s; (*b*) annealing was done at 62°C for 60 s; and (*c*) extension was done at 70°C for 60 s. The product was purified by electrophoresis in a 2.5% Nusieve agarose/1% agarose gel (wt/vol) (FMC Corp., Rockland, ME). PCR fragments of expected size were cut out of the gel and electroeluted in 0.5× TAE (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0) for 1.5 h at 180 V. DNA was desalted and concentrated to 70 μl by ultrafiltration centrifugation in Centricon X-100 tubes as recommended by the supplier (Amicon Corp., Danvers, MA). Single strand DNA was produced from the purified double strand DNA by the method of Gyllenstein and Erlich (52) using the same reaction and purification parameters as above. A second primer was not used. After reduction of the sample volume to 10 μl in an evaporation centrifuge, the sequencing reaction was done directly using T7 polymerase (Pharmacia-LKB) and the above-described 5′-primer as a sequencing primer. Electrophoresis was performed in a 6% polyacrylamide gel (29:1 acrylamide:bisacrylamide), 135 mM Tris, 45 mM boric acid, and 2.5 mM EDTA. Films were exposed overnight.

Results

Description of the apo C-III variant. During a study of apo E polymorphism in 200 students by IEF of VLDL apolipoproteins, a sample of a 27-yr old woman (P.Sch.) was found to exhibit two additional bands localized anodic from normal apo C-III$_2$ (Fig. 1 *a*). Two-dimensional SDS electrophoresis revealed that the two bands exhibited a molecular weight identical to that of apo C-III (Fig. 1 *b*). Both bands were shown to be apo C-III immunoreactive by immunoblotting of the proband's plasma subsequent to IEF (Fig. 1 *c*) and by two-dimensional immunoelectrophoresis of the proband's apo VLDL (Fig. 2). IEF performed subsequently to treatment of VLDL with neuraminidase made the additional abnormal bands disappear and generated a new band that was in a pI position slightly anodic from normal apo C-III$_1$. This band was assumed to contain the desialyated apo C-III variant. In native (i.e., untreated with neuraminidase) serum, apo VLDL, and apo HDL this apo C-III$_0$ variant was detectable neither by IEF and subsequent immunoblotting (Fig. 1 *c*) nor by two-dimensional immunoelectrophoresis (Fig. 2).

After IEF of apo VLDL and subsequent protein staining as well as by IEF of whole plasma and subsequent immunoblotting, the relative concentration of the variant apo C-III isoforms appeared to be markedly lower than that of normal apo C-III. This observation was confirmed both by two-dimensional immunoelectrophoresis of apo VLDL (Fig. 2) and densitometry of apo VLDL and apo HDL subsequent to IEF (Table I). In VLDL and HDL, the ratios of normal apo C-III$_1$/normal apo C-III$_2$ and variant apo C-III$_1$/variant apo C-III$_2$, respectively, were identical in heterozygous variant carriers. These ratios were similar to those in unaffected family members and PROCAM controls (not shown). However, the concentrations of the normal apo C-III isoforms were increased compared with the respective variant apo C-III isoproteins, namely by

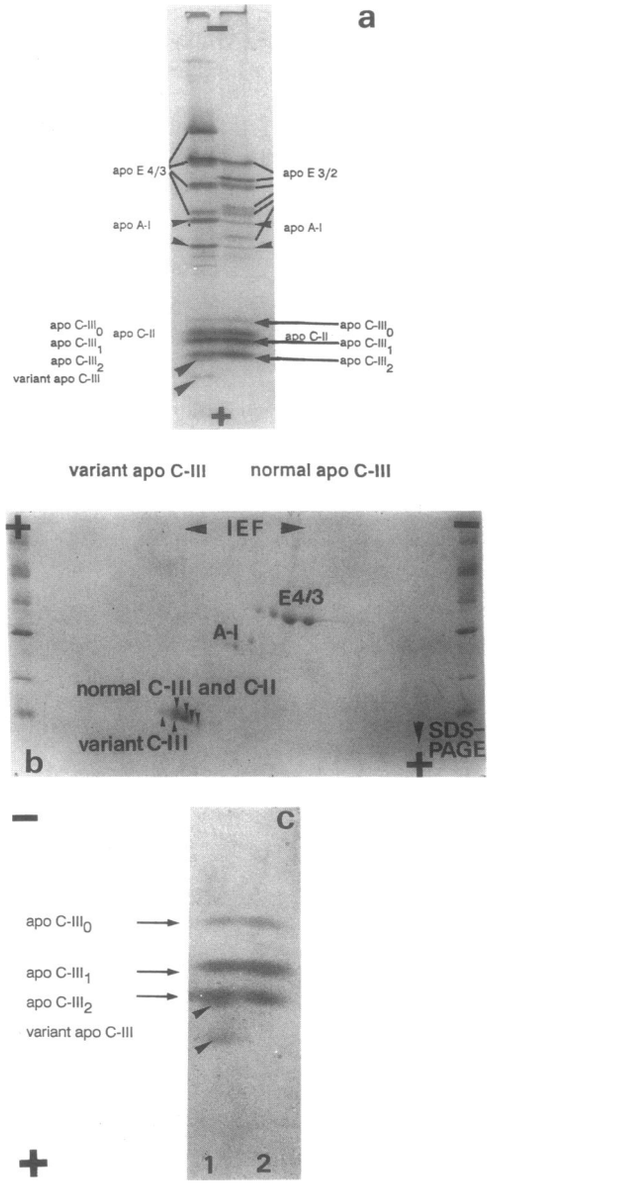

Figure 1. Demonstration of apo C-III isoproteins in apo VLDL by (*a*) IEF and (*b*) two dimensional electrophoresis, and (*c*) in serum by IEF and subsequent immunoblotting. For a description of methods see the text. Variant and normal isoforms of apo C-III and isoproteins of apo A-I, apo E, and apo C-II are marked.

factors of 7.5 in VLDL and 4 in HDL (Table I). In desialyated apo C-III obtained by neuraminidase treatment the ratios of normal apo C-III/variant apo C-III were ~ 6.5 in VLDL and 4 in HDL. When density gradient ultracentrifugation was used, we observed that at densities > 1.15 g/ml variant apo C-III but no normal apo C-III was detectable (Fig. 3).

Lipid values and apolipoprotein concentrations. A family study showed that the mother of the propositus also presented the apo C-III variant. Lipid values and apolipoprotein concentrations of the family members are given in Tables II and III. Plasma concentrations of HDL cholesterol and apo A-I were substantially higher in variant carriers than in unaffected members. They ranged above the 95th percentiles of sex matched PROCAM controls, which in female PROCAM par-

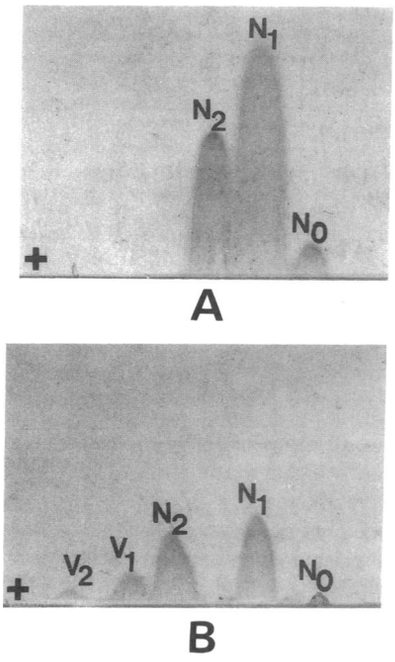

Figure 2. Two-dimensional immunoelectrophoresis of apo C-III isoproteins from a normal subject (*A*) and from P.Sch. a heterozygote for an apo C-III variant (*B*). IEF of apo VLDL was performed as in Fig. 1. For immunoelectrophoresis, the gel strips were transferred to a 1% agarose gel containing 8% rabbit anti-apo C-III antiserum. N_0, N_1, and N_2 denote the normal apo C-III isoforms, V_1 and V_2 the respective variant apo C-III isoforms.

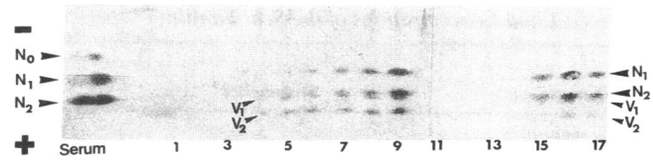

Figure 3. IEF and subsequent immunoblotting of apo C-III isoforms in density fractions of plasma from the heterozygous apo C-III variant carrier P.Sch. Fractions were obtained by density gradient ultracentrifugation. The numbers (1, 3, 5 . . . 17) refer to the fractions depicted in Fig. 4. *Serum* denotes the serum of a normal subject. N_0, N_1, and N_2 denote the normal apo C-III isoforms apo $C\text{-}III_0$, apo $C\text{-}III_1$, and apo $C\text{-}III_2$, V_1, and V_2 the respective variant apo C-III isoforms. Note that in fractions 8 and 9 and in fractions 15, 16, and 17 the concentration of the normal apo C-III exceeds that of variant apo C-III, whereas in fraction 4 the variant apo C-III, but not the normal apo C-III isoforms, were detectable.

ticipants are 83 mg/dl (HDL cholesterol) and 185 mg/dl (apo A-I), respectively (33). Triglyceride concentrations of the variant carriers were low normal compared with PROCAM controls and unaffected family members (Table III). The plasma concentrations of apo C-III were low in the variant carriers (6.65±0.78 mg/dl) compared with the unaffected sister of P.Sch. (13.4 mg/dl) as well as with 58 randomly selected women from the PROCAM study (10.2±1.9 mg/dl; $P < 0.02$, two-tailed t test). None of the variant carriers took drugs that increase plasma HDL cholesterol concentrations (estrogens, fibrates, β-hydroxy-β-methylglutaryl CoA reductase inhibitors, ion exchange resins).

Analysis of lipoproteins by density gradient ultracentrifugation and gradient gel electrophoresis. The distribution of lipids and apo A-I within the lipoprotein classes was analyzed after density gradient ultracentrifugation of plasma samples from the variant carriers and the two unaffected family members (Fig. 4). Marked differences were found in the HDL density range. In the two unaffected family members, most of the HDL lipids (i.e., 62±4% total cholesterol, 63±4% free cholesterol, 66±5% esterified cholesterol, 57±3% triglycerides, 58±5% phosphatidylcholine, and 73±5% sphingomyeline) and 70±4% of apo A-I were found in the density class of HDL_3 (> 1.120 g/ml). In contrast, in the variant carriers only 41±3% total cholesterol, 36±3% free cholesterol, 42±4% esterified cholesterol, 30±3% triglycerides, 45±3% phosphatidylcholine, and 30±4% sphingomyeline and 60±3% of apo A-I were found in HDL_3. Compared with unaffected family members the two variant carriers exhibited relatively high concentrations of apo E in both the HDL_2 and the HDL_3 density ranges. This atypical distribution of lipids and apo A-I in HDL was accompanied by an atypical particle size distribution. As analyzed by polyacrylamide gradient gel electrophoresis of fractions from density gradient ultracentrifugation (Fig. 4) and by densitometry of HDL particles separated by ultracentrifugation and subsequent gradient gel electrophoresis (Fig. 5), HDL from the variant carriers mainly consisted of HDL_{2b}. In contrast, control preparations contained mostly HDL_{3a}. In addition, the apo C-III variant carriers exhibited an unusually large HDL subclass with a Stokes diameter of 13–18 nm (Fig. 5) that corresponds to that described for HDL_c (47).

Table I. Relative Concentrations of Normal and Mutant Apo C-III Isoproteins in Heterozygous Apo C-III Variant Carriers

	VLDL		HDL	
	P	M	P	M
Normal/variant apo $C\text{-}III_1$	7.6±1.5	7.8±1.6	3.8±1.4	4.0±0.6
Normal/variant apo $C\text{-}III_2$	7.4±1.4	7.5±1.4	4.1±1.0	4.1±0.8
Normal/variant apo $C\text{-}III_{neuraminidase}$	6.5±0.9	6.5±1.2	4.0±1.0	4.0±1.3

The ratios of normal and variant apo C-III isoforms were obtained after integration of densitograms from apo C-III separated by IEF of samples from the proposita (P) and her mother (M) who are both heterozygous for the apo C-III variant. Means±SD; $n = 3$.

Structural analysis of the apo C-III variant. For the structural analysis of the defect underlying the mutant apo C-III, the most anodic band of the variant apo C-III was isolated and digested with trypsin. The resulting chromatogram was grossly identical to that of normal apo $C\text{-}III_2$, but the retention time of the fraction normally containing T6 was slightly prolonged (peptide T* in Fig. 6). TOF-SIMS analysis of this abnormally migrating peptide revealed a molecular mass of 1,141 D, while the normal peptide T6 consisted of a molecular mass of 898 D. The only possible single nucleotide substitution in APOC3 that can explain both the molecular mass of the T* peptide and the altered pI of the variant protein is $Lys_{58} \rightarrow Glu$. Sequence analysis revealed a sequence Asp-Tyr-Trp-Ser-Thr-Val-Glu-Asp-Lys (residues 52 → 60) instead of a normal Asp-Tyr-Trp-Ser-Thr-Val-Lys (residues 52 → 58), thus verifying the predicted amino acid substitution $Lys_{58} \rightarrow Glu$.

Analysis of family members for the presence of the $Lys_{58} \rightarrow Glu$ mutation. To verify the $Lys_{58} \rightarrow Glu$ mutation in the proposita and her mother and to exclude its presence in the other two family members, we directly sequenced exon 4 of the apo

Table II. Lipid Concentrations of the Sch. Family

Subjects	Triglycerides	Total cholesterol	HDL cholesterol	LDL cholesterol
(age, mutant)			*mg/dl*	
Father (53, −)	169	225	53 (>75)	138
Mother (50, +)	60	263	103 (>99)	148
Proposita (27, +)	106.5±3.2	273.0±20.8	112.5±5.2 (>99)	139.3±23.6
Sister (24, −)	162	169	48 (>29)	89

Values in parentheses give percentiles for HDL cholesterol from sex matched PROCAM controls (33). The data presented for the proposita represent average values of four analyses performed over a period of 2 yr.

C-III gene subsequent to PCR amplification. This method provides information for both alleles simultaneously. Heterozygosity for an A to G exchange in the first nucleotide of codon 58, which results in a substitution of lysine with glutamic acid, was found in the samples of the proposita and her mother but not in those of her father and sister (Fig. 7). No further deviations from the wild type sequence of apo C-III were observed.

Discussion

Hyperalphalipoproteinemia can be monogenic as well as multifactorial and polygenic in origin. Only little is known on the genetic basis of familial hyperalphalipoproteinemia: Deficiencies of cholesterol ester transfer protein in three Japanese families (53–55) and of hepatic triglyceride lipase in two families of Swedish and Japanese descent (56, 57) have been identified as causes of extremely elevated HDL cholesterol plasma concentrations. In another study, Menzel et al. (58) found that the apo A-IV polymorphism modulates the HDL cholesterol plasma concentration. The less frequent apo A-IV isoform, which differs from the more frequent by a $Gln_{360} \rightarrow$ His exchange (59), was found associated with higher serum levels of HDL cholesterol. In this report, we describe an apo C-III variant that resulted from the replacement of a lysine in position 58 by a glutamic acid residue. This variant was identified in two women with plasma concentrations of HDL cholesterol and apo A-I that were significantly higher than in unaffected family members. In fact, they were above the 95th percentiles of sex matched controls (33). The presence of secondary factors that increase HDL cholesterol plasma concentration (alcohol consumption, treatment with estrogens, fibrates, nicotinic acid, β-hydroxy-β-methylglutaryl CoA reductase inhibitors, or ion exchange resins) was ruled out. Since the family was too small,

Table III. Apolipoprotein Concentrations in the Sch. Family

No.	Apo A-I	Apo A-II	Apo B	Apo C-III
		mg/dl		
Father	153 (>71)	43	94	10.0 (>60)
Mother	195 (>96)	38	85	6.1 (<5)
Proposita	247.3±15.3 (>99)	53.5±5.4	114.5±21.2	7.2 (<5)
Sister	142 (>50)	40	64	13.4 (>85)

Values in parentheses give percentiles of apo A-I and apo C-III concentrations from sex matched PROCAM controls (33). Samples of the proposita were analyzed on four occasions and the data for apo A-I, apo A-II, and apo B represent mean values. Apo C-III concentrations determined in 61 men and 58 women participating in the PROCAM study were 8.9±2.3 mg/dl (median: 8.5 mg/dl) and 10.2±1.9 mg/dl (median: 10.1 mg/dl), respectively. The mean apo C-III concentration of the two heterozygous variant carriers (6.65±0.78 mg/dl) is significantly lower than that of women from the PROCAM study ($P < 0.02$, t test).

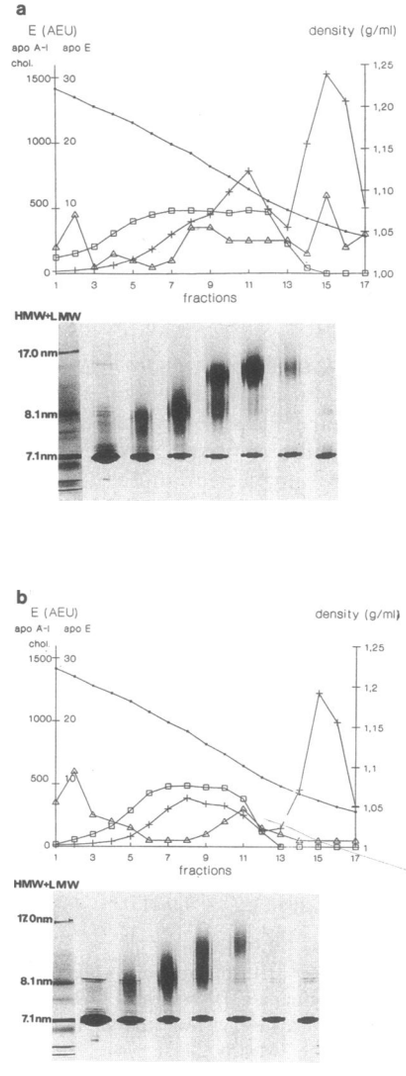

Figure 4. Density gradient ultracentrifugation profiles and polyacrylamide gradient gel electrophoresis patterns of plasma from P.Sch. heterozygous for the apo C-III variant (*a*) and from her unaffected sister D.Sch. (*b*). Most of the heterozygous proband's HDL cholesterol (+) and apo A-I (□) were present in a density range < 1.120 g/ml whereas most of her normal sister's HDL lipids occurred in a density range > 1.120 g/ml. Similar observations were made for free cholesterol, esterified cholesterol, triglycerides, phosphatidylcholine, and sphingomyeline (not shown). All HDL fractions of the apo C-III variant carrier were enriched in apo E (△) compared to the unaffected subject. By nondenaturing gradient gel electrophoresis (lower parts of the figures), particles of the HDL_2 density range were found to be larger in the heterozygous variant carrier than those in her normal sister.

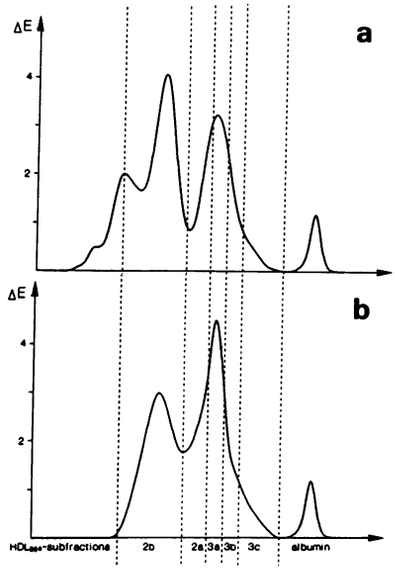

Figure 5. Densitometry of HDL from the heterozygote for apo C-III(Lys$_{58}$ → Glu) (*a*) and from her unaffected sister (*b*). In contrast to the normal sister, where HDL$_{3a}$ were predominating, in the heterozygous variant carrier, HDL$_{2b}$ were predominant and contained atypically large particles with a Stokes diameter of 13–18 nm that corresponds to that of HDL$_c$ (47).

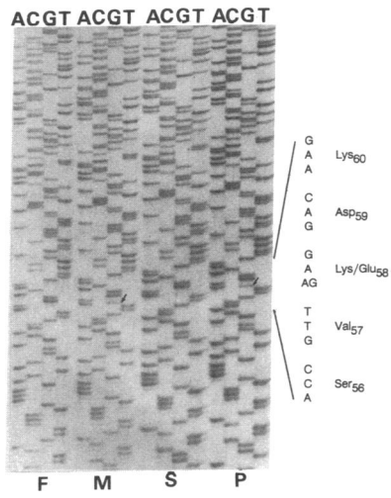

Figure 7. Demonstration of heterozygosity for the A to G exchange underlying apo C-III(Lys$_{58}$ → Glu) in affected family members. Exons 4 of the apo C-III genes of all family members were directly sequenced subsequent to PCR amplification (50, 51) so that sequence information for both alleles was obtained. *F*, father; *M*, mother; *S*, sister; and *P*, proposita. The heterozygously present mutant Gs are marked by arrows.

the association of the variant apo C-III and elevated HDL cholesterol levels could not be demonstrated unequivocally by genetic linkage analysis. However, some major biochemical differences between affected and unaffected family members on the one hand and control subjects from the PROCAM study on the other hand are in support of a causal relationship between the apo C-III mutation and hyperalphalipoproteinemia.

(*a*) In addition to high levels of HDL cholesterol and apo A-I, apo C-III(Lys$_{58}$ → Glu) heterozygotes exhibited low plasma concentrations of apo C-III when compared with unaffected family members or PROCAM controls. This was due to the diminished concentration of variant relative to normal apo C-III. The concentration of the variant apo C-III isoproteins was especially decreased in VLDL and amounted to only 15% of the respective normal isoproteins (Fig. 2 and Table I). Whether this diminished concentration of the mutant apo C-III is a consequence of decreased synthesis, of alterations in lipoprotein assembly, or of enhanced catabolism cannot be discerned at present. However, apo C-III is a major protein component of triglyceride-rich lipoproteins as well as the putative inhibitor of their lipolysis (16–19) and of their hepatic uptake (20–22). Therefore, it is conceivable that the large reduction in the content of apo C-III in VLDL causes enhanced lipolysis and/or hepatic clearance and, secondarily, increases supply of surface remnants from chylomicrons/VLDL to HDL (60, 61). Thus, the diminished concentration of apo C-III may account for both hyperalphalipoproteinemia and the relatively low levels of triglycerides in apo C-III(Lys$_{58}$ → Glu) heterozygotes. This assumption is further supported by two other previous findings. (*i*) In contrast to apo A-I deficient patients (62), both apo A-I/C-III- and apo A-I/C-III/A-IV deficient patients exhibit low plasma concentrations of triglycerides (29, 30). This has been attributed to the absence of apo C-III as an inhibitor of lipoprotein lipase (19). (*ii*) The triglyceride plasma concentration lowering drug MEDICA 16 was shown to exert its effect in rats by enhancing the plasma clearance of chylomicrons. The observed increased fractional catabolic rate results from enhanced liver uptake of chylomicrons. This has been attributed to the 10-fold decrease in the apo C-III content of chylomicrons in MEDICA 16-treated animals (63).

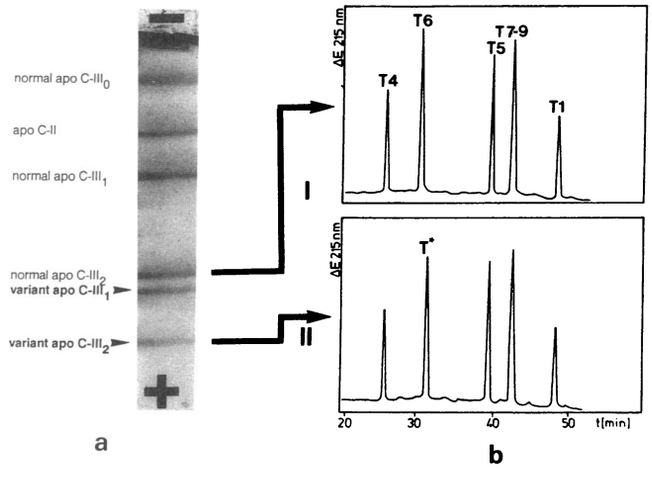

Figure 6. Demonstration and structural analysis of the apo C-III variant. (*a*) Gel strip with an immobilized pH-gradient pH 3.75–5.25 demonstrating apo C-III from apo VLDL of P.Sch. heterozygous for the apo C-III variant. (*b*) HPLC chromatograms of tryptic peptides obtained from normal apo C-III$_2$ (*I*) and from the variant apo C-III$_2$ (*II*). Digestion with trypsin in a protein/protease ratio of 40:1 for 24 h at 37°C. Proteolytic peptides were eluted with a gradient from 0 to 45% acetonitrile within 55 min. Retention times and peak areas were grossly identical in both chromatograms. The molecular masses of peptides T6 and T* as determined by TOF-SIMS were 898 D and 1,141 D, respectively.

(*b*) Alternatively, the structural defect in apo C-III(Lys$_{58}$ → Glu) rather than the diminished concentration of apo C-III might cause less inhibited clearance of triglyceride rich particles in the heterozygous apo C-III variant carriers. The structural requirements for the putative functions of apo C-III are, however, poorly understood. Further in vitro and in vivo studies are needed to distinguish between these two possibilities. It is interesting that the substituted amino residue in apo C-III(Lys$_{58}$ → Glu) is located in the thrombin fragment of apo C-III in which the protein's lipid binding properties reside (64). Furthermore, in a secondary structure model using the Chou-Fasmann algorithm (65, 66) residues 58 → 68 of normal and variant apo C-III form an amphipathic alpha-helix. The alpha

helical domain is retained in the mutant protein. However, the Edmundson wheel presentation of both normal and mutant alpha helices reveals that the substitution of residue Lys_{58} abolishes the only positive charge at the helix's hydrophilic site. Although apo C-III is little conserved inter species, the positive charge of amino acid 58 is present in the apo C-III sequences of man, rat and dog (67). Thus, there is reason to assume that Lys_{58} occupies a crucial position in apo C-III.

(*c*) Another observation in support of a causal relationship between apo C-III(Lys_{58} → Glu) and hyperalphalipoproteinemia is our finding that HDL particles in heterozygous individuals were unusual in size, in their apo E content and in the ratio of normal to variant apo C-III. Nondenaturing gradient gel electrophoresis was used to demonstrate that HDL of apo C-III(Lys_{58} → Glu) heterozygotes mainly consisted of HDL_{2b} and a subclass of large HDL particles with a Stokes diameter of 13–18 nm corresponding to the size of HDL_c (46) (Figs. 4 and 5). These particles were enriched in apo E (Fig. 4). The concentration of the mutant apo C-III was diminished relative to normal apo C-III not only in VLDL but also in HDL. However, with increasing density of HDL the concentration of the variant apo C-III increased relative to that of normal apo C-III and was still present at a density of > 1.15 g/ml where normal apo C-III was undetectable (Fig. 3). We cannot yet explain the metabolic origin of these unusual HDL subfractions in apo C-III(Lys_{58} → Glu) heterozygotes. However, their abundance seems to reflect the high plasma concentration of HDL cholesterol and apo A-I in affected individuals.

Apo C-III(Lys_{58} → Glu) is the third apo C-III mutant to be described. The two other variants differed from normal apo C-III by their degree of sialyation (14, 26–28). One was oversialyated (14), the other was not sialyated at all because of a Thr → Ala replacement at the glycosylation site (27, 28). Both of these apo C-III variants were not found to be associated with a dyslipoproteinemic phenotype. The observed association of apo C-III(Lys_{58} → Glu) with apo C-III deficiency and hyperalphalipoproteinemia, however, suggests that the apo C-III gene may affect lipoprotein metabolism profoundly.

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