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Research Article

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Apolipoprotein $CII_{St.\ Michael}$

Familial Apolipoprotein CII Deficiency Associated with Premature Vascular Disease

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Abstract

A 60-yr-old woman and her brother, products of a consanquinous mating, were chylomicronemic. The chylomicronemia in both subjects was found to be due to the absence of functional apoCII. A mutant form, designated $apoCII_{St.\ Michael}$ ($apoCII_S$), was identified by two-dimensional electrophoresis and Western blot using anti-apoCII antiserum. The isoelectric point of $apoCII_S$ was similar to that of normal apoCII, but its apparent molecular weight was 3,000 greater. Tryptic peptides of $apoCII_S$ were identified that had retention times in reverse-phase high pressure liquid chromatography and amino acid compositions indistinguishable from that of residues 1 to 48 and 51 to 55 of normal apoCII. The complete sequence of $apoCII_S$ was deduced from a combination of the sequence analysis of tryptic peptides corresponding to residues 56 through 96 and the known sequence of the apoCII gene. $ApoCII_S$ differed from apoCII at residue 70 where Gln^{70} was replaced by Pro^{70} and the sequence terminated with Pro^{96}. This is consistent with a base insertion in the codon for Asp^{69} or Gln^{70} in the apoCII gene and a subsequent translation reading frame shift. Both patients were homozygous for apoE-4. This and the absence of normal apoCII is consistent with homozygozity at the apoE–CII gene locus on chromosome 19. Both siblings and several relatives had premature ischemic vascular disease, in contrast with its apparent absence in other apoCII-deficient families.

Introduction

apoCII is known to be an activator of lipoprotein lipase (LPL),[1] the primary enzyme involved in the lipolysis of plasma triglyceride (TG) of chylomicrons and VLDL (1, 2). The discovery in this laboratory of a subject with fasting chylomicronemia caused by a deficiency of apoCII (3) established the physiological importance of the apolipoprotein as an activator of LPL. The deficiency was identified as an autosomal recessive trait (4). The phenotypic deficiency of functional apoCII has been reported in eight other families (5–10).

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1. *Abbreviations used in this paper:* $apoCII_S$, $apoCII_{St.\ Michael}$; $apoCII_T$, $apoCII_{Toronto}$; HL, hepatic lipase; LPL, lipoprotein lipase; PY, pyridylethylated; S_f, Svedberg units of flotation; TG, triglyceride.

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We previously reported that the 14 homozygotes and 23 obligate heterozygotes from the apoCII-deficient family described by this laboratory are characterized by the presence of a mutant, nonfunctional apoCII, $apoCII_{Toronto}$ ($apoCII_T$) (11). $ApoCII_T$ was shown to differ from normal apoCII in its amino acid sequence starting at residue 69 (12). Two other different apoCII-deficient families have been reported to have trace amounts of mutant forms of apoCII, $apoCII_{Bethesda}$ and $apoCII_{Padua}$, in their plasma (13). The precise structure of these mutants has not been described; however, their pI and migration in two-dimensional electrophoresis distinguish them from normal apoCII and $apoCII_T$. A variant of apoCII, $apoCII_2(Gln^{55})$, is present in 12% of individuals of African descent (14) and has been reported to activate bovine milk lipoprotein lipase in vitro as efficiently as normal apoCII (15).

We now report the discovery of a new family with apoCII deficiency characterized by the presence of a mutant form of apoCII that we have designated $apoCII_{St.\ Michael}$ ($apoCII_S$). It differs from normal apoCII at residue 70, where Gln^{70} is replaced by Pro^{70} and the sequence terminates with Pro^{96}. This is consistent with a base insertion in the apoCII gene and a subsequent translation reading frame shift. These subjects present with fasting hyperchylomicronemia and significant ischemic vascular disease, a finding that contrasts with the absence of premature ischemic vascular disease in other homozygotes for apoCII deficiency (16).

Methods

Clinical data

Proband. The proband, a 58-yr-old white Anglo-Saxon female, was referred to the Lipid Clinic at St. Michael's Hospital on December 4, 1984 with a ten-year history of lipemia. While on oral contraceptives she had two attacks of epigastric pain with markedly elevated serum amylase activity that was diagnosed as pancreatitis. She had six full-term uncomplicated, normal deliveries, including a pair of twins. However, tonsillectomy, tooth extractions, and hemorrhoidectomy were complicated by several days of bleeding. The menopause occurred at age 50. Her average adult weight was 68 kg. She did not use tobacco or alcohol. She took 1 g clofibrate twice daily until five weeks ago.

Her parents were first cousins once removed. Her father had three heart attacks in the sixth decade and died at 70 yr of age; the mother had non-insulin-dependent diabetes mellitus and died at 84; a maternal uncle died of a heart attack at age 60. The proband had seven siblings; one died of "internal bleeding" at 28 yr, one of a heart attack at 56, one of hypertension and a heart attack at 68, one was alive and well at 57, one had a heart attack at 53 and was alive at 60, one brother described below was alive at 61 with lipemia, recurrent acute pancreatitis, and diabetes mellitus, and one, age 62, had angina pectoris and hypertension. One son was reported to have elevated serum TG levels and six other children and eight grandchildren were alive and well.

Examination showed a healthy-appearing woman, 166 cm in height and weighing 67.6 kg. There was no corneal arcus or skin or

tendon xanthomata. The fundi were normal. Her pulse was 78 per min and blood pressure 140/80 mmHg. The heart, lungs, and peripheral pulses were normal except for a left carotid systolic bruit, absent dorsalis pedis, and diminished posterial tibial pulses bilaterally. There were no enlarged abdominal organs. The remainder of the examination was normal.

Ultrasound studies showed a 50–60% narrowing of the left internal carotid artery and a smaller plaque near the origin of the right carotid artery. Her electrocardiogram was normal. Radiograms showed calcification of the aortic arch compatible with atherosclerosis. Ultrasonography of the abdomen revealed a normal pancreas, liver, gall bladder, and bile ducts with no stones.

Routine laboratory tests showed normal values for urinary protein and sugar, for blood glucose, fasting and 2 h after 75 g of glucose per os, for serum uric acid, electrolytes, glutamic oxaloacetic transaminase activity, creatinine, urea, bilirubin, calcium, phosphate, amylase, tests of thyroid function, albumin, and globulin. Mild normochromic, normocytic anemia was evident. The hemoglobin was 11.8/ml, the red cell count was 4.16×10^6/ml, platelet count was 192×10^3/ml, and white blood cell count 3.9×10^3/ml. The blood smear showed normal red blood cells except for a few microcytes, polychromatic cells, and target cells. The peripheral blood had a reticulocyte count of 13%, neutrophils 81%, lymphocytes 17%, and eosinophils 2%. The following blood studies were normal: red blood cell osmotic fragility test, serum haptoglobin, prothrombin time, activated partial thromboplastin time, fibrinogen titers, euglobulin lysis, bleeding time, and Factor XIII.

The fasting plasma after standing overnight at 4°C had diffuse lipemia throughout and a chylomicron layer on top. The total cholesterol concentration was 190 mg/dl and TG was 1,325 mg/dl. She was instructed in an American Heart Association phase III diet that allowed 1,200 kcal/d, 20% of energy as fat, < 150 mg of cholesterol and a ratio of polyunsaturated to saturated fatty acids of 1:1, and she was permitted to take 1 g of clofibrate twice daily. During follow up, every few months for the next two years, the plasma lipid concentrations for cholesterol have ranged from 132 to 360 mg/dl and for TG from 960 to 3,220 mg/dl, and there has been no recurrence of pancreatitis.

Brother. The 62-yr-old brother of the proband was examined on May 21, 1985. Elevated serum lipids were detected during a routine examination in 1970. In 1971 acute abdominal pain resulted in a laparotomy and diagnosis of pancreatitis, type V hyperlipoproteinemia, chemical diabetes, and slight splenomegaly. He was treated with a low fat diet and briefly with clofibrate and then with *d*-thyroxine. In 1974 the serum TG was 6,400 mg/dl. After several drinks of alcohol in 1975, he had acute pancreatitis with markedly elevated serum amylase activity and a TG of 776 mg/dl. Clofibrate and a still lower fat content in the diet was substituted for *d*-thyroxine. Despite his abstinence from alcohol since 1975 a third attack occurred in 1977 when his serum cholesterol was 569 mg/dl and TG was 5,880 mg/dl. This led to the discovery of cholelithiasis and cholecystectomy. The serum TG fell to 206 mg/dl on intravenous glucose and saline but as an outpatient it was 3,590 mg/dl. The onset in 1979 of angina pectoris and ischemic electrocardiographic changes after exercise led to the use of a beta blocker, a still lower fat diet, the later addition of gemfibrozil, and then diet plus lorelco, 500 mg twice daily, that continues to the present time. In 1980, pancytopenia was noted with hemoglobin levels down to 7 g/dl, a white blood cell count of 3.2×10^3/ml, platelet count of 60×10^3/ml, a low serum iron concentration of 9 nM/liter, hypochromia, microcytosis, and occult blood in the stool. The only gastrointestinal tract abnormality was severe gastritis that was seen on gastroscopy. The anemia responded to transfusions, iron therapy, and treatment of the gastritis. There was no evidence of hemolysis. Red cell survival studies were normal. The marrow had no iron stores, but was infiltrated with lipid-filled macrophages that decreased in number with lower fat diets and lower serum lipid values. The liver was normal on palpation, but the spleen was felt 4 cm below the costal margin. Diabetes mellitus, discovered in 1980, was treated with oral medication and later with lente insulin up to 30 U daily. Coronary angiography showed severe proximal right coronary artery stenosis. Hospital records of his earlier illnesses included appendectomy at age 7, malaria, amebic dysentery and hepatitis at age 21, pneumonia at age 29, and hernial repair at age 42, with no report of lipemia or blood lipid values.

Physical examination showed a man of stated age, 180 cm tall, and weighing 76 kg. The fundi were normal and there was no corneal arcus. There were no skin or tendon xanthomata. His pulse was 80 per min and blood pressure 90/70 mmHg. The lungs and heart were normal except for a grade 2/6 apical and basal systolic murmur. The peripheral arteries were normal except for a bruit of the right carotid. The liver was smooth and firmer than normal, 7 cm below the ninth costal cartilage, and a firm spleen extended 8 cm below the left costal margin. Follow up by his personal physician, since the time of this examination, showed the development of a caput medusa and venous bruit from dilated veins radiating from the umbilicus.

Routine laboratory tests showed a serum cholesterol of 87 mg/dl, TG of 788 mg/dl, a fasting blood glucose of 164 g/dl, a serum glutamic oxaloacetic transaminase of 49 U/liter (normal, 4–28 U/liter), alkaline phosphatase of 106 U/liter (normal, 25–96 U/liter), globulin 45 g/liter (normal, 15–30 g/liter), and normal values for albumin, creatinine, bilirubin, calcium, phosphate, amylase, uric acid, and thyroid indices. The overnight standing plasma had diffuse lipemia and a chylomicron layer on top. The urine had negative tests for protein and sugar. The blood hemoglobin was 12.6 g/dl, red cell count 4.38×10^6/ml, white blood count 2.8×10^3/ml, platelet count 58×10^3/ml, the erythrocyte sedimentation rate was 52 mm/h, and the peripheral blood had a reticulocyte count of 6%, neutrophils 75%, lymphocytes 21%, monocytes 2%, eosinophils 1%, and basophils 1%. Red cell agglutination and white blood cells that were not intact were noted in the blood film.

The procedures followed in these studies were in accordance with the ethical standards of the University of Toronto Committee on Human Experimentation.

Plasma lipids and lipoproteins

The plasma lipid and lipoprotein profile was determined after the Lipid Research Clinic's protocol (17). Blood samples were obtained from subjects after a 12–16-h fast and collected into tubes containing Na_2-EDTA. The plasma was centrifuged using a Beckman 50.3 rotor in a L8-80 ultracentrifuge at 20,000 rpm for 30 min to isolate the chylomicron fraction. The remaining plasma was centrifuged at 45,000 rpm for 18 h to isolate the VLDL. The cholesterol and TG of the LDL and HDL was determined after precipitation of the LDL with heparin and manganese chloride. The total cholesterol and TG of these fractions were measured using the Technicon AA-II method, which was standardized with the assistance of the Lipid Standardization Laboratory of the Centers for Disease Control (Atlanta, GA).

The plasma lipoproteins were also fractionated by sequential ultracentrifugation as follows: chylomicrons, 20,000 rpm for 30 min; VLDL, 45,000 rpm for 18 h; LDL, isolated from plasma, adjusted to a density of 1.063 g/ml, by centrifugation at 45,000 rpm for 18 h; and HDL, isolated from the remaining plasma after adjusting the density to 1.21 g/ml and centrifugation at 45,000 for 48 h (18). The chylomicrons and VLDL were washed one time by centrifugation under the conditions of isolation. The cholesterol, phospholipid, cholesteryl ester, and TG of each fraction was quantitated by temperature-programmed gas chromatography (19). The protein content of each fraction was determined using a modification of the Lowry method (20). The stoichiometry of lipid molecules per lipoprotein particle was calculated using the equations of Shen et al. (21).

Electrophoretic analysis of the apolipoproteins of chylomicrons and VLDL

The isolated chylomicrons and VLDL were dialyzed against 0.01% Na_2-EDTA, pH 7.4, aliquoted, lyophilized, and delipidated with ethanol/diethyl ether (3:1) (22). The apolipoproteins were separated by isoelectric focusing using pH 4 to 6 ampholines (LKB, Sweden) as previously described (11). Aliquots of 50 μg were electrophoresed on 3-mm diam gels and separated in a second dimension of sodium dodecyl sulfate–glycerol gel electrophoresis (23). The gels were either

stained with Coomassie Blue G-250 or the proteins were transferred to nitrocellulose filters and immunoblotted with rabbit antihuman apoCII as previously described (11, 24).

The apolipoproteins were tested for the presence of cysteine residues by alkylation with iodoacetate or by pyridylethylation with 4-vinyl pyridine. Alkylation of cysteine residues with sodium iodoacetate introduces a negative charge (25), while pyridylethylation introduces a positive charge. 50–100 μg of chylomicron apolipoprotein or VLDL apolipoprotein was dissolved in 100 μl of 0.1 M NH_4HCO_3, pH 8.0/0.2% decylsodium sulfate/20 mM DTT, and incubated at 37°C for 30 min. Sodium iodoacetamide or sodium iodoacetate (0.5 M, pH 8.0) was then added to a final concentration of 0.1 M and the samples were incubated at room temperature for an additional 30 min. Alternatively, 4-vinylpyridine was added to a final concentration of 40 mM (26). The samples were then evaluated by analytical isoelectric focusing.

Purification of apoCII$_S$

Chylomicronemic plasma (1 liter) was collected by plasmapheresis from an apoCII$_S$ homozygous patient. The chylomicrons and VLDL were isolated by ultracentrifugation of 500 ml of plasma in a zonal Ti 15 rotor as described previously (12). The isolated lipoproteins were dispersed in 0.15 M NaCl/0.01% Na_2-EDTA, reisolated by centrifugation in an 80 Ti rotor for 18 h at 45,000 rpm, and dialyzed against 0.01% Na_2-EDTA, pH 8.2. The lipoproteins were then lyophilized and delipidated with ethanol/diethylether (22).

The total apolipoprotein was treated with 4-vinylpyridine to pyridylethylate the cysteine residue of apoCII$_S$. About 20 mg of total protein was dissolved in 0.1 M NH_4HCO_3, pH 8.2/0.5% decylsodium sulfate/400 mM DTT, and incubated at 37°C for 30 min. 4-Vinylpyridine was added to a final concentration of 330 mM and the sample incubated at room temperature for 30 min.

Pyridyethyl-apoCII$_S$ (PY-apoCII$_S$) was purified by preparative flatbed isoelectric focusing using Ultradex granulated gel and pH 4 to 6 ampholines as previously described for apoCII$_T$ (12) with the following modifications introduced to minimize oxidation of apolipoproteins during electrophoresis. The gel solution contained 15 mM DTT, and after the gel slurry was poured into the Hoeffer Isobox apparatus, excess liquid was removed by capillary action using paper wicks. The apparatus was also modified to allow a N_2 atmosphere to be established over the gel during electrophoresis by making inlet and outlet holes in the plexiglass cover. Finally, light was excluded by covering the electrophoresis chamber with black plastic. The isolated PY-apoCII$_S$ was found to give a single band in analytical isoelectric focusing. Immunoblot showed that it reacted only with antiserum specific for apoCII and not with antiserum to apoCIII or apoAII.

PY-apoCII$_S$ (10-μg aliquot) was hydrolyzed with 6 N HCl at 110°C for 20 h. The amino acid composition was determined by using a Beckman 120C amino acid analyzer and standard elution conditions (27).

Tryptic digestion and isolation of peptides by reverse-phase HPLC

Approximately 100 μg of each of apoCII and PY-apoCII$_S$ was digested with trypsin (protein/trypsin; 50:1 wt/wt) by incubation in a buffer of 0.1 M NH_4HCO_3, pH 8.2/0.2% decylsodium sulfate for 18 h at 37°C. HPLC was performed as previously described (12) using a 15 × 0.46-cm Altex ODS column (particle size, 5 μm).

Each peptide was collected and the amino acid composition determined as described above. The amino acid sequence of selected peptides was determined in an Applied Biosystems 470A sequenator. The phenylthiohydantoin derivatives of the amino acids were identified by reverse-phase HPLC according to the manufacturers' specifications.

Assay of apoCII activation of bovine milk LPL

The ability of the patient's whole plasma to activate bovine milk LPL was assayed essentially as described by Kashyap et al. (28). [^{14}C]Triolein emulsified with Triton X-100 was used as the substrate and the release of FFA was determined after a 30-min incubation. The assay was linear from 0.1 to 1 ml of normal plasma. Activities were expressed as micromoles FFA × 10^{-3} released per milliliter of plasma per minute.

Assay of LPL and hepatic lipase (HL)

LPL and HL were released into the blood by the administration of a bolus of heparin (100 U/kg body wt). The postheparin plasma was collected one-half hour after injection of heparin and the activity of LPL and HL were determined using [^{14}C]triolein emulsified with Triton X-100 (29, 30) as a substrate. The total lipolytic activity was assayed in the presence of normal plasma which served as a source of apoCII. The activity of HL was determined after inhibition of LPL by preincubation with protamine sulfate. LPL activity was taken as the difference between the total lipolytic activity and the HL activity. The linearity of the assay was established for each subject. The activities are expressed as micromoles FFA released per milliliter plasma per hour.

Results

Lipoprotein analysis. The proband and her brother both presented with fasting chylomicronemia (Table I). Their initial total serum cholesterol values were 190 and 87 mg/dl and TG values were 1,325 and 788 mg/dl, respectively. The brother had consistently lower concentrations of chylomicrons, possibly due to prior treatment from his referring physician with diet and lorelco. The plasma LDL and HDL of both subjects were TG rich. The LDL and HDL cholesterol concentrations were significantly below the fifth percentile for subjects of the same age and sex (31). These results were similar to those that we have observed for homozygotes for apoCII$_T$ (1).

The average number of molecules per particle of the lipoproteins of the proband and her brother are presented in Table II. The chylomicrons from these subjects had a free cholesterol content that exceeded the amount of phospholipid. Since it has been estimated that a 1:1 mole ratio of free cholesterol to phospholipid is the maximum physically possible in the surface monolayer, this suggests that a significant percentage of the free cholesterol would be dissolved in the core of the parti-

Table I. Plasma and Lipoprotein Cholesterol and TG Concentrations of the apoCII-deficient Proband and Brother

	Proband	Brother*
	mg/dl	*mg/dl*
Plasma		
Chol	190	87
TG	1,325	788
Chylo		
Chol	79	52
TG	745	627
VLDL		
Chol	69	24
TG	393	127
LDL		
Chol	25	6
TG	51	21
HDL		
Chol	17	5
TG	40	13

Chol, Total cholesterol; Chylo, chylomicrons.
* The brother was taking probucol at time of analysis.

Table II. Molecules per Average Lipoprotein Particle in apoCII$_S$ Homozygotes

Sample	Diam	FC	PL	CE	TG
Proband					
Chylo	1,428	104,843	83,142	113,175	743,151
VLDL	422	3,889	5,314	2,405	11,519
LDL	337	4,151	3,036	2,196	4,076
HDL	153	55	239	41	91
Brother					
Chylo	1,989	183,485	166,759	223,729	2,179,239
VLDL	387	2,315	4,287	1,533	8,495
LDL	267	606	1,585	874	1,532
HDL	150	44	223	54	71

Diam, Calculated diameter in angstroms; FC, free cholesterol; PL, phospholipid; CE, cholesteryl ester; Chylo, chylomicrons.

cle or exist as separate domains in the surface as has been suggested by others (32, 33). The LDL fractions of the proband and her brother had significantly increased amounts of TG and relatively normal amounts of cholesteryl ester. Their HDL fraction also contained significantly increased amounts of TG and relatively more TG than cholesteryl ester.

Postheparin LPL and HL activity. Both patients were found to have heparin releasable LPL and HL (Table III). LPL activity of each patient was normal when assayed in the presence of plasma from a control subject with normal apoCII. The activities of HL were significantly below normal values, similar to a previous report on subjects homozygous for apoCII$_T$ (34).

ApoCII activation of bovine milk LPL. The plasma from each patient was tested as a source of activator of bovine milk LPL (Table III). The patient's plasma activated bovine milk LPL to 14% of the normal control value. The activities obtained for the apoCII$_S$ homozygotes were identical to those of patients homozygous for apoCII$_T$ (3, 12), consistent with the absence of functional apoCII.

Isoelectric focusing of apochylomicron and apoVLDL. The apolipoproteins of the chylomicrons and the VLDL were investigated by isoelectric focusing. All results shown were obtained using the apolipoproteins of the proband. Identical results were seen for the apolipoproteins of the brother. We noted that the isoelectric focusing pattern was sensitive to oxidation. The pattern in the absence of DTT (Fig. 1, DT−) was complex in the pH 4 to 5 range where the C apolipoproteins normally focus. This resolved to an apparently normal pattern when the sample was reduced with DTT (Fig. 1, DT+), giving a band with the pI of normal apoCII. However, the ratio of apoCII and apoCIII in the reduced sample was 0.15, as determined by densitometry of the Coomassie Blue–stained gels, which is about one-half the normal ratio of 0.25–0.30 (4).

The unusual apoprotein bands were positively identified as apoCII by immunoblot with a rabbit antiserum to human apoCII (Fig. 1 *B*). At least six different bands could be identified by immunoblot analysis of the nonreduced sample (DT−), with two major bands in the region near normal apoCII. Two isoforms of apoCII were identified by immunoblot of the reduced apolipoproteins (DT+). The major form had a pI indistinguishable from that of normal apoCII. The minor isoform was more acidic than the major isoform. These results suggested that the apoCII of these patients was a mutant that contained a cysteine residue, in contrast to normal apoCII, which does not contain cysteine. To demonstrate the presence of cysteine, the reduced apolipoproteins were carboxyamidomethylated with iodoacetamide, carboxymethylated with iodoacetic acid, which introduces one negative charge per cysteine residue, or pyridylethylated with 4-vinylpyridine, which introduces one positive charge per cysteine residue. The results are shown in Fig. 1. Treatment with either iodoacetate or 4-vinylpyridine resulted in the disappearance of apoCII from its normal position and, as identified by immunoblot, resulted in a charge shift in apoCII that was consistent with the presence of a single cysteine residue per mole.

The apoE of both patients had the pI of apoE-4, which is known to differ from apoE-3 and apoE-2 by the absence of cysteine (35). There was no charge shift of the apoE in these experiments (Fig. 1 *A*), confirming that these subjects were homozygous for apoE-4.

Two-dimensional electrophoresis of apoVLDL. The mutant apoCII was further characterized by two-dimensional electrophoresis with isoelectric focusing in the first dimension and sodium dodecyl sulfate–glycerol polyacrylamide gel electrophoresis in the second dimension. The patterns of reduced apoVLDL from a subject with normal apoCII and reduced

Table III. Lipolytic Activities and Plasma CII Activation of Bovine LPL

	Proband	Brother	Normal*
LPL‡	7.6	8.8	8–20
HL‡	2.9	2.6	5–11
Plasma CII§	5	5	35

* Normal ranges as in reference 29.
‡ Postheparin LPL and HL activities are expressed as micromoles FFA released per milliliter plasma per hour.
§ Plasma CII is the activation of bovine skim milk LPL by plasma. Activity is expressed as micromoles FFA released times 10^{-3}/ml plasma per minute.

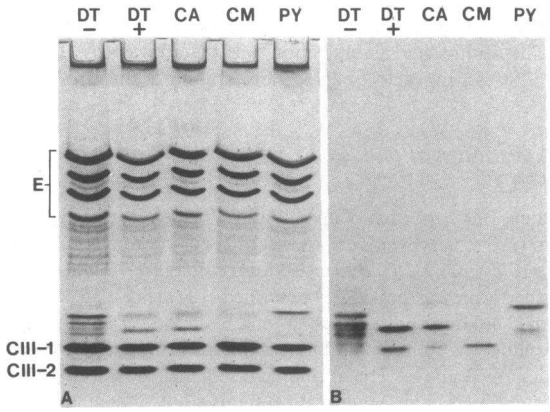

Figure 1. Isoelectric focusing of apoVLDL from the proband. (*A*) Coomassie Blue–stained gel. (*B*) Immunoblot with anti-apoCII of apoVLDL bound to a nitrocellulose membrane. CA, Carboxyamidomethylation; CM, carboxymethylation; PY, pyridylethylation; E, apoE isoforms; DT−, without DTT; DT+, with DTT; CIII-1, apoCIII-1; CIII-2, apoCIII-2.

apochylomicrons from the proband with nonfunctional apoCII are shown in Fig. 2. Normal apoCII (Fig. 2 *1*) can be identified as one major isoform, $apoCII_1$, with a molecular weight of 8,900 and a pI of 4.88, as a minor acidic isoform with an identical molecular weight and a pI of 4.74, and as another minor isoform, $apoCII_{1A}$, with a molecular weight estimated to be 7,840 and a pI of 4.82. $ApoCII_{1A}$ appears to correspond to $apoCII_{-1/2}$ as described by Fojo et al. (36). The pattern of the reduced apolipoproteins of the $apoCII_S$ proband (Fig. 2 *2*) showed that normal apoCII was absent and that all of the immunologically identifiable apoCII had a molecular weight estimated to be 12,300, with a major isoform with a pI of 4.88 and a minor isoform with a pI of 4.74. The presence of a major and a minor isoform may be analogous to the major and minor isoforms that we observed for normal apoCII and for $apoCII_T$ (11). The pI 4.74 isoform is most likely the result of a posttranslational modification of the pI 4.88 isoform.

Two-dimensional electrophoresis of a nonreduced sample is shown in Fig. 3. The mutant apoCII was difficult to detect by Coomassie Blue stain. The apoCII was positively identified by immunoblot. It was observed to have three different molecular weights that were estimated to be 12,400, 20,800, and 24,600. The 12,400-mol-wt form consisted of one major spot and one minor spot that corresponded to the forms seen for the reduced apolipoprotein. The 24,600-mol-wt form had one major spot detectable by Coomassie Blue stain and three spots detectable by immunoblot. The 20,800-mol-wt form also had one major spot detectable by Coomassie Blue stain and two spots detectable by immunoblot. These seven isoforms corresponded well with the isoforms observed by one-dimensional isoelectric focusing (Fig. 1 *A*, DT−). The dimerization of the two isoforms of the mutant apoCII would result in three isoforms of ~ 24,600-mol-wt. This corresponds to the higher molecular weight form. The 20,800-mol-wt forms are consistent with apoCII/apoAII dimers. ApoAII was identified by immunoblot with anti-apoAII antiserum (data not shown). We cannot determine from these experiments whether $apoCII_S$ exists in vivo as a monomer or a disulfide bridged dimer. It is possible that the formation of homodimers and heterodimers is an artifact of the isolation procedure.

Thus the mutant nonfunctional apoCII was distinguished from normal apoCII by the presence of cysteine and an apparently higher molecular weight. The absence of normal apoCII in these subjects is consistent with their being homozygous for a mutation in apoCII.

Amino acid composition and sequence analysis of $apoCII_S$ and its tryptic peptides. The amino acid composition of PY-$apoCII_S$ and normal apoCII are compared in Table IV with the amino acid composition derived for normal apoCII from the published amino acid sequence (37). There were numerous differences, including an increase in proline, glycine, alanine, isoleucine, phenylalanine, and arginine. The lower content of tyrosine in PY-$apoCII_S$ compared with normal apoCII was due to the loss of tyrosine during the acid hydrolysis as a result of the relatively small amounts of PY-$apoCII_S$ analyzed. A similar loss of tyrosine was observed in the amino acid analyses of the isolated peptides (Tables V and VI).

Tryptic peptides of PY-$apoCII_S$ and apoCII were separated by reverse-phase HPLC (Fig. 4). Six major peaks were identified in the profile of normal apoCII. Seven major peaks were identified in the profile of PY-$apoCII_S$. On the basis of retention time and amino acid composition (Table V), peaks P1′,

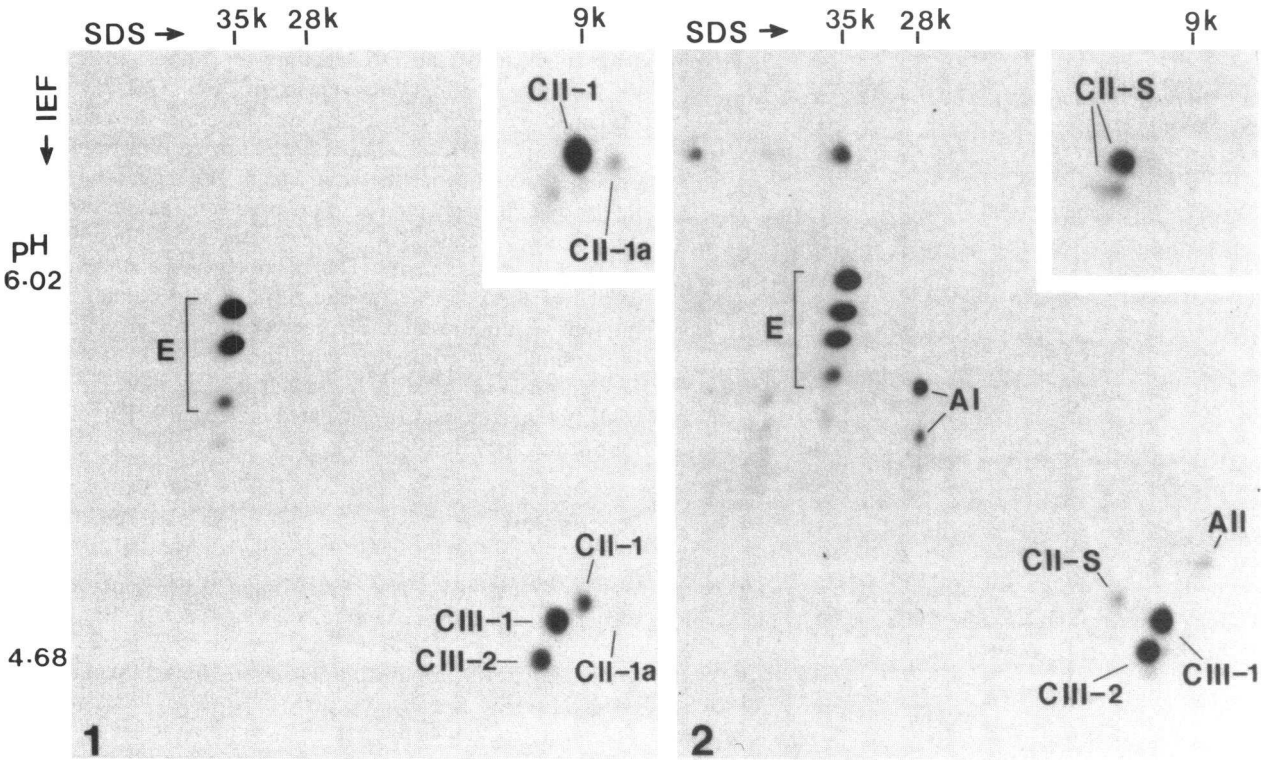

Figure 2. Two-dimensional electrophoresis of normal apoVLDL (*1*) and of apochylomicron from the proband (*2*). (*Insets*) Results of immunoblot with anti-apoCII. IEF, Isoelectric focusing.

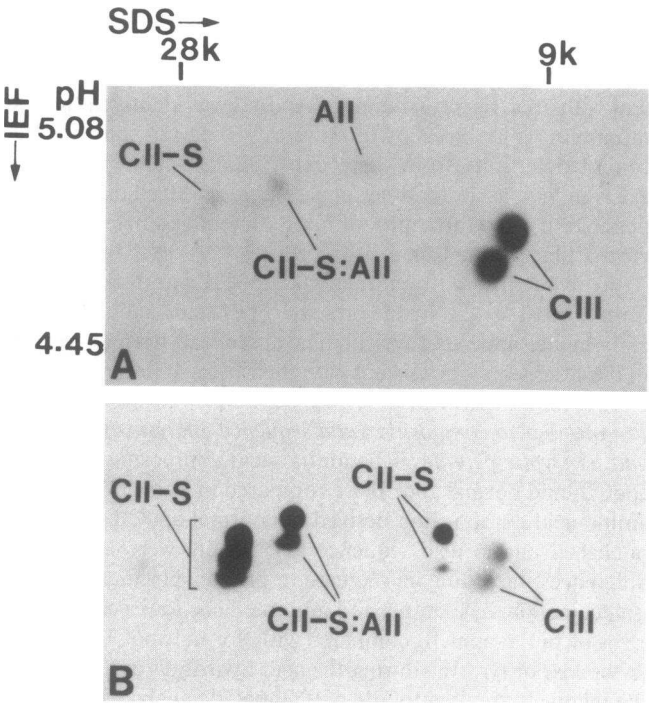

Figure 3. Two-dimensional electrophoresis of nonreduced apoVLDL from the proband. (*A*) Coomassie Blue–stained gel. (*B*) Immunoblot with anti-apoCII. The area shown represents the portion of the gel containing the C apoproteins. ApoAII was identified by immunoblot with anti–apoAII antiserum (data not shown).

P2′, P3′, P4′, and P5′ of PY-apoCII$_S$ were determined to be identical, within experimental error, to peaks P1, P2, P3, P4, and P5 of normal apoCII. These peaks correspond to residues 1 to 48 and 51 to 55 of normal apoCII. The dipeptide, Leu^{49}Arg^{50}, was not detected. The profile of PY-apoCII$_S$ did not contain a peak corresponding to P6 of normal apoCII. Two new peaks, identified as P3.1′ and P6′, were observed. Their amino acid composition (Table VI) did not correspond to any tryptic peptide predicted for normal apoCII. Peak P6′ was determined to contain pyridylethyl-cysteine using the relative absorbance at 254 and 226 nm (25). The unlabeled peak eluting between P2′ and P3′ of the chromatogram of PY-apoCII$_S$ tryptic peptides (Fig. 4) did not yield any amino acids upon analysis and was apparently not an authentic peptide. The peaks eluting later than P6′ appear in that part of the chromatogram that typically contains incompletely cleaved products of the tryptic digest of apoproteins.

Table IV. Amino Acid Analysis of apoCII and PY-apoCII$_S$

Amino acid	PY-CII$_S$	CII	CII*
Asx	5.9	6.6	6.4
Thr	9.7	12.1	11.5
Ser	10.9	11.0	11.5
Glx	15.1	18.6	17.9
Pro	9.2	5.3	5.1
Gly	6.2	3.1	2.6
Ala	8.7	7.9	7.7
Cys	ND	ND	0
Val	3.4	4.8	5.1
Met	0.1	2.0	2.6
Ile	1.9	1.3	1.3
Leu	8.4	10.4	10.3
Tyr	3.9	6.2	6.4
Phe	3.3	2.5	2.6
Trp	ND	ND	1.3
His	0	0	0
Lys	6.1	7.0	7.7
Arg	3.0	1.2	1.3

ND, Not determined. Values presented as moles amino acid per 100 mol of total amino acids.
* From the sequence reported by Hospattanker et al. (37).

The amino acid sequences determined for the peptides in peaks P3.1′ and P6′ are shown in Table VII. The first residue of P6′ was identified as either Asp or Ser. The next 13 residues were identical with residues 57 to 69 of normal apoCII. However, the sequence differed starting at residue 70 of normal apoCII, where Gln^{70} was replaced by Pro^{70}. The residue at cycle 17 was tentatively identified as Ser. No amino acid was identified at cycle 19. Gly at cycle 22 was the last residue that could be positively identified.

The sequence of P3.1′ did not correspond to any known sequence of normal apoCII. No residue was detected at cycle 3. The residues at cycles 5 and 7 could not be unambiguously identified. The Ser at cycle 14 was the last residue that could be detected.

By combining the results of the amino acid composition and sequence analysis of P6′, it was possible to conclude that this peptide consisted of 23 amino acids corresponding to residues 56 to 78 of the mutant protein. The presence of pyridylethyl-cysteine in this peptide was established by its ultraviolet absorption characteristics. Since P6′ was a peptide produced by trypsin, this implied that the single Arg residue (Table VI) would be the COOH-terminal amino acid. Thus, by elimination, the cysteine could be assigned to position 74.

Inspection of the DNA sequence of the apoCII gene (38–40) indicated that the sequence of P6′ and P3.1′ could be explained by the insertion of a nucleotide in the codon for Asp^{69} or Gln^{70}. The subsequent translation reading frame shift predicted the sequence shown in Fig. 5. The sequence of tryptic peptide P6′ was identical to the predicted sequence of residues 56 to 78. Peptide P3.1′ corresponded to residues 79 to 96 of the predicted sequence. This peptide was the combination of two of the predicted tryptic peptides and contained one internal arginine residue. The lack of proteolytic cleavage at this residue was consistent with the resistance of Arg-Pro bonds to trypsin. ApoCII$_S$ had a calculated molecular weight of 10,524, in contrast to a calculated molecular weight of 8,915 for normal apoCII.

Discussion

We have identified a new type of apoCII deficiency on the basis of the absence of structurally normal apoCII, the absence of functional apoCII, and the presence of a new, mutant form of apoCII that we propose to call apoCII$_S$. This mutant apoCII showed considerable heterogeneity in isoelectric focusing gels when analyzed under nonreducing conditions. The heterogeneity was due to the presence of a cysteine residue that contrasted with the absence of cysteine in normal apoCII. Determination of the primary structure showed that the mutation

Table V. Amino Acid Composition of Peptides P1', P2', P3', P4', and P5' PY-apoCII$_S$

Amino acid	P1'	P1*	P2'	P2	P3'	P3	P4'	P4	P5'	P5
Asx	21.8	20	9	11	11.6	11	1.2	0	7.1	5.3
Thr	0.0	0	10	11	10.8	11	0	0	16.6	15.8
Ser	21.6	20	2	0	1.6	0	36.4	36.4	4.9	5.3
Glx	4.2	0	23	22	12.0	11	21.5	18.2	32.0	31.6
Pro	0.0	0	0	0	11.4	11	0	0	17.9	15.8
Gly	5.2	0	3	0	2.0	0	2.4	0	0.6	0
Ala	0.0	0	22	22	11.8	11	11.7	9.1	0.4	0
Cys	0.0	0	0	0	0	0	0	0	0	0
Val	0.0	0	1	0	11.2	11	0	0	5	5.3
Met	0.0	0	0	0	0	0	0	0	0	5.3
Ile	0.0	0	1	0	0	0	0.6	0	0	0
Leu	21.6	20	12	11	12.0	11	10.9	9.1	5.5	5.3
Tyr	2.5	20	1	11	1.5	11	3.4	9.1	0	0
Phe	0.0	0	1	0	0	0	0	0	4.8	5.3
Trp	ND	0	ND	0	ND	0	ND	0	ND	0
His	0.0	0	1	0	0	0	0	0	0	0
Lys	22.9	20	13	11	11.7	11	10.3	9.1	5.1	5.3
Arg	0.0	0	1	0	2.4	0	1.35	0	0	0

* The composition of the corresponding sequences of normal apoCII: P1, residues 51 to 55; P2, residues 31 to 39; P3, residues 40 to 48; P4, residues 20 to 30; and P5, residues 1 to 19. Values presented as moles amino acid per moles of total amino acids.

was consistent with a translation reading frame shift beginning at residue 70. ApoCII$_S$ terminated after 96 residues due to the presence of a new stop codon at this point. Thus, it differed from normal apoCII starting at residue 70 and contained 17 additional amino acids. The molecular weight of apoCII$_S$ as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis differed from the calculated molecular weight by ~ 1,900. This is within the accuracy of this method for proteins with molecular weights < 12,000 (41).

The concentration and composition of the lipoproteins in

Table VI. Amino Acid Composition of Peptides P3.1' and P6' of PY-apoCII$_S$

Amino acid	P3.1'	P6'	P6*
Asx	0.6	5.3 (1)‡	4.8 (1)
Thr	5.2 (1)	18.3 (4)	19.0 (4)
Ser	8.8 (2)	16.2 (4)	14.3 (3)
Glx	12.8 (2)	5.8 (1)	4.8 (1)
Pro	23.4 (4)	5.7 (1)	0
Gly	15.7 (3)	10.4 (2)	4.8 (1)
Ala	5.7 (1)	14.6 (3)	9.5 (2)
Cys	0	0	0
Val	5.5 (1)	0	9.5 (2)
Met	0	1.2	4.8 (1)
Ile	5.1 (1)	4.9 (1)	4.8 (1)
Leu	11.2 (2)	0	9.5 (2)
Tyr	0	1.6	4.8 (1)
Phe	0.3	9.9 (2)	4.8 (1)
Trp	ND	ND	0
His	0.1	0	0
Lys	0.4	0	4.8 (1)
Arg	5.1 (1)	4.9 (1)	0

Values presented as moles amino acid per moles of total amino acids.
* P6, Residues 56 to 76 of normal apoCII.
‡ Values in parentheses represent the minimum number of residues per mole of peptide. ND, Not determined.

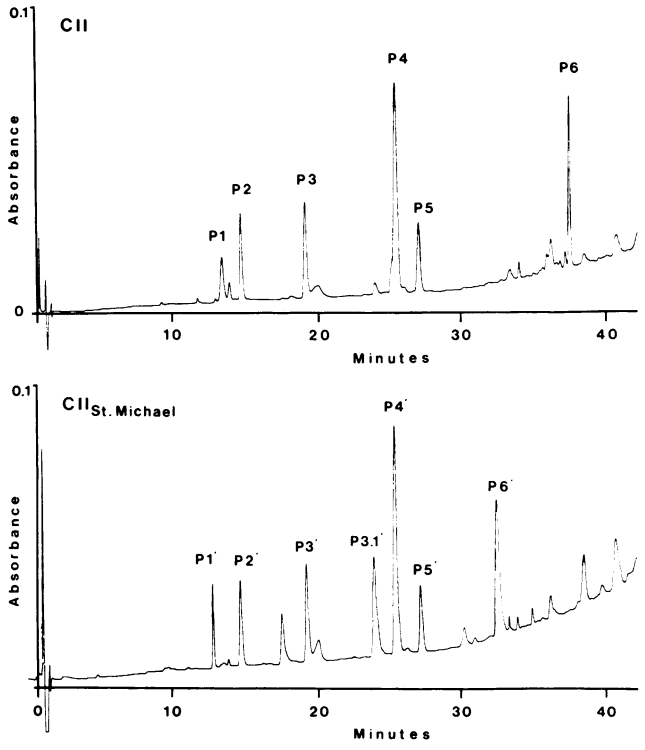

Figure 4. Elution profiles of tryptic peptides of apoCII (*top*) and PY-apoCII$_S$ (*bottom*) after separation by reverse-phase HPLC. The peptides were detected by their absorbance at 226 nm using an absorbance range of 0.1. Peak P6' was determined to contain pyridyethylcysteine by simultaneous monitoring of the absorbance at 254 nm (26).

Table VII. Amino Acid Sequence Analysis of Tryptic Peptides P3.1' and P6'

P3.1'		P6'	
Cycle	Residue	Cycle	Residue
1	Gly	1	Asp*
2	Val	2	Thr
3	ND	3	Ala
4	Ala	4	Ala
5	(Arg)‡	5	Met
6	Pro	6	Ser
7	(Pro)	7	Thr
8	Ile	8	Tyr
9	Ser	9	Thr
10	Gly	10	Gly
11	Gln	11	Ile
12	Gly	12	Phe
13	Glu	13	Thr
14	Ser	14	Asp
		15	Pro
		16	Ser
		17	(Ser)
		18	Phe
		19	ND
		20	Ala
		21	Glu
		22	Gly
		23	ND

* Asp was identified as the main residue and Ser was identified as a minor residue at cycle 1.
‡ Identification of residues in parentheses is tentative due to low yields at these cycles. ND, No residue detected.

the patients with apoCII$_S$ was consistent with those reported for other apoCII-deficient patients (16). The patients presented with fasting chylomicronemia and a variable elevation of VLDL. The concentrations of LDL and HDL were uniformly low. Analytical ultracentrifugation has been used to study the lipoproteins of several apoCII-deficient patients previously described by this laboratory (34). The Svedberg units of flotation (S_f) 20 to 400 fraction was present in the homozygotes in amounts four- to sixfold greater than normal. The S_f 0 to 20 fraction lipoproteins of the homozygotes had two maxima between S_f 0 to 12, at S_f 3 to 4, and at S_f 7 to 10, compared with a single maximum in the S_f 0 to 12 fraction lipoproteins at S_f 6 to 7 from a normal subject. A similar pattern was reported by Baggio et al. (42) in an independent kindred with apoCII deficiency. Miller et al. (7) reported the concentration of lipoproteins of S_f 0 to 12 and S_f 12 to 20 fractions as determined by analytical ultracentrifugation, but did not comment on the heterogeneity of these lipoproteins.

The lack of function of apoCII$_S$ may be due to the absence of residues 70 to 79 or it may be the result of the properties of the mutant portion of residues 70 to 96. The relationship between the structure and function of apoCII as an activator of LPL has been investigated through the use of peptide fragments of apoCII, synthetic peptides, and chemical modification of lysine or arginine residues (43–46). Three functional domains, a lipid binding domain (residues 1 to 52), a LPL activation domain (residues 56 to 67) and a LPL binding domain (residues 65 to 75) have been postulated (43). Ser^{61} in the LPL activation domain was thought to be essential for the function of apoCII; however, it has recently been shown that synthetic peptides with Gly^{61} function equally well in vitro (47). The LPL activation domain has been found to be essential for the function of apoCII in studies with artificial substrates (43). Residues 44 to 79, which contain a portion of the lipid binding domain and the complete LPL activation and LPL binding domains, has been found to have optimal activity with a broad spectrum of substrates (46). However, the region of apoCII required to activate LPL is in part a function of the substrate (46).

The lipid binding domain of apoCII$_S$ is intact, consistent with its association with chylomicrons and VLDL. The LPL activation domain is also intact. The mutation has occurred in the putative LPL binding domain of apoCII. It is interesting to note that the mutation which produced apoCII$_T$ occurred in nearly the identical position. Both mutant apoproteins contain new amino acid residues and a single cysteine residue. Further studies of the carboxyl-terminal residues of normal apoCII are needed in order to define the significance of residues 69 to 79.

The presence of premature ischemic vascular disease has not been described in other subjects with apoCII deficiency (15) and is not common in subjects with familial lipoprotein lipase deficiency (48). It is not possible at present to discern the cause for the ischemic vascular disease in the apoCII$_S$ patients

```
CII
56                                                    70                  76
Ser Thr Ala Ala Met Ser Thr Tyr Thr Gly Ile Phe Thr Asp Gln Val Leu Ser Val Leu Lys
AGC ACA GCA GCC ATG AGC ACT TAC ACA GGC ATT TTT ACT GAC CAA GTT CTT TCT GTG CTG AAG

CII_S
56                                                                            78
Ser Thr Ala Ala Met Ser Thr Tyr Thr Gly Ile Phe Thr Asp Pro Ser Ser Phe Cys Ala Glu Gly Arg
                                                      CCA AGT TCT TTC TGT GCT GAA GGG AGA

79                                                    96
Gly Val Thr Ala Arg Pro Pro Ile Ser Gly Gln Gly Glu Ser Pro Leu Leu Pro
GGA GTA ACA GCC AGA CCC CCC ATC AGT GGA CAA GGG GAG AGT CCC CTA CTC CCC TGA
```

Figure 5. The amino acid sequence of residues 56 to 76 of normal apoCII and the corresponding DNA sequence (38–40) and of residues 56 to 96 of apoCII$_S$ with the predicted DNA sequence of the gene for apoCII$_S$.

and family. They are unusual in two respects, firstly, due to the presence of a mutant apoCII in plasma, and secondly, because they are homozygous for apoE-4. Homozygozity for both of these apoproteins is consistent with homozygozity for the apoE–CII gene locus that has been established to be on chromosome 19 (49), and agrees with earlier findings from this laboratory (50) that the family members with $apoCII_T$ were always homozygous for apoE-3. It has been suggested on the basis of surveys of several populations that apoE-4 predisposes individuals to hyperlipidemia (51–53). Further investigation of the other relatives with $apoCII_S$ who unfortunately are remote from our laboratory, will be undertaken in order to reveal whether the incidence of premature vascular disease in this family is associated with the apoE–apoCII gene locus.

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