Genetic heterogeneity in familial dysbetalipoproteinemia. The E2(lys146→gln) variant results in a dominant mode of inheritance

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Abstract As determined by isoelectric focusing, most patients with familial dysbetalipoproteinemia (FD) exhibit the homozygous apolipoprotein (apo) E2E2 phenotype. Only rarely does FD develop in the more common heterozygous phenotypes E3E2 or E4E2. In fact, only 1 to 4% of the E2E2 homozygotes will develop FD. We wondered whether this reduced penetrance of FD in E2E2 homozygotes could be due to additional heterogeneity in the APOE*2 allele. In the literature a number of different mutations causing an E2 isoelectric focusing variant have been described. To study the genetic heterogeneity of the APOE gene, hybridization of enzymatically amplified genomic DNA with mutation-specific oligonucleotide probes was applied. All FD patients (n = 40) with the E2E2 phenotype appeared to be homozygous for the common E2(arg158→cys) mutation. However, all three unrelated patients with the E3E2 phenotype exhibited the rare E2(lys146→gln) mutation due to an A→C substitution at nucleotide position 3,847 of the APOE gene. This mutation was not found among normolipidemic individuals with the E2E2 (n = 13) or E3E2 phenotype (n = 120) selected from a random population sample. Family studies of the three probands heterozygous for the E*2(lys146→gln) allele showed that this rare allele predisposes to FD with high penetrance. We conclude that FD is a genetically heterogeneous disease entity, displaying a recessive mode of inheritance with strongly reduced penetrance in case of the common E2(arg158→cys) variant and with a dominant mode of inheritance with high penetrance in case of the rare E2(lys146→gln) mutant. It should be noted that in this dominant form presymptomatic diagnosis is possible. —**Smit, M., P. de Knijff, E. van der Kooij-Meijs, C. Groenendijk, A. M. J. M. van den Maagdenberg, J. A. Gevers Leuven, A. F. H. Stalenhoef, P. M. J. Stuyt, R. R. Frants, and L. M. Havekes.** Genetic heterogeneity in familial dysbetalipoproteinemia. The E2(lys146→gln) variant results in a dominant mode of inheritance. *J. Lipid Res.* 1990. **31:** 45-53.

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In normal individuals the chylomicron remnants and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver (1). In familial dysbetalipoproteinemia (FD) or type III hyperlipoproteinemia, the increased plasma cholesterol and triglyceride levels are due to an impaired clearance of chylomicron and VLDL remnants. The accumulation of these remnants in the circulation often results in xanthomatosis and premature coronary and/or peripheral vascular disease (2).

The apolipoprotein (apo) E present on the lipoprotein remnants plays an important role as ligand for the high affinity hepatic lipoprotein receptors (3). With isoelectric focusing apoE can be separated into three common isoforms, i.e., E2, E3 and E4 (4, 5) encoded by codominant alleles at a single APOE gene locus on chromosome 19 (6). The apoE 3 isoform is the most frequently occurring (or wild type) isoform.

The vast majority of all FD patients exhibits the E2E2 phenotype, as defined by isoelectric focusing (7, 8). Only rarely does FD develop in the heterozygous phenotypes E3E2 or E4E2. It has been shown that the E2 isoform displays defective binding of the remnants to the hepatic lipoprotein receptors (9, 10) and delayed clearance from plasma (11). However, only a small percentage (1-4%) of the E2E2 homozygotes develops familial dysbetalipoproteinemia, suggesting that additional genetic and/or environmental factors are required for expression of this disease.

ApoE4 differs from E3 by an amino acid substitution (cys→arg) at position 112 and is designated E4(cys112→arg). At present four different mutations, giving a band at the

Abbreviations: apoE, apolipoprotein E; FD, familial dysbetalipoproteinemia; PCR, polymerase chain reaction; VLDL, very low density lipoprotein; SSC, saline sodium citrate buffer; SSPE, saline sodium phosphate-EDTA buffer.

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E2 position with isoelectric focusing, have been described. These are designated E2(arg158→cys), E2(lys146→gln), E2(arg145→cys), and E2-Christchurch(arg136→ser) (9, 12–14). E2(arg158→cys) is the most common E2 mutation. In addition, double mutations have also been described: E3(cys112→arg; arg142→cys) (15), E3(ala99→thr; ala152→pro) (16) E1(gly127→asp; arg 158→cys) (17), and E1-Harrisburg(lys146→glu) (18). Recently, we encountered three unrelated FD patients with the E3E2 phenotype (19). These patients exhibit a rare E2 variant containing only one cysteine residue. This E2 variant cosegregates with FD in their families, suggesting that it is a dominant trait in the expression of FD.

The reduced penetrance of FD in subjects with E2E2 homozygosity, as well as the dominance hypothesis of the rare E2 variant containing one cysteine residue, prompted us to study the genetic heterogeneity of the APOE gene in FD patients and healthy controls in more detail. For this purpose we used the technique of genomic DNA amplification (polymerase chain reaction, PCR) followed by hybridization with mutation-specific synthetic oligonucleotide probes.

MATERIALS AND METHODS

Patients

Patients with familial dysbetalipoproteinemia (FD) were diagnosed on the basis of the presence of elevated plasma cholesterol and triglyceride levels, concomitant with floating beta lipoproteins and an elevated VLDL cholesterol/plasma triglyceride ratio (>0.69 on a mmolar basis). Furthermore, palmar and tuberous elbow xanthomas were frequently present in the patients.

ApoE phenotyping

EDTA plasma was obtained after venapuncture and stored at −20°C until use. ApoE phenotyping was performed using a rapid micro-method based on isoelectric focusing of delipidated plasma followed by immunoblotting using a polyclonal anti-apoE antiserum (20).

Genomic DNA isolation

DNA was isolated from leukocytes of whole blood by standard methods (21).

Polymerase chain reaction (PCR)

The procedure used for DNA amplification in vitro was a modification of the original procedure described by Saiki et al. (22). Two sets of amplimers (AE1/AE2 and L1/L2) have been used. The nucleotide sequence and the position of these amplimers in exon 4 of the APOE gene is presented in **Fig. 1**.

The reaction mixture contained 1 μg of genomic DNA, 1 μM of both amplimers, 0.1 mM dNTPs, 67 mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 6.7 mM EDTA, 10 mM β-mercaptoethanol, 6.7 mM $(NH_4)_2SO_4$, 170 μg/ml BSA, and 10% v/v dimethylsulfoxide DMSO) in a total volume of 100 μl.

The mixture was first incubated for 8 min at 95°C to denature the DNA, followed by a 1-min incubation at room temperature, allowing the amplimers to anneal to the DNA. One unit of Taq polymerase (Beckman or Cetus) was added to each sample and the chain elongation was performed at 65°C for 2 min. Subsequent amplification rounds of 1 min denaturation (95°C), 1 min annealing (room temperature), and 2 min elongation (65°C) continued for 35 cycles, with 1 unit Taq polymerase extra added at rounds 10 and 20, and 0.5 units at round 30.

Hybridization with variant-specific oligonucleotide probes

The variant-specific oligonucleotides were synthesized by the solid phase triester method as described previously (23). The sequences of the different oligonucleotides were deduced from the sequences of a number of known apoE variants and are presented in **Table 1**. For each mutant we designed sets of two oligonucleotides, one for the mutant allele and the other for the normal E*3 allele.

The oligonucleotides were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase according to the conditions recommended by the manufacturer (Pharmacia). The reaction was stopped by the addition of 10 μl formamide and the mixture was loaded onto a 10% polyacrylamide–7 M urea sequence gel to separate the end-labeled oligonucleotide from the unlabeled oligonucleotide and free nucleotides. The labeled oligonucleotide was visualized by autoradiography and the excised band was eluted in 600 μl 1 mM EDTA for 90 min at 65°C. The eluate was used directly for hybridization. The specific activity of the ^{32}P labeled oligonucleotides ranged between 10^8 and 10^9 cpm/μg.

Five μl of the amplified DNA was separated by electrophoresis on a 1.5% agarose gel for 1 h and blotted to GeneScreen Plus filters (New England Nuclear) in 0.4 M NaOH/0.6 M NaCl.

Dot-blots were prepared on GeneScreen Plus filters pretreated by successive incubations in distilled water and 10 × SSC and dried at 60°C. Three μl of the amplified (denatured) DNA was spotted onto the filters (24).

Hybridization was performed as described by den Dunnen et al. (25). The temperature during the hybridization and the washing conditions for the different sets of oligonucleotides were experimentally determined and are presented in Table 1. In general, the hybridization temperature was chosen 10–12 degrees below the melting tempera-

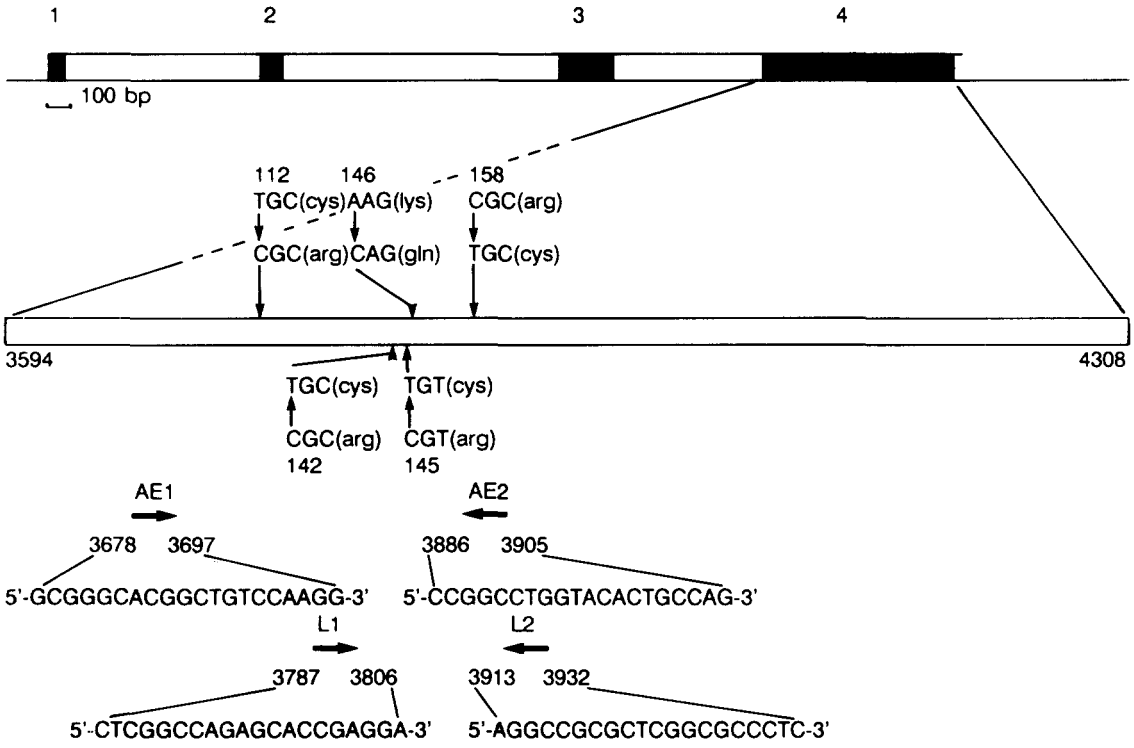

Fig. 1. Schematic representation of the APOE gene; exon 4 enlarged. The most common mutations are indicated. Localization and sequence of the two sets of oligonucleotides/amplimers (AE1/AE2 and L1/L2) used for the amplification are indicated. The AE1/AE2 amplimers are identical to those described by Smeets et al. (32). Numbering of nucleotides is according to Paik et al. (34).

ture (Tm), which was calculated according to the formula Tm (^{0}C) = 4(G + C) + 2(A + T), where G, C, A, and T indicate the number of the corresponding nucleotides in the oligomer (26).

Finally, the filters were exposed to Konica medical X-ray films at − 70°C for 1 to 3 h using intensifying screens.

DNA sequencing of the APOE gene

For DNA sequencing of the relevant part of the APOE gene, genomic DNA was amplified by PCR using 20-mer oligonucleotides L3 (nucleotides 3,555–3,574 of the coding strand) and L2 (nucleotides 3,932–3,913 of the

TABLE 1. Sequence and hybridization and washing conditions of the different oligonucleotide probes

Oligonucleotide[a]	Sequence[b]	Hybridization Temperature	Washing Procedure and Temperature[c]
		°C	°C
112-Arg	5'-AGGCGGCCGCGCACGTCCTCC-3'	64	1 73.5
112-Cys	5'-AGGCGGCCGCACACGTCCTCC-3'	64	1 73.5
146-Gln	5'-GGAGCCGCTGACGCAGCTT-3'	53	1 63.5
146-Lys	5'-GGAGCCGCTTACGCAGCTT-3'	53	1 63.5
158-Cys	5'-CTGCCAGGCACTTCTGCAG-3'	53	2 63.5
158-Arg	5'-CTGCCAGGCGCTTCTGCAG-3'	53	2 63.5
145-Cys	5'-AGCCGCTTACACAGCTTGCGC-3'	57	1 63.5
142-Cys	5'-TCCCACCTGTGCAAGCTGC-3'	53	1 61

[a]Nomenclature: 112-arg stands for the oligonucleotide specific to the allele coding for an apoE variant with an arginine residue at position 112, etc.

[b]Sequence: the sequence is deduced from Paik et al. (34). The substituted nucleotide is underlined.

[c]Washing procedures: procedure 1: 2 × 5' at room temperature in 5 × SSPE, 0.3% SDS; 10' at hybridization temperature in 1 × SSPE, 0.3% SDS; 10' at specific wash temperature in 1 × SSPE, 0.3% SDS; procedure 2: 2 × 30' at room temperature in 1.5 × SSC, at 0.1% SDS; 15' at hybridization temperature in 3 × SSC, 0.1% SDS; 10' at specific wash temperature in 3 × SSC, 0.1% SDS.

noncoding strand) as amplimers. The conditions applied for PCR were as described before. Pst I-digested amplified DNA, containing nucleotides 3,700–3,873 of the APOE gene, was cloned into Pst I-digested M13mp19. The 174 base pairs inserts in M13 were sequenced by the Sanger method with the ^{T7}Sequencing^{TM} Kit (Pharmacia) using [α-^{32}P]dATP.

RESULTS

APOE genotyping

For a routine screening of the various mutant alleles (genotyping), amplified DNA was subjected to electrophoresis on a 1.5% agarose gel, blotted to GeneScreen Plus filters, and hybridized with the respective mutation-specific oligonucleotide probes (for nucleotide sequences and hybridization and washing conditions of the oligonucleotides see Table 1). After autoradiography for 1 to 3 h strong signals were obtained (see **Fig. 2 left** for examples). As the hybridization signals corresponded with the amplified fragments visible on the ethidium bromide-stained gel (not shown), the electrophoresis step could be substituted by a direct dot blot procedure (Fig. 2 right). The results presented in Fig. 2 are schematically presented in **Table 2**. Two rare variants are presented. Sample E represents a subject heterozygous for the wild type or E3 isoform and the rare E2(lys146→gln) variant, whereas sample G is heterozygous for the common E2(arg158→cys) variant and another as yet unknown E2 variant.

Screening of patients and healthy controls

The method of DNA amplification followed by hybridization with mutant-specific oligonucleotides (Fig. 2) was applied for APOE genotyping of a population sample of 40 FD patients with the E2E2 phenotype and 3 FD patients with the E3E2 phenotype. The amplified DNA samples were hybridized separately with the respective mutation-specific oligonucleotides presented in Table 1. All FD patients with the E2E2 phenotype appeared to be homozygous for the common E2(arg158→cys) mutation, whereas all 3 patients with the E3E2 phenotype were shown to carry the E2(lys146→gln) mutation (**Table 3**).

To definitely prove that these patients carry the E2(lys-146→gln) mutation, we sequenced the relevant part of the APOE gene of one of these patients. Therefore, a 174 base pairs Pst I fragment of amplified APOE gene DNA was cloned into Pst I-digested M13mp19 (see Materials and Methods). Five independent clones were analyzed by DNA sequencing. The sequence of two clones was identical to the normal E*3 allele, whereas three clones indeed showed an A→C substitution at position 3,847 leading to an amino acid substitution lys→gln at position 146 (**Fig. 3**).

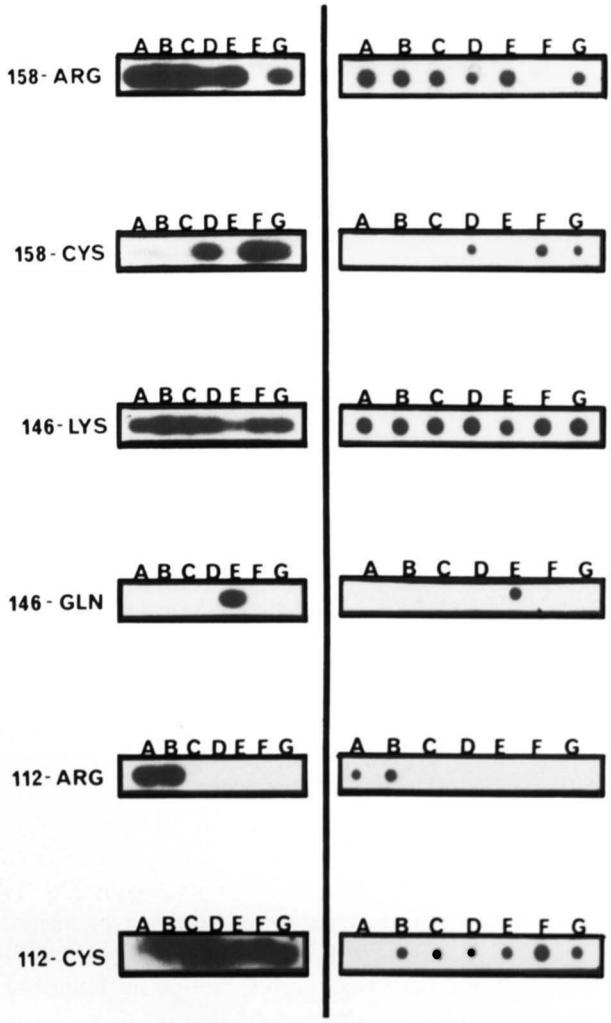

Fig. 2. Detection of the APOE mutants using DNA amplification and hybridization with mutant-specific oligonucleotide probes (APOE genotyping). For detection with the 112-arg and 112-cys oligonucleotide probes, the samples were amplified with the AE1 and AE2 amplimers. For detection with the other oligonucleotides, the same samples were amplified with the L1/L2 amplimers. The positions of the different amplimers in APOE exon 4 are shown in Fig. 1. Left: 5 μl of the amplified DNA was separated by electrophoresis on a 1.5% agarose gel, blotted to GeneScreen Plus filters, and hybridized with the different oligonucleotide probes. Right: 3 μl of the amplified DNA was spotted on pretreated GeneScreen Plus filters. The respective hybridization and washing conditions of left and right were identical and are presented in Table 1. In both experiments the same series of samples were investigated: A: E4E4; B: E4E3; C: E3E3; D: E3E2; E: E3E2; F: E2E2; G: E2E2. A schematic representation of the results shown in this figure is given in Table 2.

Recently we performed apoE phenotyping by IEF in a random population sample of 2,000 35-year-old males (27, 28). In this population study, 13 E2E2 homozygotes were found. Eleven out of these 13 E2E2 individuals appeared to be homozygous for the E2(arg158→cys) mutation, whereas the remaining 2 appeared to be heterozygous for this common E2(arg158→cys) mutation and

TABLE 2. Schematic representation of the results of the APOE genotyping by hybridization of APOE exon 4 amplified DNA with mutant-specific oligonucleotides as shown in Fig. 2

	Phenotype by Isoelectric Focusing						
Oligonucleotide	A 4/4	B 4/3	C 3/3	D 3/2	E 2/2	F 2/2	G 2/2
158-Arg	+	+	+	+	+	–	+
158-Cys	–	–	–	+	–	+	+
146-Lys	+	+	+	+	+	+	+
146-Gln	–	–	–	–	+	–	–
112-Arg	+	+	–	–	–	–	–
112-Cys	–	+	+	+	+	+	+
145-Cys[a]	nd	nd	–	–	nd	–	–
142-Cys[a]	nd	nd	–	–	nd	–	–
Genotype							
Allele 1:	arg112	arg112	wt	wt	wt	cys158	cys158
Allele 2:	arg112	wt	wt	cys158	gln146	cys158	?

[a]The hybridization results using the 145-cys and 142-cys oligonucleotides are not shown in Fig. 2; nd, not determined; wt, wild type or E*3 allele.

another, so far unknown, E2 mutation (Table 3). From this random population sample we also selected 50 individuals with the E3E2 phenotype. All these individuals appeared to be heterozygous for the common E2(arg158→cys) mutation. The E2(lys146→gln) variant was not found in these control individuals.

The results in Table 3 show that the E*2(lys146→gln) allele is very rare and so far found exclusively in FD patients with the E3E2 pheontype. This suggests that the E*2(lys146→gln) mutation is dominant in the predisposition of an individual to FD. To sustain this hypothesis we performed detailed family studies.

Family studies of the three FD probands carrying the E*2(lys146→gln) allele.

Fig. 4 shows the pedigrees of the three probands carrying the E*2(lys146→gln) allele. The clinical and genetic parameters of these families are presented in **Table 4**. In family V the two brothers (proband represents II-4) carrying the E*2(lys146→gln) allele display manifest FD with elevated plasma cholesterol and triglyceride levels. They both exhibit typical FD-associated xanthomas. Because of ethical rules, we were not allowed to study the young children of proband II-4. The two sisters (II-1 and II-2) are homozygous E3E3. Although they have slightly elevated plasma lipid levels, they were not classified as FD.

From family D we studied 10 siblings of the proband (II-9). As presented in Table 4, 5 out of 7 individuals carrying the E*2(lys146→gln) allele display FD. Two siblings carrying the E*2(lys146→gln) allele were not FD. Subjects II-6 was very lean, whereas subject II-14 was a female before menopause, two conditions that may prevent or delay the expression of FD. The four siblings not carrying the rare E*2(lys146→gln) allele had normal plasma lipid levels. The remaining family members were not available for analysis.

In family N we were able to study three generations (proband II-3). From the results presented in Table 4 it is obvious that in this family, again, all family members carrying the E*2(lys146→gln) allele displayed elevated plasma cholesterol and triglyceride levels and clinical symptoms of FD, except the 19-year-old female III-4. All family members not carrying the rare E*2(lys146→gln) allele showed normal plasma lipid levels except subject I-1 who had an increased plasma triglyceride level.

DISCUSSION

Most FD patients have the E2E2 phenotype. In the normal population the frequency of the E2E2 phenotype is about 1% (28), whereas the prevalence of FD in the general population has been estimated as 1–4 in 10,000. Hence, only 1 to 4% of all individuals with the E2E2 phenotype develop FD. Utermann et al. (29) suggested that another gene and/or environmental factors are re-

TABLE 3. APOE genotyping of FD patients and healthy controls

Subjects	Phenotype	Genotype
FD patients	E2E2 (n = 40)	E2(arg158→cys) / E2(arg158→cys)
FD patients	E3E2 (n = 3)	E3 / E2(lys146→gln)
Controls	E2E2 (n = 11)	E2(arg158→cys) / E2(arg158→cys)
Controls	E2E2 (n = 2)	E2(arg158→cys) / E2(?)
Controls	E3E2 (n = 50)	E3 / E2(arg158→cys)

ApoE phenotypes were determined by isoelectric focusing. APOE genotypes were determined by separate hybridization of amplified genomic DNA with the variant-specific oligonucleotides presented in Table 1; n, number of subjects.

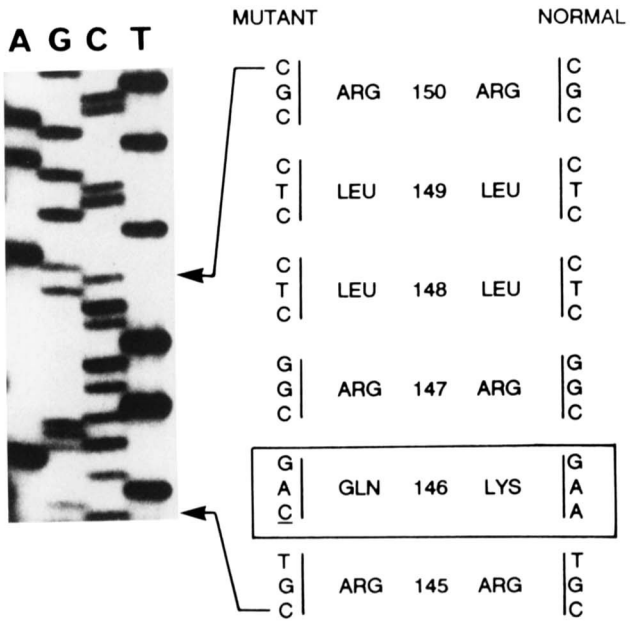

Fig. 3. Sequence of the relevant part of the mutant APOE*2 allele of an FD patient (pedigree #II-3 of family N in Fig. 4). The square denotes the codon with the substitution A→C, leading to amino acid substitution lys146→gln. The corresponding sequence of the normal allele is derived from Paik et al. (34).

quired in addition to the E2E2 phenotype for the expression of FD. However, it is also possible that additional heterogeneity in the APOE*2 allele is responsible for the apparent reduced penetrance of FD in E2E2 homozygotes; i.e., a subvariant of apoE2 may be predominant in FD patients. Four apoE2 variants have been described thus far: E2(arg158→cys), E2(lys146→gln), E2(arg145→cys), and E2-Christchurch(arg136→ser) (9, 12–14). Unequivocal detection of each of these different apoE2 variants cannot be performed simply by isoelectric focusing. Therefore, in order to discriminate between the different apoE2 variants, we used DNA hybridization with mutation-specific oligonucleotides.

For mutation-specific oligonucleotide hybridization an "in gel" hybridization procedure of restriction enzyme-digested genomic DNA with variant-specific synthetic oligonucleotides could be used (30). Detection of apoE variants with this procedure has recently been described by Funke, Rust, and Assmann (31). However, in our hands this method was less suitable for screening purposes since relatively large amounts of genomic DNA (10 μg) and long exposure times were needed. Furthermore, additional (nonspecific) bands were visible.

To improve the sensitivity and specificity of the method for the detection of known mutations in the APOE gene,

TABLE 4. Clinical and genetic parameters of the families carrying the E*2(lys146→gln) allele

Subject	Age/Sex	TC	TG	FD	Xanthomas	E Phenotype	E*2(Lys146→Gln)
Family V							
II-1	60/F	7.60	3.27	no	no	E3E3	–
II-2	56/F	6.45	2.18	no	no	E3E3	–
II-3	50/M	7.19	3.05	yes	yes	E3E2	+
II-4	37/M	14.45	8.30	yes	yes	E3D2	+
Family D							
II-1	54/F	9.50	3.9	yes	no	E3E2	+
II-3	50/M	3.44	1.83	no	no	E3E3	–
II-6[a]	47/M	6.20	1.77	no	no	E3E2	+
II-7	42/F	5.90	1.58	no	no	E3E3	–
II-8[a]	49/M	7.88	1.91	yes	no	E3E2	+
II-9	46/M	9.87	4.69	yes	yes	E3E2	+
II-10	42/M	4.64	1.08	no	no	E3E3	–
II-11	45/M	7.18	6.77	yes	no	E3E2	+
II-12	30/M	13.86	5.30	yes	yes	E3E2	+
II-13	37/M	6.81	1.55	no	no	E3E3	–
II-14	40/F	6.36	2.35	no	no	E3E2	+
Family N							
I-1	79/M	4.64	3.36	no	no	E3E3	–
I-2	75/F	8.87	3.75	yes	yes	E3E2	+
II-1	43/M	21.88	17.31	yes	yes	E3E2	+
II-2	49/M	5.50	2.20	no	no	E3E3	–
II-3	40/F	18.44	16.15	yes	yes	E3E2	+
III-1	25/M	6.67	2.25	yes	no	E3E2	+
III-2	27/M	4.50	1.15	no	no	E3E3	–
III-3	23/F	5.42	3.26	yes	no	E3E2	+
III-4	18/F	6.43	2.65	no	no	E3E2	+

Age, represents age at the time of blood sampling; TC, total cholesterol (mmol/l); TG, triglyceride (mmol/l); FD, clinical symptoms of FD, including the presence of βVLDL and increased ratio VLDL-cholesterol/plasma triglyceride level.

[a]Very lean subject.

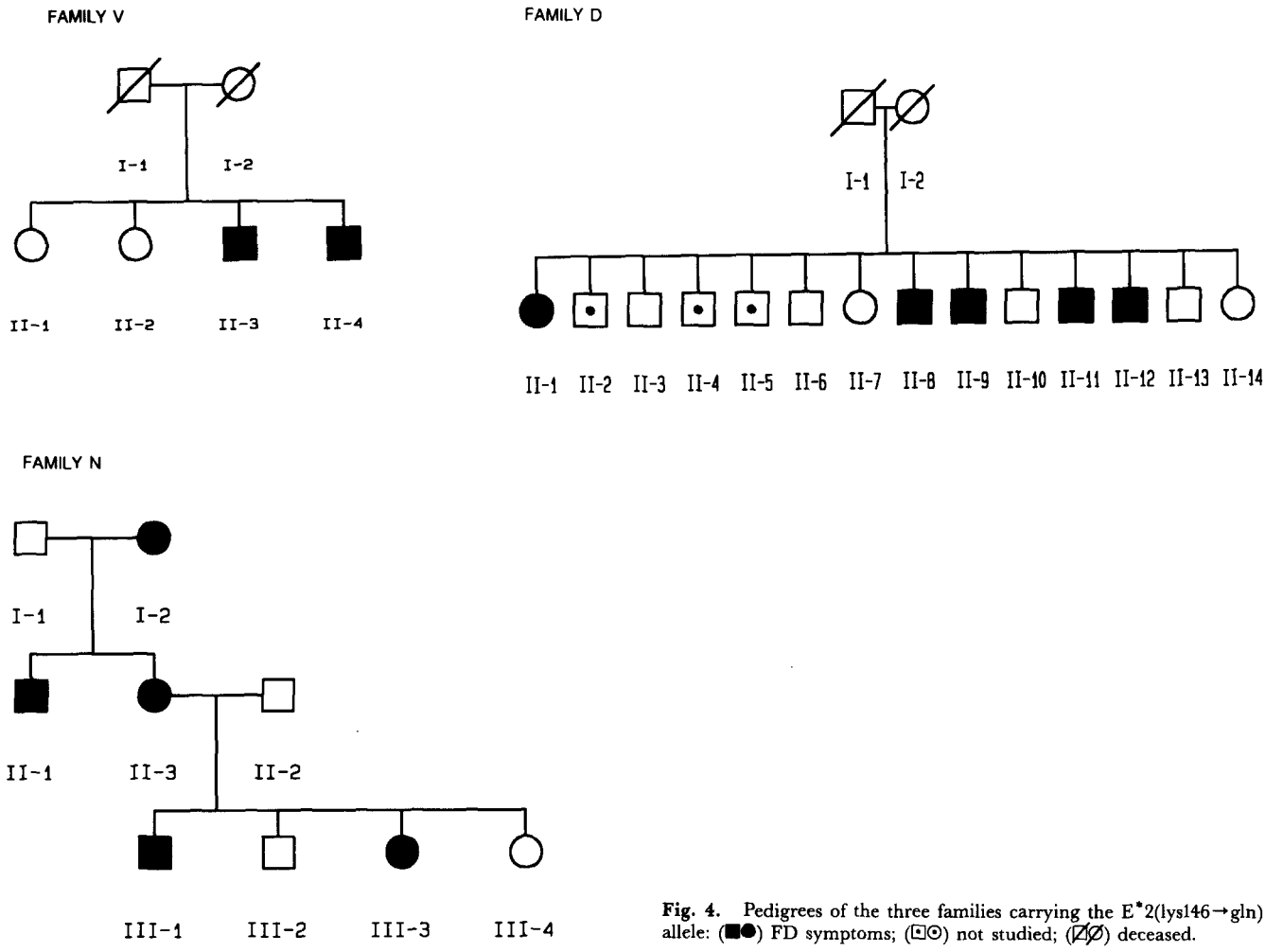

Fig. 4. Pedigrees of the three families carrying the E*2(lys146→gln) allele: (■●) FD symptoms; (⊡⊙) not studied; (⊠⊘) deceased.

we included an in vitro amplification step applying the heat-stable Taq polymerase. Starting with 1 μg of genomic DNA, this procedure produces strong and specific signals within 1 to 3 h of exposure and is therefore suitable for routine screening. A similar procedure, but using Klenow polymerase, was recently published by Smeets et al. (32) for the detection of the E2(arg158→cys) and E4(cys112→arg) mutations.

Using this method, we evaluated the presence of the known E4(cys112→arg), E2(arg158→cys), E2(lys146→gln), E2(arg145→cys), and E3(cys112→arg;arg142→cys) variants in FD patients and in control subjects. We demonstrated that all 40 FD patients with the E2E2 phenotype were homozygous for the mutation arg 158→cys. The same was true for 11 out of 13 clinically normal individuals with the E2E2 phenotype. In addition, 50 normal subjects with the E3E2 phenotype also displayed heterozygosity for the arg158→cys mutation. Since these heterozygotes do not express FD, it is obvious that the E2(arg158→cys) mutation behaves like a recessive trait in the expression of FD (both alleles need to be defective). Furthermore, since both patients and controls with the E2E2 phenotype appeared to be homozygous for the common E2(arg158→ys) variant, it seems that the reduced penetrance of FD in individuals with the E2E2 homozygosity is not due to heterogeneity in the E*2 allele, although the existence of additional unknown mutations within the APOE gene of E2E2 homozygous FD patients cannot be excluded by this approach. For a definite conclusion in this respect, it is necessary to determine the complete nucleotide sequences of the APOE genes of both E2E2 homozygous FD patients and E2E2 homozygous control subjects.

In a previous paper (19) we described three unrelated FD patients with the E3E2 phenotype. The E2 variant present in these patients contains only one cysteine residue and we suggested that it was the E2(lys146→gln) variant previously found in two E3E2 heterozygous FD patients (siblings) by Rall et al. (12). All other known E2 variants contain two cysteine residues (9, 13, 14). In the present study, using the method of amplification followed by hybridization with variant-specific oligonucleotide probes, we were able to prove the presence of the E2(lys146→gln) mutation in these E3E2 heterozygous FD

patients. In one of these FD patients with the E3E2 phenotype we confirmed the E2(lys146→gln) mutation by DNA sequence analysis (Fig. 3). None of the 53 individuals (40 FD patients and 13 controls) with the E2E2 phenotype tested in the present study appeared to carry the E2(lys146→gln) mutation (see Table 3). The same was true for 50 unrelated E3E2 normolipidemic individuals randomly selected from a large population sample previously screened for apoE phenotypes (27, 28). They all appeared to contain the E2(arg158→cys) mutation (Table 3). Additionally, another 70 E3E2 individuals selected from the same population sample were studied by the cysteamine modification followed by isoelectric focusing (19). Two cysteine residues were found in all E2 isoforms tested. Thus, in a total number of 120 E3E2 subjects selected from a random population sample, the presence of the E2(lys146→gln) mutation could not be detected. Based on these results, we conclude that the E*2(lys146→gln) allele is very rare with an allele frequency of less than 0.001 and, so far, exclusively found in FD patients with the E3E2 phenotype.

The E2(lys146→gln) mutation has now been found in several apparently unrelated families; one in the U.S.A. (12) and three in the Netherlands (present study). For the Dutch families, genealogical studies are presently being conducted in order to find out whether these three families share a common ancestor. The two E3E2 heterozygous subjects (siblings) exhibiting the rare E2(lys146→gln) variant found by Rall et al. (12) also express FD. These facts, taken together, strongly suggest that heterozygosity for the lys146→gln mutation is sufficient for the expression of FD. To sustain this dominance hypothesis, we performed detailed family studies. In all three kindreds studied (Fig. 4; Table 4) we found that the E*2(lys146→gln) allele indeed cosegregates with FD except for three subjects (III-4 in family N and II-6 and II-14 in family D). The absence of FD in these E3E2(lys146→gln) heterozygotes might be due to a "healthy" life-style and/or young age. It should be noted in this respect that the FD patients carrying the E*2(lys146→gln) allele also respond to diet and medical treatment. We conclude that, in contrast to the common E2(arg158→cys) variant, the E2(lys146→gln) mutation behaves like a dominant trait in the expression of FD (one defective allele is sufficient for the expression of the disease). This dominance hypothesis does not necessarily imply that in the case of this rare variant the level of penetrance of FD is 100%. Nevertheless, our results show that in the case of the E2(lys146→gln) variant, FD is inherited with a high level of penetrance; whereas in the case of homozygosity for the E2(arg158→cys) mutation, the recessively inherited FD disease is commonly assumed to be inherited with a strongly reduced penetrance (1 to 4%) (29).

Recently, Mann et al. (18) described a kindred in which heterozygosity for an E1 variant (E1-Harrisburg) also cosegregates with the expression of FD. Strikingly, in E1-Harrisburg the mutation is also localized at amino acid position 146 (lys146→glu) suggesting that this lysine residue plays a crucial role in the removal of chylomicron and VLDL remnants in vivo.

Previously, we described the E3-Leiden mutation which also behaves like a dominant trait in the expression of FD (33). Recently, a second family has been discovered in which the E3-Leiden variant cosegregates with familial dysbetalipoproteinemia (Havekes et al., unpublished data). Similarly, the E3(cys112→arg; arg142→cys) also cosegregated with FD in the family in a dominant manner (15).

In summary, we conclude that FD is a genetically heterogenous disease with a recessive form with reduced penetrance in case of E2E2 homozygosity and with a dominant form with high penetrance in case of heterozygosity for a number of different rare apoE variants. The finding of different apoE variants predisposing to FD in a dominant fashion should caution against the use of the E2E2 phenotype as an obligatory diagnostic criterion for FD. In the case of the dominant form, presymptomatic diagnosis is possible.

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