

# Three Novel Mutations in the Apolipoprotein E Gene in a Sample of Individuals with Type 2 Diabetes Mellitus

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**Background:** Apolipoprotein E (apoE) is found in association with triglyceride-rich lipoproteins and is the ligand for the removal of these particles from the plasma. Genetic variations in exon 4 lead to three common gene variants: E2, E3, and E4.

**Methods:** We performed apoE genotyping in 765 individuals with type 2 diabetes.

**Results:** We identified three new variant heteroduplex patterns. Sequencing of these variants revealed three novel mutations that were related to biochemical and clinical characteristics. One mutation produced a frameshift at amino acid position 166, which predicted termination of protein synthesis. This individual had a heteroduplex pattern and sequence of E3E3, which was associated with a change in the plasma isoelectric focusing pattern and a 70% lower plasma concentration of apoE compared with healthy individuals. The other mutations were both single base changes. A CGC>CAC change at amino acid position 150 predicted a substitution of Arg>His. This individual had a heteroduplex pattern and sequence of E2E2, which was not associated with major changes in plasma lipids or apoE concentration. The third individual had a CGC>CCC base change at amino acid position 114, which predicted an Arg>Pro change. This person had a heteroduplex pattern and

sequence of E3E3, higher plasma total cholesterol, and moderately decreased plasma apoE.

**Conclusions:** The frequency of new mutations in this sample (1 in 255) is higher than that of a healthy population (1 in 7900). Further screening for common apoE gene variants in individuals at risk for dyslipidemia may reveal abnormal heteroduplex patterns and uncover further mutations in this important lipid-regulating gene.

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Mature apolipoprotein E (apoE)<sup>5</sup> is a 299-amino acid glycoprotein (1) synthesized in the liver and intestine and is found in association with triglyceride-rich lipoproteins (2). It is the ligand for removal of these particles from the plasma and thus in determining the metabolic fate of these lipoproteins. Of the candidate genes involved in determining plasma lipid concentration and coronary heart disease (CHD) risk, apoE is the most comprehensively studied (3).

The human apoE gene is located on chromosome 19 and consists of 3.7 kb and four exons. Genetic variations in exon 4 produce three common gene variations, E2, E3, and E4, which have strong and consistent influences on plasma lipids (4) and CHD risk (5). Each isoform differs by one amino acid (4), an Arg (CGC) or Cys (TGC) at amino acid positions 112 and 158; the resulting amino acid variations are Cys112/Arg158 in apoE3, Arg112/Arg158 in apoE4, and Cys112/Cys158 in apoE2 (6). With respect to receptor binding activity, apoE3 and apoE4 bind with equal affinity, whereas apoE2 is defective, with 1–2% of the binding activity of the other isoforms (7). E3 is the most common isoform with a frequency of 0.77 in Cauca-

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<sup>5</sup> Nonstandard abbreviations: apoE, apolipoprotein E; CHD, coronary heart disease; TG, triglyceride; IEF, isoelectric focusing; and LDL-C, LDL-cholesterol.

sian populations, whereas E4 and E2 are present at frequencies of 0.15 and 0.08, respectively. Carriers of the E2 allele, who represent ~12% of the population, have cholesterol concentrations ~10% lower than E3 homozygotes, whereas E4 carriers, who represent ~25% of the population, have cholesterol concentrations ~5% higher than E3 homozygotes. Compared with men homozygous for the E3 allele, those carrying E2 show protection from both CHD and stroke (8), whereas those with the E4 allele have a higher risk (9). The most likely explanation for the increased risk associated with the E4 allele comes from the fact that these individuals have a preponderance of small dense LDLs, which are more prone to oxidation. The protection against oxidation in vitro is apoE2 > apoE3 > apoE4 (10).

ApoE2 is also associated with increased triglyceride (TG) concentrations compared with the other isoforms. Homozygosity for E2 predisposes to the development of type III hyperlipoproteinemia attributable to delayed metabolic clearance of apoE-containing lipoproteins. The result is that there is an accumulation of chylomicron and VLDL remnants in the plasma (7).

To date, ~30 apoE variants have been characterized (11). Fourteen variants have been found to be associated with familial dysbetalipoproteinemia, a genetic lipid disorder characterized by increased plasma cholesterol and TG concentrations and an increased risk of atherosclerosis, whereas 7 were found to be associated with other forms of hyperlipoproteinemia (11). In this report, we describe three new mutations identified during apoE genotyping of a sample of 765 individuals with type 2 diabetes mellitus. These mutations were related to clinical characteristics as well as apoE phenotype, as determined by isoelectric focusing, and plasma apoE concentration.

## Materials and Methods

### PATIENTS

Ethical approval was granted by the institutional ethics committee, and all participants gave written informed consent before recruitment. Patients were recruited from the University College London Diabetes and Cardiovascular Study, described elsewhere (12, 13). Briefly, this comprises 1011 consecutive patients recruited from the diabetes clinic at University College London Hospitals NHS Trust between the years 2001 and 2002. All patients had diabetes according to WHO criteria (14). Analyses were confined to individuals with type 2 diabetes mellitus only (n = 765).

### ApoE GENOTYPING AND SEQUENCING OF MUTATIONS

*ApoE genotyping.* ApoE genotyping was performed on 2.5  $\mu$ L of DNA (at a concentration of 5 ng/ $\mu$ L) by use of a universal heteroduplex generator (15). Briefly, the method involves PCR amplification of the region containing the two polymorphic sites at amino acid positions 112 and 158, followed by hybridization of this PCR product to a universal heteroduplex generator. The universal heteroduplex generator is used to induce heteroduplex formation, which is visualized by ethidium bromide on a 10% nondenaturing polyacrylamide minigel.

*ApoE sequencing.* PCR products were purified by use of the GFX<sup>TM</sup> PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Subsequently, automated sequence analysis was performed with the ABI Prism 377 DNA Sequencer (PE Applied Biosystems) with the Big Dye Terminator v 3.1 Cycle Sequencing Kit.

*Confirmation of sequence changes by restriction endonuclease digestion for R150H and 166delG mutations.* After sequence analysis, restriction site assays were developed for the identified mutations. Of the three mutations identified, the sequence changes at positions amino acids 150 and 166 were subsequently confirmed by restriction digestion of the wild-type PCR products with *Bst*uI and *Bs*II.

### ApoE PHENOTYPE

*Isoelectric focusing.* ApoE phenotyping was performed on 10  $\mu$ L of serum from the three patients with the newly identified mutations by isoelectric focusing (IEF) followed by Western blotting using monoclonal antibodies specific for apoE (Dako) (16).

*Plasma apoE measurement.* Plasma apoE was measured with a commercially available immunoturbidimetric assay from Wako Chemical.

## Results

We successfully genotyped 765 individuals with type 2 diabetes (100%) for the apoE variants. The genotype distribution was in Hardy–Weinberg equilibrium, as shown in Table 1.

### ApoE GENOTYPING AND SEQUENCING OF MUTATIONS

We identified three new variant heteroduplex patterns in the 765 patients. The expected heteroduplex patterns for the apoE gene variants are shown in Fig. 1. All three were

**Table 1. Frequency distribution for apoE genotype in individuals with type 2 diabetes.**

	ApoE genotype <sup>a</sup>					
	22	23	24	33	34	44
Number of individuals (%)	5 (0.7)	96 (12.5)	19 (2.5)	490 (64.1)	148 (19.3)	7 (0.9)

<sup>a</sup> ApoE genotypes were in Hardy–Weinberg equilibrium,  $\chi^2 = 2.70$ ;  $P = 0.26$ . The allele frequencies were as follows: E2 = 0.08; E3 = 0.81; E4 = 0.12.

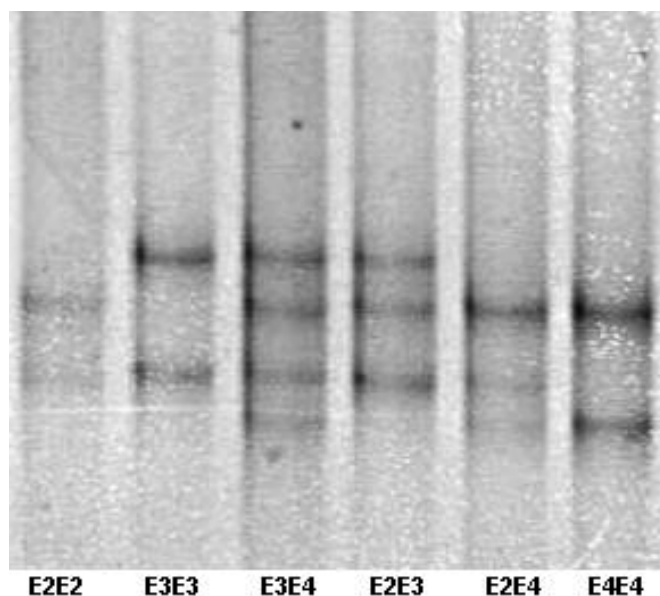


Fig. 1. Heteroduplex patterns for the common apoE gene variants.

seen as the presence of an additional band on gel electrophoresis (Fig. 2). This pattern was confirmed on repeat PCR and heteroduplex genotyping. All variants were observed in males. Patient 1 was genotyped as E2E2 + 1 extra band, patient 2 as E3E3 + 1 extra retarded band, and patient 3 as E3E3 + 2 extra bands. In view of this, the three samples were sequenced. Three novel mutations were identified and are summarized in Table 2. Patients 1 and 3 had single base changes that were predicted to alter amino acids Arg150His and Arg114Pro, respectively, but patient 2 had a frameshift mutation predicted to cause premature termination of the gene product and hence a change in plasma apoE concentration (see Fig. 1 of the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol51/issue1/>). These mutations were heterozygous in all three patients.

#### IEF

To study the phenotypes of these mutations further, we performed IEF, the results of which are shown in Table 2

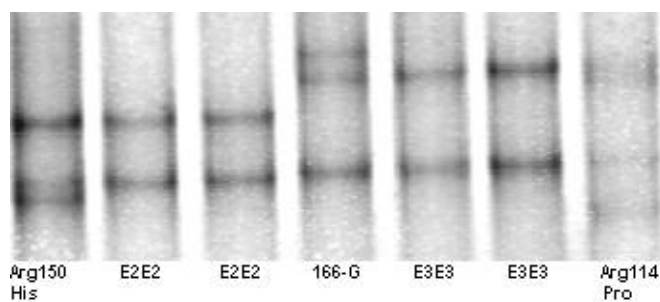


Fig. 2. Heteroduplex patterns for the newly identified mutations (Arg150His, 166-G, and Arg114Pro).

and in Fig. 2 of the Data Supplement. In patients 1 and 3, the apoE genotype as determined by heteroduplex analysis (E2E2 and E3E3, respectively) corresponded to that determined by IEF. In patient 2, the IEF phenotype was E2E3 with a genotype of E3E3. Interestingly, in this patient we observed none of the smaller protein fragments that usually appear below the main bands. When the genotypes were determined by sequencing, patient 1 was E2E2, patient 2 was E3E3, and patient 3 was E3E3.

#### PLASMA apoE CONCENTRATION

As shown in Table 3, patient 2 with the frameshift mutation (166delG) had a plasma apoE concentration of 19.8 mg/L. The respective plasma apoE concentrations for patient 1 (Arg150His) and patient 3 (Arg114Pro) were 72.6 and 21.7 mg/L, respectively.

As shown in Table 3, the apoE concentration in plasma has been shown previously to range from 50 to 70 mg/L (17) and by genotype to range from a mean (SD) of 138 (38) mg/L in E2E2 to 55 (20) mg/L in E3E3 and 44 (20) mg/L in E4E4.

#### OTHER CLINICAL ASSOCIATIONS

Other routine clinical measures for the three patients are shown in Table 3. Patient 1 (Arg150His) had a total plasma cholesterol of 3.0 mmol/L on the first visit and recently had a total cholesterol of 5.0 mmol/L, LDL-cholesterol (LDL-C) of 2.3 mmol/L, and TGs of 1.4 mmol/L. Patient 2 (frameshift mutation) is 56 years of age and to date has not had a clinically detectable cardiovascular event. There was no apparent family history of CHD events. This individual had the highest recent plasma TG concentration of the three patients (1.8 mmol/L). Before treatment, patient 2 had a baseline total cholesterol of 4.5 mmol/L and a high HDL of 1.5 mmol/L. Patient 3 (Arg114Pro) had the highest baseline plasma total cholesterol concentration (6.8 mmol/L). Interestingly, after treatment with a relatively low dose of simvastatin (10 mg/day), his recent cholesterol was 2.0 mmol/L (with a LDL-C of 0.6 mmol/L and TGs of 0.8 mmol/L).

#### Discussion

This study reports the identification of three novel mutations in the apoE gene in a sample of 765 patients with type 2 diabetes, one of which was a novel frameshift mutation. In the patient with that mutation, plasma apoE was ~70% lower than in healthy individuals, and there was a discrepancy between apoE phenotype, as determined by IEF, and genotype, as determined by heteroduplex analysis and sequencing.

ApoE contains two important structural domains: an amino-terminal (residues 1–164) and a carboxyl-terminal domain (residues 201–299). The amino-terminal domain, which has been studied extensively, contains the receptor-binding domain of apoE. This domain encompasses residues 130–150 and contains nine positively charged amino acids, six of which face outward. These positively charged

**Table 2. ApoE genotypes and phenotypes in patients with novel mutations**

	Patient		
	1	2	3
Predicted apoE variant by heteroduplex pattern	E2E2 + 1 extra band	E3E3 + 1 extra retarded band	E3E3 + 2 extra band
Predicted apoE genotype by sequencing	E2E2	E3E3	E3E3
Base change <sup>a</sup>	503G>A	550-G	395G>C
Amino acid change	Arg150His	Premature termination after 166	Arg114Pro
Predicted apoE variant by IEF	E2E2	E2E3	E3E3
ApoE concentration, mg/L	72.6	19.8	21.7
Reported mean (SD) apoE concentration, mg/L (17)	138.0 (20.0)	44.0 (20.0)	44.0 (20.0)

<sup>a</sup> Bases are numbered from the sequence where A of the initiator methionine is +1.

residues interact with the negative charges present in the ligand domain of the LDL and LDL-receptor-related protein receptors (18) and hence facilitate the uptake and removal of apoE-containing lipoproteins (VLDL and intermediate-density lipoprotein remnants) from the circulation. The carboxyl-terminal domain of apoE has a strong  $\alpha$ -helical character containing a heparin-binding domain (19). This domain plays an important role in the interaction of apoE with proteoglycans on the arterial wall, anchoring the lipoprotein particle to the endothelium, thus allowing the enzyme lipoprotein lipase to hydrolyze TGs to free fatty acids and glycerol. The frameshift mutation seen in patient 2, despite decreasing plasma apoE concentrations below normal, did not appear to alter the total cholesterol or LDL-C markedly. This deletion is outside the receptor binding domain for LDL-C; therefore, although we would expect that the apoE concentration

would be reduced because of premature termination of protein synthesis, we might expect receptor binding activity to be within normal limits. This may explain the relatively normal total cholesterol and LDL-C concentrations in this patient. Interestingly, however, this individual had the highest TG concentrations of the three patients with novel mutations. Because this mutation would lead to premature termination of apoE synthesis and loss of the carboxyl-terminal domain, there may be loss of interaction between proteoglycans and apoE on the endothelium, with subsequent decreased lipoprotein lipase hydrolysis of plasma TGs. With such a mutation, one might expect this patient to be at higher cardiovascular risk, but at 56 years of age, he has had no such clinically manifest events.

The Arg150His amino acid change observed in patient 1 would not be expected to produce a significant change

**Table 3. Clinical and biochemical measures.**

	Patient (mutation)		
	1 (Arg150His)	2 (166-G)	3 (Arg114Pro)
Most recent measurement			
Age, years	64.7	56.3	70.4
Duration DM, <sup>a</sup> years	16	9	18
Ethnic origin	African	Caucasian	Caucasian
Family history of CHD	No	No	Yes
CHD	No	No	Yes
Statin therapy	No	No	Simvastatin (10 mg/day)
Diabetes therapy	Insulin	Metformin	Insulin and metformin
BMI, kg/m <sup>2</sup>	29.4	28.5	31.6
Glucose, mmol/L	16.8	9.6	8.6
HbA <sub>1c</sub> , %	9.4	7.2	6.6
Cholesterol, mmol/L	5.0	4.2	2.0
LDL-C, mmol/L	2.9	2.3	0.6
HDL, mmol/L	1.5	1.1	1.0
TGs, mmol/L	1.4	1.8	0.8
Creatinine, mmol/L	106	110	113
Proteinuria	No	No	Yes
CRP, mg/L	10.29	1.84	4.52
Baseline measures before therapy (first visit to clinic)			
Cholesterol, mmol/L	3.0	4.5	6.8
HDL, mmol/L	1.1	1.5	1.1

<sup>a</sup> DM, diabetes mellitus; BMI, body mass index; Hb, hemoglobin; CRP, C-reactive protein.



in amino acid charge (both Arg and His being hydrophilic positively charged polar molecules) in the amino-terminal domain. This mutation would therefore not be expected to alter the IEF pattern of the protein, which was E2E2, the same as that determined by heteroduplex analysis and sequencing. In addition, receptor binding and, hence, plasma cholesterol, LDL-C, and TG concentrations would be expected to be unchanged. The plasma cholesterol concentration at this patient's first clinic visit was 3.0 mmol/L (most recent cholesterol, 5 mmol/L; LDL-C, 2.3 mmol/L; TGs, 1.4 mmol/L).

The Arg114Pro amino acid substitution would produce a change from a hydrophilic positively charged, polar amino acid on the amino-terminal domain (residues 1–164) to a hydrophobic nonpolar amino acid. This might then affect receptor binding. This patient had the highest plasma total cholesterol concentration (6.8 mmol/L at first visit). Interestingly, after treatment with a relatively low dose of a statin (simvastatin, 10 mg/day), his cholesterol decreased markedly to 2.0 mmol/L (LDL-C, 0.6 mmol/L; TGs, 0.8 mmol/L). This patient (now 70 years of age) has clinical evidence of CHD and is also an ex-smoker with a family history of CHD in a first-degree relative.

The patterns of apoE isoforms observed on IEF gels are attributable to variations in charge as a result of the amino acid sequence encoded at the apoE gene locus. Nonenzymatic posttranslational modification may also alter isoform mobility (20). With respect to apoE, the usual degree of migration toward the anode is E2 (Cys112/Cys158) > E3 (Cys112/Arg158) > E4 (Arg112/Arg158) because Arg has a positive charge and Cys is uncharged. This pattern of migration is therefore dependent on the charge of the amino acids at positions 112 and 158. With respect to the frameshift mutation, the apoE phenotype as determined by IEF was E2E3. This individual would have had an abnormal protein after position 166 as a result of the frameshift mutation (analysis of the sequence after this point revealed the presence of a premature stop codon at position 232). Because of the heterozygous nature of this mutation, it is therefore not surprising that one of the alleles determined by IEF is the same as that determined by heteroduplex analysis and sequencing (E3). The other allele determined by IEF was an E2 allele. This may represent the mutant allele because if transcription were interrupted, it could produce an abnormal, smaller protein; this smaller protein would migrate further on the IEF gel and may migrate at a position similar to that of E2.

With respect to the Arg150His change, the heteroduplex pattern (E2E2) was similar to the IEF phenotype (E2E2). This mutation would not cause a change in amino acid charge or in size. Therefore, as expected, the apoE genotype as determined by IEF and heteroduplex were the same.

In the patient with the Arg114Pro mutation, the heteroduplex pattern was of an E3E3 individual (with two extra bands) and the IEF of an E3E3 individual. The

Arg114Pro change would lead to the loss of a charge, but this did not appear to alter the IEF pattern significantly.

Previously, a discrepancy between apoE phenotype as determined by IEF and genotype has been described in individuals with diabetes (21). One possible explanation for this is that, in diabetic individuals, sialic acid derivatives present in the plasma lead to posttranslational modification and nonenzymatic glycosylation of apoE (22–24). Although this may occur, in our current study, the expected IEF phenotypes in the samples appear to be identified by the described mutations.

In summary, we describe three new mutations among 765 patients with type 2 diabetes screened for the common apoE gene variants. Family studies would be desirable in these individuals, but no relatives are available. Each of these three mutations is, of course, rare, and we would not advocate their routine screening when genotyping for the common apoE gene variants. The frequency of newly identified mutations in our sample of patients with type 2 diabetes is higher than was detected in healthy individuals by the same heteroduplex method [in the Whitehall-II Study of UK individuals, the frequency of new mutations was 1 in 7900 (S.E. Humphries, unpublished observation)]. Dyslipidemia is common in individuals with diabetes (particularly type 2 diabetes/metabolic syndrome), and further screening for common apoE variants in those at risk of dyslipidemia, such as patients with the metabolic syndrome, type 2 diabetes, and a family history of premature CHD, may reveal abnormal heteroduplex patterns and uncover further mutations in this important lipid-regulating gene.

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