J Med Genet 1994;31:622–626

Apolipoprotein CII-Padova (Tyr^{37}→stop) as a cause of chylomicronaemia in an Italian kindred from Siculiana

Suat Tuzgöl, Saskia M Bijvoet, Taco Bruin, Johannes J P Kastelein, Michael R Hayden

Abstract

In this paper we report on the molecular defect underlying apolipoprotein CII (apoCII) deficiency in an Italian kindred. ApoCII serves as cofactor for lipoprotein lipase (LPL) in triglyceride hydrolysis of chylomicrons and very low density lipoproteins. Homozygous apoCII deficiency manifests with type I hyperlipoproteinaemia and is a rare disorder of lipoprotein metabolism. Until now, only 10 kindreds with apoCII deficiency have been published and all underlying mutations were unique.

The proband was the offspring of a consanguineous mating. Sequencing of cloned DNA from the proband presented in this report showed homozygosity for a C→A substitution at position 3002 in the apoCII gene, resulting in the introduction of a premature stop codon at residue 37 of the mature apoCII protein. Therefore, a truncated apoCII is synthesised, lacking the part of the apolipoprotein that activates LPL.

This mutation has previously been described in another Italian family and is known as $apoCII_{Padova}$. We propose that $apoCII_{Padova}$ is a frequent cause of apoCII deficiency in persons of Italian descent.

(J Med Genet 1994;31:622–626)

The hydrolysis of the triacylglycerol core of circulating chylomicrons and very low density lipoproteins (VLDL) requires the presence of the plasma enzyme lipoprotein lipase (LPL) and its cofactor apolipoprotein (apo) CII.[1]

LPL is synthesised in adipose, muscle, and several other tissues and is transported to the luminal surface of capillary endothelial cells where it can interact with circulating triacylglycerol rich lipoproteins.

ApoCII is an apolipoprotein, necessary for efficient LPL mediated triglyceride hydrolysis by binding, and thereby activating, LPL. Chylomicrons acquire apoCII from high density lipoproteins (HDL) upon entering the circulation.[2] This apolipoprotein is initially synthesised in the liver as a preproapoprotein of 101 amino acids length, and undergoes subsequent cotranslational cleavage of a 22 amino acid signal peptide. The major form of apoCII in plasma ($apoCII_0$) contains 79 amino acids and is not glycosylated. Sialic acid containing isoforms ($apoCII_1$ and CII_2) and a 73 amino acid protein, lacking the six amino-terminal residues, constitute the remainder of apoCII mass in plasma.[3]

Studies using either proteolytic fragments or synthetic peptides are suggestive of the presence of different functional domains in the mature protein. The N-terminal part of the protein seems to mediate lipoprotein binding, probably through amphipathic helix interaction with phospholipids. The C-terminal domain of apoCII has been suggested as the main site for LPL activation (residue 54 to 63) and for binding to LPL (residue 64 to 79).[4] Not all studies, however, support these conclusions.[5]

The gene encoding for apoCII maps to the long arm of chromosome 19 (19q13) and the complete nucleic acid sequence and genomic organisation have been elucidated.[6,7]

ApoCII deficiency is a rare hereditary disorder, inherited as an autosomal recessive trait. The clinical manifestations of apoCII deficiency are identical to those of LPL deficiency and include upper abdominal pain, eruptive xanthomatas, lipaemia retinalis, and recurrent pancreatitis. ApoCII deficiency was first described in 1978.[8] Since then 10 different genetic defects underlying apoCII deficiency in different parts of the world have been determined.[9]

Here we report the molecular basis of functional apoCII deficiency in an Italian kindred from Sicily. We show a C→A transition, resulting in the introduction of a stop codon for Tyr^{37}, which is identical to a previously reported mutation from another family with origins in a distant region. We suggest therefore that this mutation might be a frequent cause of apoCII deficiency in patients of Italian descent.

Material and methods

CASE 1

The index patient (II.5) is a 43 year old male of Sicilian origin. The patient was first seen when he was 24 years old at the Lipid Research Clinic at Shaugnessy Hospital (University of British Columbia) when he experienced his first episode of severe abdominal pain. On physical examination he had mild splenomegaly and laboratory tests showed hyperlipidaemia, while serum amylase was normal. Medication for hyperlipidaemia, including clofibrate and nicotinic acid, was not effective nor well tolerated. Between the ages of 24 and 34 the patient suffered from severe recurrent upper abdominal pain, two to three times per year.

No definite diagnosis was made at that time and an exploratory laparotomy was performed and an enlarged spleen was removed. There-

Centre for Vascular Medicine, Academic Medical Centre, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
S Tuzgöl
S M Bijvoet
T Bruin
J J P Kastelein

Department of Medical Genetics, University of British Columbia, 416–2125 East Mall, Vancouver, BC, V6T 1Z4, Canada
M R Hayden

Correspondence to
Dr Bijvoet.

Received 20 December 1993
Revised version accepted for publication 30 March 1994

after he continued to suffer from numerous episodes of acute pancreatitis and abdominal pain. Lipoprotein electrophoresis and lipoprotein analysis at that time showed a type V lipoprotein pattern with the following lipoprotein levels: total cholesterol 5·57 mmol/l, HDL cholesterol 0·49 mmol/l, triglycerides 36·18 mmol/l, apolipoprotein B_{100} 0·76 g/l, apolipoprotein AI 0·74 g/l. The apoE phenotype was E3/E3. When an optimal amount of normolipidaemic control plasma was used to activate the substrate emulsion, the patient showed LPL activity within the normal range. When the patient's plasma was used as the source of activator, no LPL activity was detected. This confirmed the presence of normal LPL and the absence of LPL activator in the plasma of the index patient. Hepatic lipase activity was normal. Isoelectric focusing of chylomicron/VLDL apoproteins showed absence of apoCII in the plasma of the index patient and intermediate levels in both parents.[10] The patient was put on a fat restricted diet (less than 20% of total calories provided by fat), and bouts of abdominal pain did not recur.

The index patient comes from a sibship of six. His parents, three of his sibs, and his ancestors live in the small village of Siculiana on the island of Sicily. His parents are first cousins and there has been little migration into or from this area over the past 150 years.

LIPOPROTEIN ANALYSIS

Lipoproteins were separated by ultracentrifugation as previously described[11] and washed once at the upper density limit. Cholesterol and triglycerides were measured by standard enzymatic techniques,[12 13] using reagents from Boehringer-Mannheim (Dorval, Quebeq, Canada). The Corning ACI system was used for lipoprotein electrophoresis.

Apolipoprotein B was measured in isolated lipoproteins by tetramethylurea (TMU) treatment or by electroimmunoassay.[14 15] Apolipoprotein AI and AII standards were purified by gel chromatography in the presence of 6 mol/l urea.[16]

DNA ANALYSIS

Polymerase chain reaction

Genomic DNA was extracted from the buffy-coat of 30 ml EDTA anticoagulated blood of the index patient and his available family members, for analysis by the polymerase chain reaction (PCR).

The reactions were performed in a DNA thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA) using the buffer recommended by the manufacturer in conjunction with 1·5 mmol/l Mg^{++}, 200 µmol/l deoxynucleotide triphosphates (dNTPs), 500 ng of each primer, 500 ng genomic DNA, and 2·5 units of Amplitaq (Cetus) in 100 µl reactions. The primers were derived from intronic sequences of the apoCII gene[6] and constructed to include intron-exon boundaries and complete exons in the amplified products.

The oligonucleotide primers were synthesised on the Gene Assembler Plus (Pharmacia, Uppsala, Sweden). The sequence of the primers and the annealing temperatures in the PCR reactions are given in table 1. Each exon of the apoCII gene of the index patient was individually amplified from 500 ng of genomic DNA. The samples were denatured at 94°C for one minute and annealed for one minute at the temperatures given in table 1 for the different oligomers. The reactions were extended at 72°C for one minute for a total of 30 cycles.

Sequence analysis

The PCR products from the four exons of the apoCII gene of the index patient were precipitated with NH_4Ac/ethanol and size fractionated on a 2% agarose gel. The band, corresponding to the amplified exon size was excised and purified using Spinbind DNA Extraction Units (FMC Bioproducts, Denmark) and resuspended in 30 µl H_2O.

Taking advantage of the A base overhang added to all templates during the PCR reaction, each amplified and purified fragment was directionally ligated into the TA Cloning pCR vector (TA Cloning System, Invitrogen, San Diego, CA, USA).

The ligation products were then transformed into the recommended INVαF' cells and incubated for 12 to 14 hours at 37°C on LB agar plates containing kanamycine and X-gal. The following day, six independent white colonies were isolated and grown overnight in LB medium for DNA isolation purposes. Small amounts of the extracted plasmid DNA were digested with *Hin*dIII and *Eco*RI to determine the size of the inserted fragments.

Positive samples were subjected to double stranded DNA sequencing using the Sequenase version 2.0 (US Biochemicals, Cleveland, OH) after annealing of 1 pmol of the required "nested" PCR primer according to the ligated exon. Annealing was performed by heating for two minutes at 65°C and cooling down until room temperature was reached. The labelling reaction took place at 4°C for five minutes followed by termination for five minutes at 39°C. These samples were loaded on a 6% polyacrylamide 5 mol/l urea sequencing gel and electrophoresed at 55°C for two hours at 1900 V.

Mutation detection using RsaI restriction endonuclease

Exon 3 of all family members was separately

Table 1 Sequence of PCR primers for exons 1, 2, 3, and 4 of the apoCII gene

Exon	*Sequence of PCR primers*	*Annealing temp*
1	5′ primer: 5′-GTGGCTGTGGAGCGGAAGTG-3′ 3′ primer: 5′-TTATCGATCTTTGCCCTCT-3′	50°C
2	5′ primer: 5′-CACACAGAGCAGGATCTCAG-3′ 3′ primer: 5′GAGGCAGAGGTAAGAAGGGC-3′	55°C
3	5′ primer: 5′-CTTCCTCCTTTCCCCCTGCT-3′ 3′ primer: 5′GCCAGACCCCATTTCTCCAG-3′	55°C
4	5′ primer: 5′-CCCTCTAACCATCTGTGCTT-3′ 3′ primer: 5′TCTCCCCTTGTCCACTGATG-3′	55°C

Table 2 Lipid and lipoprotein data of the proband and two sibs

Subject	Age (y)	BMI (Kg/m²)	Plasma Chol	Plasma Trig (mmol/l)	VLDL Chol	VLDL Trig (mmol/l)	LDL Chol (mmol/l)	HDL Chol (mmol/l)
Proband	43	22	5·13	8·47	2·51	7·07	0·96	0·52
Sister II.2	47	27	4·38	1·98	0·34	0·78	1·66	1·19
Sister II.6	42	21	3·08	1·33	0·28	0·40	0·65	1·14
Reference*								
Males	30–50		5·17	1·96	0·90	1·58	3·20	1·09
Females	30–50		5·17	1·75	0·58	1·03	3·26	1·33

* Values based on findings in 80 healthy Canadians (data not published).

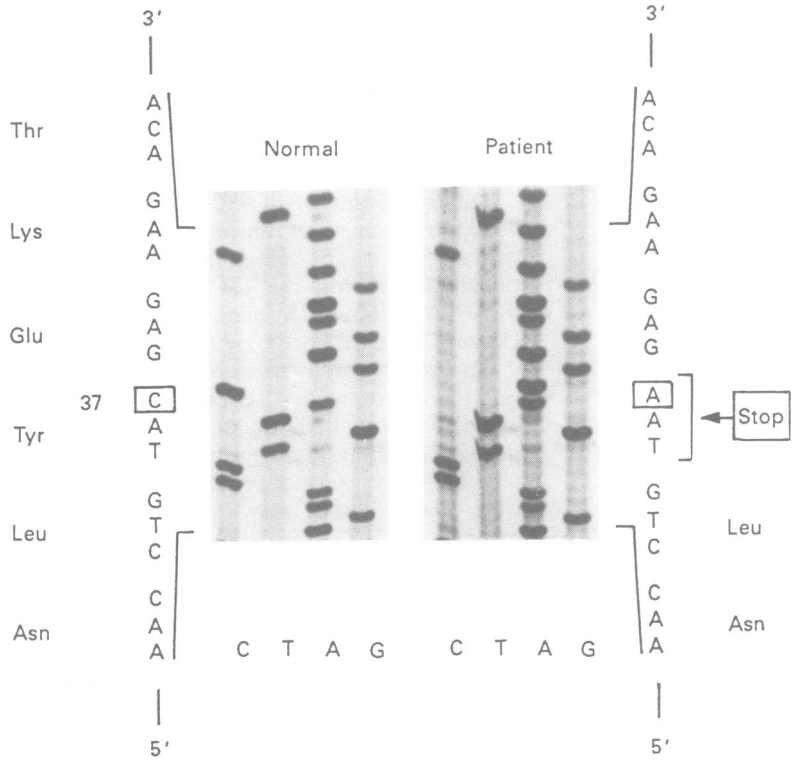

Figure 1 Autoradiogram showing the DNA sequence of the normal and the mutant exon 3 surrounding nucleotide 3002. The C→A transition, resulting in substitution of a stop codon for Tyr at residue 37, is indicated in boxes.

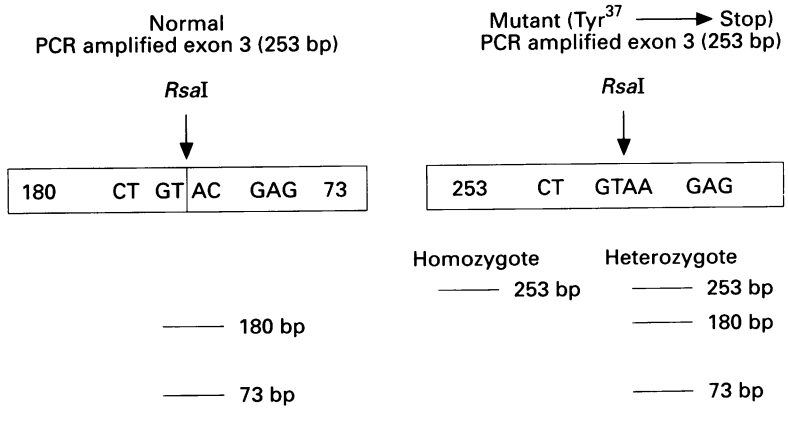

Figure 2 A schematic representation of the loss of the RsaI recognition site in mutant exon 3 of the apoCII gene. Numbers indicate fragment sizes and lines indicate expected electrophoresis patterns after RsaI digestion of exon 3 of the apoCII gene in control subjects and in subjects who are homozygous or heterozygous for apoCII$_{Padova}$.

amplified. PCR amplified exon 3 DNA (50 ng) was subsequently mixed with 2 µl of recommended buffer and 20 units of *Rsa*I restriction enzyme (Boehringer Mannheim) and incubated at 37°C for three hours. The DNA was then subjected to electrophoresis on 2% agarose gels.

Results

LIPIDS AND LIPOPROTEINS

Values for lipids and lipoproteins for the index patient and two sisters, II.2 and II.6, are summarised in table 2.

In the proband, TG levels are raised, mainly owing to high VLDL concentrations. LDL and HDL cholesterol are slightly decreased. Lipoprotein profiles in the two sisters exhibit no hypertriglyceridaemia; however, VLDL, LDL, and HDL plasma concentrations are decreased in both sisters.

MUTATION ANALYSIS

DNA sequence analysis was performed for all four exons from the index patient. A substitution of A for C was detected at position 3002 in exon 3 (fig 1). This mutation leads to the introduction of a premature stop codon (TAA) at the position corresponding to amino acid 37 of mature apoCII. The index patient represents a true homozygote for this nucleotide substitution, previously reported in another apoCII deficient kindred from Italy as apoCII$_{Padova}$.[17 18]

As previously described, this mutation leads to the loss of an *Rsa*I restriction enzyme site (GTAC→GTAA) in the normal apoCII gene.[18] Amplification of exons from all family members was performed by PCR. Digestion of amplified normal exon 3 with *Rsa*I should result in the formation of two fragments of 180 and 73 bp, respectively. However, digestion of amplified exon 3 from the proband homozygous for this mutation or his heterozygous sibs should yield a single band of 253 bp or a combination of the 253, 180, and 73 bp bands, respectively, as indicated in fig 2.

Fig 3 illustrates the restriction endonuclease analysis of amplified exon 3 of all family members, indicating true homozygosity for the index patient ((II.5), heterozygosity for both parents (I.1, I.2), and for two sisters and a brother (II.1, II.2, and II.6). One brother (II.3) did not carry the nucleotide substitution in exon 3. No other DNA alterations were detected in any other exons or in exon-intron boundaries of the apoCII gene.

Discussion

In this study we have identified a nonsense mutation in the apoCII gene, underlying apoCII deficiency and chylomicronaemia in a patient of Italian descent.

Sequence analysis of the apoCII gene of the proband showed, on both alleles, a substitution of cytosine for adenosine (C→A) at nucleotide 3002 in exon 3, resulting in the introduction of a premature stop codon at the position corresponding to residue 37 of the mature apoCII protein. As a consequence, a truncated 36

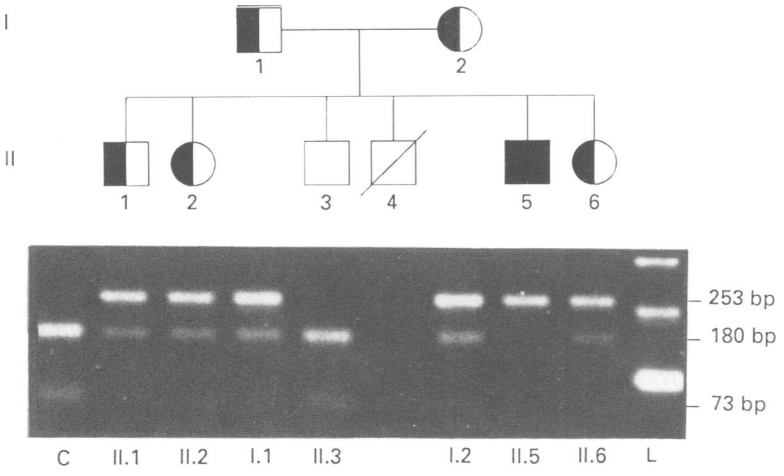

Figure 3 Family pedigree and results of restriction enzyme digestion with RsaI, indicating presence (black) and absence (white) of the mutant allele (top). C, control exon 3. II.5, proband. L, 123 bp ladder (as a DNA size marker).

residue apoCII is synthesised, lacking the carboxy-terminal part of the protein which is crucial for activation of lipoprotein lipase. Absence of apoCII mass on isoelectric focusing of VLDL protein, as detected in the index patient, may indicate diminished excretion of apoCII, enhanced degradation of an unstable apoCII, or, lastly, defective binding to apoCII by the anti-apoCII antibody, owing to absence of the epitope in the truncated protein. This mutation does not interfere with mRNA production as similar levels of mRNA transcript were detected in normal and affected persons.[19] The available data cannot conclusively show which of the above mentioned mechanisms is responsible for the absence of apoCII protein in plasma.

The identified mutation is identical to the one described by Crepaldi *et al*[17 18] in a family from Padova. In this paper we report on the second kindred with apoCII$_{Padova}$. The parents of the proband from our family originate from Siculiana, a small village in Sicily, Italy, and are first cousins. In the kindred from Padova, a Sicilian history or a history of consanguineous marriage was not reported. However, since the father of the two affected probands in that publication was adopted, the presence of Sicilian ancesters cannot be excluded, and is even likely in light of the fact that the mutation occurred on the same, albeit common, allele in both families. It was previously shown that in these families the mutant allele is present on a 3·8 kb *Taq*I fragment of the apoCII gene.[10 20]

Recently, the molecular defect underlying apoCII deficiency in a third patient also originating from Siculiana, in whom a genetic relationship with any of the other families is not known, was also shown to be identical to apoCII$_{Padova}$ (A Notarbartolo, personal communication). Another nonsense mutation in the apoCII gene, apoCII$_{Bari}$, has been reported in a patient of Sicilian descent.[21] In this defect cytosine$_{3002}$ is substituted for by guanine, resulting in the introduction of a premature termination codon at precisely the same nucleotide as in apoCII$_{Padova}$.[21 22] The exact reason for aggregation of two apparently different mutations at the same nucleotide in persons of a similar ancestry is uncertain. The nucleotides surrounding this mutation are GC rich and the presence of GC dinucleotides may serve as a potential hotspot for mutations.[23]

Our proband with apoCII deficiency presented with type V hyperlipoproteinaemia. Heterozygosity for the apoCII defect, diagnosed in two sisters of the proband, did not result in overt hypertriglyceridaemia, but rather in low plasma concentrations of all lipoprotein classes, which we cannot explain at this point. Heterozygous apoCII deficiency can influence lipoprotein metabolism and may manifest under stressful conditions or if additional factors are present that compromise lipoprotein metabolism. Hegele *et al*[24] reported that hypertriglyceridaemia in heterozygous apoCII deficient persons with an apoE3/E4 genotype occurred with a significantly higher frequency than among their heterozygous family members with an apoE3/E3 gentoype[24] and, similarly, in a case of homozygous LPL deficiency, pregnancy was shown to convert a mild phenotype in severe hypertriglyceridaemia, whereas the lipoprotein profile became normal upon delivery.[25] The low LDL and HDL cholesterol levels measured in heterozygotes from this family may reflect compromised lipolysis. Their low VLDL cholesterol and triglyceride concentrations, however, indicate that the mechanisms underlying the lipoprotein abnormalities in these family members are more complex and require further investigation.

In summary, apoCII deficiency is a rare disorder, with only 10 kindreds described in whom a defect in the apoCII gene has been shown.[9] The finding of three apoCII deficient Italian probands with the same apoCII$_{Padova}$, including two of Sicilian ancestry, suggests that this is a more common mutation underlying apoCII deficiency in Italy.

This work was supported by grants from "Het Praeventiefonds", the foundation "De Drie Lichten", and "Stichting Dr Hendrik Muller's Vaderlandsch Fonds". Dr M R Hayden is an established investigator of the British Columbia Children's Hospital and an investigator of the Canadian Genetic Diseases Network. Dr J J P Kastelein is a clinical investigator of The Dutch Heart Foundation.

1 La Rosa M, Leng RI, Hubert P, Lux, SE, Fredrickson DS. A specific apoprotein activator for lipoprotein lipase. *Biochem Biophys Res Commun* 1970;**41**:57–62.
2 Havel RJ, Kane JP, Kashyap ML. Interchange of apoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man. *J Clin Invest* 1973;**52**:32–7.
3 Fojo SS, Taam L, Fairwell T, *et al*. Human preproapolipoprotein CII. Analysis of major plasma isoforms. *J Biol Chem* 1986;**261**:9591–4.
4 Wang CS, Hartsuck J, McConathy WJ. Structure and functional properties of lipoprotein lipase. *Biochim Biophys Acta* 1992;**1123**: 1–17.
5 Jackson RL, Balasubramaniam A, Murphy RF, Demal RA. Interaction of synthetic peptides of apoCII and lipoprotein lipase at monomolecular lipid films. *Biochim Biophys Acta* 1986;**875**:203–10.
6 Fojo SS, Law SW, Brewer HB Jr. The human preproapolipoprotein CII gene. Complete nucleic acid sequence and genomic organization. *FEBS Letters*1987;**213**:221–5.
7 Wei CF, Tsao YK, Robbertson DL, *et al*. The structure of the human apolipoprotein CII gene: electron microscopic analysis of RNA: DNA hybrids, complete nucleotide sequence, and identification of 5' homologous sequences among apolipoprotein genes. *J Biol Chem* 1985; **260**: 15211–21.
8 Breckenridge WC, Little JA, Steiner G, Chow A, Poapst M. Hypertriglyceridemia associated with deficiency of apolipoprotein CII. *N Engl J Med* 1978;**298**:1265–73.

9 Fojo SS. Genetic dyslipoproteinemias: role of lipoprotein lipase and apolipoprotein CII. *Curr Opin Lipid* 1992;**3**:186–95.
10 Hayden MR, Vergani C, Humphries SE, Kirby L, Shukin RJ, McLeod R. The genetics and molecular biology of apolipoproteins CII. In: Angel A, Frohlich J, eds. *Lipoprotein deficiency syndromes*. New York: Plenum Publishing Corporation, 1986:241–51.
11 Match FT, Lees RF. Plasma lipoprotein analysis. *Adv Lipid Res* 1969;**6**:33–6.
12 Allain CC, Poon L, Chan SG, Richmond W, Fu P. Enzymatic determination of total serum cholesterol. *Clin Chem* 1974;**20**:470–5.
13 Buccolo G, David H. Quantitative determination of serum triglycerides by the use of enzymes. *Clin Chem* 1973;**19**:476–82.
14 Kane JP, Sata T, Hamilton RL, Havel RJ. Apolipoprotein composition of very low density lipoproteins of human serum. *J Clin Invest* 1975;**56**:1622–43.
15 Reardon MF, Poapst M, Uttelman KD, Steiner G. Improved method for quantitative of B apoprotein in plasma lipoproteins by electroimmunoassay. *Clin Chem* 1981;**27**:892–5.
16 Lux SE, John KM, Brewer HB Jr. Isolation and characterization of n-II (apo AII), a plasma high density apolipoprotein containing two identical polypeptide chains. *J Biol Chem* 1972;**247**:7510–18.
17 Crepaldi G, Fellen R, Baggio G, Augustin J, Greten H. Lipoprotein and apoprotein, adipose tissue and hepatic lipoprotein lipase levels in patients with familial hyperchylomicronemia and other immediate family members. In: Gotto AM Jr, Smith LC, Allen B, eds. *Atherosclerosis*. New York: Springer-Verlag, 1980:250–4.
18 Fojo SS, Lohse P, Parrot C, *et al.* A nonsense mutation in the apo CIIpadova gene in a patient with apolipoprotein CII deficiency. *J Clin Invest* 1989;**84**:1215–19.
19 Fojo SS, Baggio G, Gabelli G, *et al.* Apolipoprotein CII deficiency: identification of a structural variant. ApoCII-padova. *Biochem Biophys Res Commun* 1988;**154**:73–9.
20 Humphries SE, Williams L, Myklebost O, *et al.* Familial apolipoprotein CII deficiency: a preliminary analysis of the gene defect in two independent families. *Hum Genet* 1984;**67**:151–5.
21 Catapano AL, Mills GL, Roma P, LaRosa M, Capurso A. Plasma lipids, lipoproteins and apoproteins in a case of apoCII deficiency. *Clin Chim Acta* 1983;**130**:17–27.
22 Crecchio C, Capurso A, Pepe G. Identification of the mutation responsible for a case of plasmid apolipoprotein CII deficiency. *Biochem Biophys Res Commun* 1990; **168**:1118–27.
23 Monsalve MV, Henderson H, Roederer G, *et al.* A missense mutation at codon 188 of the human lipoprotein lipase gene is a frequent cause of lipoprotein lipase deficiency in persons of different ancestries. *J Clin Invest* 1990; **86**:728–34.
24 Hegele RA, Breckenridge WC, Cox DW, *et al.* Interaction between variant apolipoproteins CII and E that affects plasma lipoprotein concentrations. *Arteriosclerosis Thromb* 1991;**11**:1303–9.
25 Ma Y, Liu MS, Brunzell JD, Hayden MR. Gene-environment interaction in the conversion of a mild to severe hypertriglyceridemia in a patient homozygous for a $Ser^{172} \rightarrow Cys$ mutation in the lipoprotein lipase gene. *Circulation* 1993;**86**:1–609.