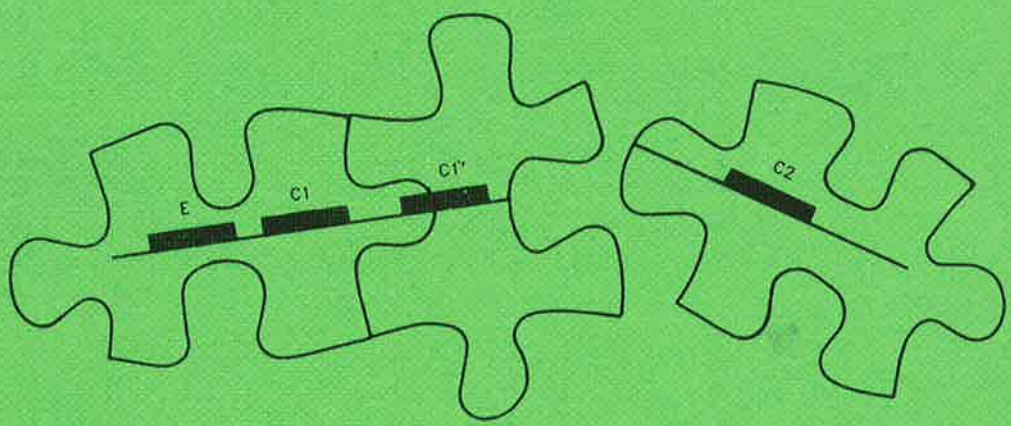
GENETIC ASPECTS OF FAMILIAL DYSBETALIPOPROTEINEMIA



MARIJKE SMIT

GENETIC ASPECTS OF FAMILIAL DYSBETALIPOPROTEINEMIA

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It was a collaborative study of the department of Human Genetics of the State University of Leiden and the Gaubius Institute, Health Research Division TNO.

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STELLINGEN

1 Het bepalen van het apolipoproteïne E genotype verdient de voorkeur boven het bepalen van het apoE fenotype bij de diagnose van familiaire dysbetalipoproteïnemie.

Dit proefschrift

2 Het beschikbaar komen van cholesterolsynthese remmers is een extra rechtvaardiging voor het vroegtijdig screenen op erfelijke hypercholesterolemieën.

3 Bij het doen van dieetstudies met betrekking tot het plasma cholesterolgehalte, is het van belang het apolipoproteïne E fenotype in het onderzoek te betrekken.

4 De suggestie van Tajima *et al.*, dat de totale netto lading van het apolipoproteïne E bepalend zou zijn voor het plasma cholesterolgehalte, is voorbarig.

S. Tajima et al. (1989) J. Biochem. 105: 249-253

5 Bij de bestudering van DNA-bindende eiwitfaktoren en hun eventuele verwantschap, dient men bedacht te zijn op de mogelijkheid, dat de voor binding belangrijke sequentie opgesplitst is.

J.C. Dorsman et al. (1988) Nucl. Acids Res. 16: 7287-7301

6 Als uitgangspunt voor medisch wetenschappelijk onderzoek dient men eerder het belang van de patiënt voor ogen te hebben, dan een mogelijke kostenbesparing in de gezondheidszorg.

7 Het (vrijwel) ontbreken van part-time post-doc plaatsen staat een volledige emancipatie van de vrouw aan de universiteiten in de weg.

8 Mede gezien de toename van derde geldstroom onderzoek aan de universiteiten verdient het aanbeveling in de doktoraalstudie reeds aandacht te besteden aan het octrooirecht.

9 Promoveren is altijd een investering voor de toekomst van de groep waaruit de promovendus afkomstig is, maar niet altijd voor de toekomst van de promovendus zelf.

10 De voordelen van geautomatiseerd literatuuronderzoek en automatische attendering worden nog niet ten volle onderkend en benut. De faciliteiten hiervoor dienen standaard deel uit te maken van een wetenschappelijke bibliotheek.

11 Tabak is een onkruid.

7 juni 1989 Marijke Smit

GENETIC ASPECTS OF FAMILIAL DYSBETALIPOPROTEINEMIA

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Aan pap en mam

Aan Bert

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Chapter 1

General Introduction

1.1 **Introduction**

Over the past few decades a great amount of scientific effort has been put in finding risk factors for atherosclerosis, since this disease is one of the major health problems in the Western society.
44 percent of all deaths in the Netherlands in 1986 was attributable to coronary and peripheral vascular disease [1], which is mainly caused by atherosclerosis. The incidence of coronary heart disease is lower in females than in males [2].
Epidemiological studies have established multiple additional (environmental) risk factors, such as high cholesterol level, high blood pressure, diabetes, diet, overweight, lack of exercise, a positive family history, age and smoking [3,4].
Nevertheless, adherence to a healthy life style, *i.e.* the avoidance of as many environmental risk factors as possible, does not always guarantee that individuals will stay free from atherosclerosis. The opposite is also true: people can live to their 80's or 90's in spite of an unhealthy life style. Besides environmental factors, genetic factors have also been shown to be involved in the susceptibility of an individual to develop atherosclerosis [2,5]. Consequently, atherosclerosis is caused by an interaction of both genetic and environmental factors.

An important risk factor for atherosclerosis is hyperlipoproteinemia, which is characterized by elevated levels of cholesterol- and/or triglyceride- carrying proteins, called lipoproteins (see 1.2). Epidemiological studies have shown that the lipoprotein levels in the plasma are strong predictors for atherosclerosis in the population [6]. In particular, the level of two of the plasma lipoproteins (HDL and LDL, see 1.2) have been shown to be strongly associated with the development of atherosclerosis. Increased LDL and decreased HDL levels are powerful predictors of atherosclerosis [7,8].
It appears that a proportion of the hyperlipoproteinemias are genetically determined. Some of them are monogenetic (*e.g.* familial hypercholesterolemia [9]), whereas others are more complex genetic disorders, in which probably more than one gene is involved (*e.g.* familial dysbetalipoproteinemia, see 1.4 for more details).

A better characterization of the genes affecting the plasma lipid levels will provide opportunities to establish the individual's susceptibility to develop atherosclerosis.

Table 1. Physical properties and composition of human plasma lipoproteins [11,12]

Lipo-protein	electro-phoretic mobility+	particle size (nm)	molecular weight (kD)	density (g/ml)	composition (% wt) tg	phos	chol	prot	Major apolipoproteins
chylo-microns	origin	75-1200	400.000	< 0.94	80-95	3-6	3-7	1-2	A1,A4,B48*,C1,C2,C3,E
VLDL	pre-ß	30-80	10.000-80.000	0.94-1.006	45-65	15-20	20-30	6-10	B100*,E,C1,C2,C3
LDL	ß	18-25	2.300	1.019-1.063	4-8	18-24	51-58	18-22	B100*
HDL	α	5-12	175-360	1.063-1.210	2-7	26-32	18-25	45-55	A1,A2,C1,C2,C3,E

+ according to the mobility of plasma α- and ß-globulins on agarose gel electrophoresis.
* The apoB48 protein corresponds to the amino-terminal half of the apoB100 protein. Both proteins are the products of a single APOB gene. ApoB48 is predominantly produced in the intestine from mRNAs which differ from the apoB100 mRNAs in the liver by a C→U change in codon 2153, resulting in an in-frame-stopcodon [164].
tg: triglycerides; phos: phospholipids; chol: cholesterol; prot: proteins; wt: weight.

1.2 **Cholesterol and triglyceride metabolism**

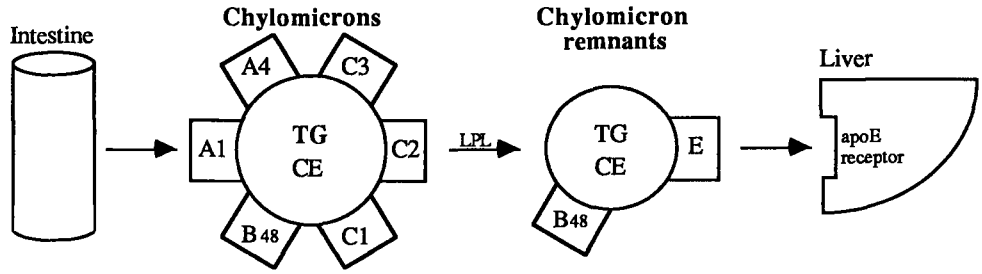
To identify the factors involved in the development of atherosclerosis, much attention has been paid to the unravelling of the lipid metabolism over the past decades.
The major plasma lipids are cholesteryl ester and triglyceride. The triglycerides are used for energy and storage in muscle and adipose tissues, respectively, whereas cholesterol (after hydrolysis of the cholesteryl esters) is used for the synthesis of plasma membranes, steroid hormones in special steroidogeneic organs and bile acids in the liver.
Cholesterol and triglycerides are hydrophobic molecules, as such unsoluble in the hydrophilic blood. Therefore, the plasma lipids are transported and metabolized in the form of lipid-protein complexes, called lipoproteins. Lipoproteins are spherical particles. The core consists of nonpolar neutral lipids, mainly cholesteryl esters and triglycerides. The surface consists of amphipathic substances, such as proteins (called apolipoproteins), phospholipids and free cholesterol [10]. Several types of apolipoproteins have been described: apoA1, apoA2, apoA4, apoB (B48 and B100), apoC1, apoC2, apoC3, apoD and apoE.
The lipoproteins are divided into four major classes according to their density, as determined by ultracentrifugation: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). They also differ in size, composition and electrophoretic mobility (see table 1).
In addition to the four major lipoproteins, two other lipoprotein particles are recognized: the chylomicron remnants and the VLDL remnants, the latter one also called intermediate density lipoproteins (IDL). These remnant particles are formed during the metabolism of the chylomicrons and VLDL particles, respectively.

The lipid metabolism can be divided into three pathways: 1) the metabolism of exogenous lipids (dietary fats), 2) the metabolism of endogenously synthesized lipids (mainly in the liver) and 3) the reverse cholesterol transport from peripheral tissues to the liver [for reviews see refs 11,13 and 14] (see figure 1).

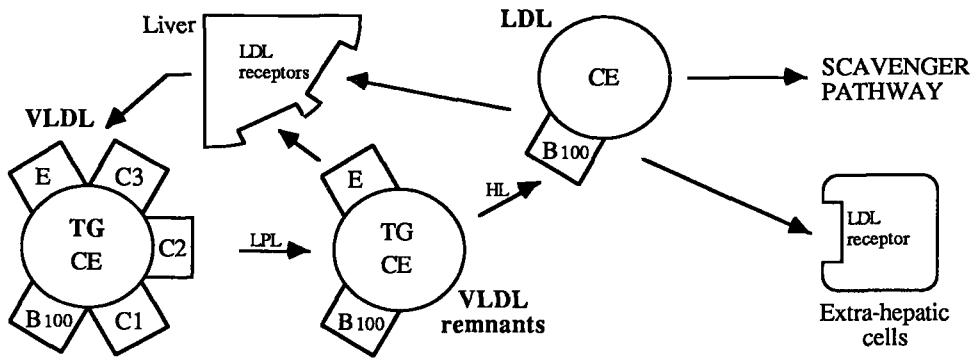
1.2.1 *Exogenous lipid transport*

Dietary lipids, in particular cholesteryl esters and triglycerides are packaged in the intestinal mucosal cells into large triglyceride-rich particles, called chylomicrons. On the surface the chylomicrons contain the apolipoproteins B48, A1 and A4.
The chylomicrons are secreted from the intestine and enter the blood stream via the lymph. Upon entering the circulation, the chylomicrons loose their apoA1 and the major part of apoA4 and acquire apoC1, apoC2 and apoC3. Subsequently, their triglycerides are rapidly lipolyzed by the enzyme lipoprotein lipase (LPL), with apoC2 acting as a co-factor. This enzyme is attached to the endothelial cells lining the blood capillaries. The products of the triglyceride hydrolysis, the fatty acids, are transported to muscle

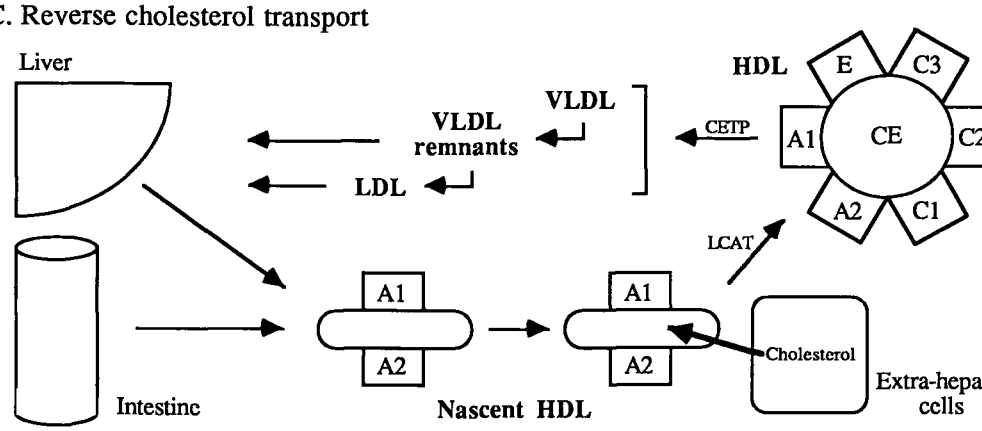
A. Exogenous lipid transport



B. Endogenous lipid transport



C. Reverse cholesterol transport



tissues (for energy) and to adipose tissue (for storage).
In the circulation and during the process of hydrolysis, excess surface components (phospholipids and apolipoproteins A and C) are transferred to HDL, and apoE is transferred from HDL to the chylomicron particle, which becomes relatively poor in triglyceride, but enriched in cholesterol (chylomicron remnant). These remnants are then rapidly taken up by the liver through receptor-mediated endocytosis. The hepatic receptors, the putative chylomicron remnant- or apoE receptors, specifically recognize apoE, present on the remnant surface. It has been suggested that premature uptake of unhydrolyzed chylomicrons by the apoE receptor is prevented by the presence of apoC3 [14,15]. Loss of apoC3 during the remnant formation would permit the subsequent binding of the remnants to the receptors. The dietary cholesterol taken up by the liver through the chylomicron (exogenous) pathway is used for the synthesis of bile acids and VLDL.

1.2.2 *Endogenous lipid transport*

In the liver, free fatty acids and carbohydrates are converted into triglycerides. These endogenously synthesized triglycerides are packaged into VLDL particles together with cholesterol. The cholesterol used for VLDL production is dietary cholesterol, that has entered the liver via uptake of chylomicron remnants as well as cholesterol synthesized by the liver (endogenous cholesterol).
VLDLs are, like chylomicrons, rich in triglycerides, and contain the apolipoproteins B100, C1, C2, C3 and E. After secretion into the circulation, the VLDL triglycerides are hydrolyzed by lipoprotein lipase, resulting in the formation of VLDL remnants (or IDL). This process is basically the same as for the chylomicrons. During this process the major surface components of the VLDL particles (phospholipids, apoC1, apoC2 and apoC3) are transferred to HDL. The VLDL particles increase in density and become enriched in cholesteryl ester and apoE. The particles retain their apoB100.
The major part of the VLDL remnants is cleared from the plasma by hepatic LDL receptors, through receptor-mediated endocytosis [11]. These receptors recognize apoE on the VLDL remnant surface. Analogous to the chylomicrons, apoC3 prevents premature uptake of VLDL by the liver [14,15].
The remaining part of the VLDL remnants is further metabolized into LDL. A second lipolytic enzyme, hepatic lipase, has been proposed to be involved in the conversion of VLDL remnants to LDL particles [16]. The formation of LDL from VLDL remnants is accompanied by a further loss of triglycerides, phospholipids, apoC and apoE. LDL is rich in cholesteryl ester and contains apoB100 as the only protein component on its surface. In humans, about 2/3 of the total plasma cholesterol is present in the LDL fraction. ApoB100 in LDL acts as a ligand for the LDL receptor and most LDL

← **Figure 1.** Schematic illustration of the lipid metabolism.
TG: triglycerides; CE: cholesteryl ester; E: apoE, etc.

particles are cleared by the liver. A smaller part is cleared through extra-hepatic tissues (*e.g.* adrenal gland, adipose tissue and muscle) [17]. The affinity of the (hepatic) LDL receptor for VLDL remnants or LDL particles is different. VLDL remnants, containing apoE, are very efficiently taken up by the liver, because they contain several molecules of apoE and hence are able to bind multivalently to the LDL receptors. LDL, in contrast, binds to the receptor monovalently via a single molecule of apoB100, resulting in a lower affinity [18].
The fraction of LDL, which is not taken up by the LDL-receptor, is cleared from the plasma by other less efficient, receptor independent, mechanisms in which cells of the reticuloendothelial system, like macrophages, are involved. The amount of LDL cleared by this "scavenger pathway" is linearly correlated with the plasma level of LDL. Thus high plasma LDL levels will result in overloading of macrophages in the tunica intima of the bloodvessels with cholesteryl esters. This process is called "foam cell formation" and is commonly assumed to be one of the initial steps in the process of atherosclerosis [19,20].

1.2.3 *The reverse cholesterol transport*

Elimination of cholesterol from the body occurs primarily through the liver via bile acid formation and secretion. It is essential therefore, that the surplus of cholesterol, which cannot be metabolized by the peripheral tissues, is transported back to the liver. This process is called the reverse cholesterol transport.
In the liver and intestine, nascent HDL particles are formed and secreted into the blood stream. These disc-like particles, containing mainly apoA1, apoA2 and phospholipids, accept free cholesterol from extra-hepatic tissues, thereby preventing overloading of these tissues with cholesterol. The cholesterol taken up from peripheral cells by nascent HDL is subsequently esterified by the lecithin cholesterol acyltransferase (LCAT) enzyme, bound to the HDL particle and utilizing apoA1 as a co-factor.
The cholesteryl ester is directed into the core of HDL and complexes with new surface components *i.e.* phospholipids and apolipoproteins (derived from the chylomicron and VLDL catabolism) from which mature spherical HDL is formed.
Subsequently, the HDL-cholesteryl ester is transferred to VLDL, VLDL remnants and probably LDL, by the action of the cholesteryl ester transfer protein (CETP) and is eventually taken up by the liver via the LDL receptors. The role in the reverse cholesterol transport is considered to be the basis of the 'anti-atherogenic' effect of HDL particles.

1.2.4 *Regulation of the LDL receptor activity*

An important aspect of the whole lipid metabolism is the regulation of the LDL receptor activity. Brown and Goldstein have elucidated this regulation mechanism and

were awarded the Nobel Prize for medicine in 1985 for their work.
They showed that the number of LDL receptors on the cell surface can be regulated by the amount of unesterified cholesterol available within the cell [21]. When cells accumulate excess cholesterol, the production of LDL receptors is down-regulated. Conversely, when cells are deprived of cholesterol, they transcribe the LDL receptor gene at a high rate and produce increased amounts of LDL receptor protein [9,22,23].
Endogenous cholesterol synthesis is also regulated by the cellular cholesterol level. When there is sufficient cholesterol present in the cell, the endogenous synthesis is suppressed as a result of inhibiting HMGCoA reductase, which is the rate limiting enzyme in the biosynthesis of cholesterol [24]. Conversely, when the amount of cellular cholesterol is decreased, the activity of the enzyme HMGCoA reductase, like the LDL receptor activity, is increased.
As the liver is by far the most important organ in both LDL catabolism and endogenous cholesterol synthesis, it is obvious that plasma LDL cholesterol levels are directly related to the hepatic demand for cholesterol.
The strategy for the treatment of patients with hypercholesterolemia is to decrease the hepatic cellular cholesterol content. This can now easily be achieved by administration of specific HMGCoA reductase inhibitors [25].

1.2.5 *Disturbances in lipoprotein metabolism: hyperlipoproteinemias*

Disturbances in lipoprotein metabolism often result in an accumulation of specific lipoproteins in the plasma, called hyperlipoproteinemia (HLP).
In 1967, Fredrickson *et al.* described a classification of hyperlipoproteinemia based on lipoprotein patterns in plasma. Six HLP phenotypes were delineated [26,27], see table 2. Later, it was observed that, not only were there specific phenotypes associated with genetically determined hyperlipidemia, but also that there could be more than one phenotype present in a given genetic form of hyperlipidemia. The recognized genetic hyperlipidemias are also summarized in table 2.

In this paragraph we have seen that apolipoproteins are very important in lipoprotein metabolism. They have three fundamental functions (see table 3) as: 1) structural components of the lipoproteins, 2) co-factors for specific enzymes involved in lipid metabolism, 3) ligands for specific receptors facilitating the binding and uptake of the lipoproteins.

Many studies have been performed to characterize the normal and variant apolipoproteins. In the next paragraph the structure and evolution of the apolipoprotein genes will be discussed as well as the use of Restriction Fragment Length Polymorphism (RFLP) studies to detect genetic heterogeneity in these genes in relation to atherosclerosis.

Table 2. Characteristics of the various hyperlipoproteinemia phenotypes, according to Fredrickson et al. [25] and their associations with genetic disorders [11,26,27]

phenotype	common name	lipoprotein and lipid abnormalities	associations with genetic disorders
I	exogenous hyperlipemia	* chylomicrons present and increased * VLDL, LDL, HDL normal or decreased * cholesterol level normal or increased * triglyceride level increased	Familial LPL deficiency ApoC2 deficiency
IIa	hypercholesterolemia	* LDL increased * VLDL normal * cholesterol level increased * triglyceride level normal	Familial hypercholesterolemia LDL receptor abnormal Polygenic hypercholesterolemia Familial combined hypercholesterolemia
IIb	combined hyperlipidemia	* LDL increased * VLDL increased * cholesterol level increased * triglyceride level increased	Familial combined hyperlipidemia Familial hypercholesterolemia
III	dysbetalipoproteinemia	* presence of ß-VLDL or VLDL remnants * cholesterol level increased * triglyceride level increased	Familial dysbetalipoproteinemia
IV	endogenous hyperlipemia	* VLDL increased * chylomicrons absent * LDL normal * cholesterol level normal or increased * triglyceride level increased	Familial hypertriglyceridemia Familial combined hypertriglyceridemia
V	mixed hyperlipidemia	* chylomicrons present and increased * VLDL increased * cholesterol level increased * triglyceride level increased	Familial hypertriglyceridemia Familial combined hyperlipidemia

Table 3. Functions and properties of the apolipoproteins [10,13,28]

apolipo-protein	molecular weight (kD)	plasma conc. (g/l)	isoelectric point (pI)	function
A1	28	1.0-1.2	5.4-5.9	tissue cholesterol efflux LCAT activation
A2	17	0.3-0.5	5.0	structural protein of HDL
A4	46	0.16	5.5	LCAT activation
B100	550	0.7-1.0	-	VLDL production ligand for LDL receptor
B48	275	0.03-0.05	-	chylomicron production
C1	7	0.04-0.06	7.5	LCAT activation (moderate)
C2	9	0.03-0.05	4.9	LPL activation
C3	9	0.12-0.14	4.7-5.0	lipase inhibition
D	33	0.06-0.07	5.0-5.2	cholesterol transport
E	34	0.03-0.05	5.7-6.0	ligand for apoE- and LDL-receptor

LCAT: lecithin cholesterol acyltransferase; HDL: high density lipoproteins; VLDL: very low density lipoproteins; LDL: low density lipoproteins; LPL: lipoprotein lipase

1.3 **Apolipoprotein genes**

1.3.1 *Chromosomal localization of the apolipoprotein genes*

Over the last few years, the genes coding for the apolipoproteins A1, A2, A4, B, C1, C2, C3, D and E have been isolated and localized to specific chromosomes using somatic cell hybrids and/or *in situ* hybridizations.
The genes coding for the nine apolipoproteins are distributed over five different chromosomes (see table 4).
APOA2 has been localized on chromosome 1. APOB has been localized on chromosome 2 and the APOD gene was recently localized on chromosome 8. On

Table 4. Chromosomal localization of genes involved in lipid metabolism [33,34]

name		chromosomal localization	references
apolipoprotein A1	APOA1	11q23-qter	35,36
apolipoprotein A2	APOA2	1q21-q23	37-39
apolipoprotein A4	APOA4	11q23-qter	29,35
apolipoprotein B	APOB	2p23-p24	40-43
apolipoprotein C1	APOC1	19q12-q13.2	37,44-46
apolipoprotein C2	APOC2	19q12-q13.2	44,46-49
apolipoprotein C3	APOC3	11q23-qter	35
apolipoprotein D	APOD	3p14.2-qter	50
apolipoprotein E	APOE	19q12-q13.2	44,46,51,52
low density lipoprotein receptor	LDLR	19p13.2-p13.1	44,53,54
cholesteryl ester transfer protein	CETP	16q12-q21	55
lipoprotein lipase	LPL	8p22	56
lecithin cholesterol acyltransferase	LCAT	16q22	57
hepatic lipase	HL	15q21	56
3-hydroxy-3-methyl glutaryl coenzyme A reductase	HMGCR	5q13.3-q14	54,58
3-hydroxy-3-methyl glutaryl coenzyme A synthase	HMGCS	5p14-p13	59,60

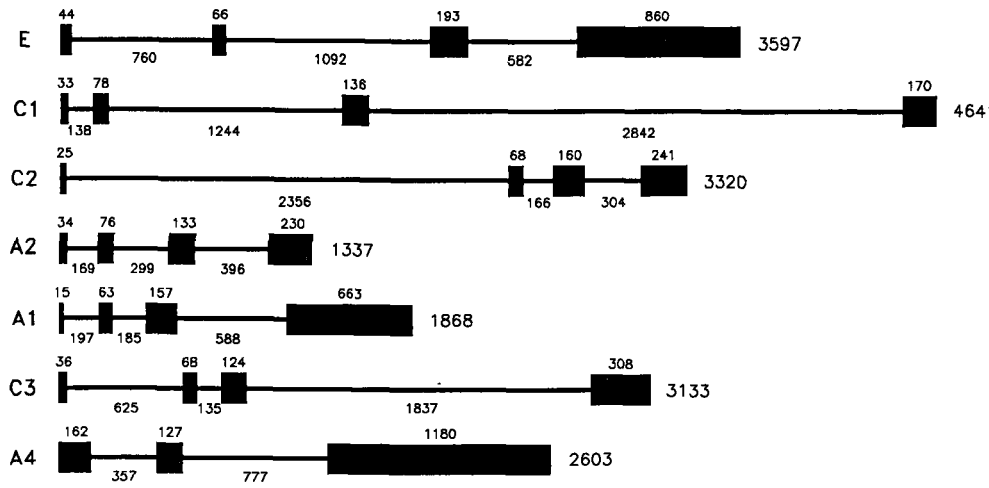
chromosome 11, there is a cluster of genes, spanning no more than 15 kb, coding for APOA1, APOC3 and APOA4. The APOC3 gene is positioned between the APOA1 and APOA4 genes and is orientated in the opposite direction [29]. Another apolipoprotein gene cluster is present on chromosome 19: APOE, APOC1 and APOC2 are located within a 45 kb stretch of DNA and are all orientated in the same direction [30-32, this thesis]. This cluster contains two copies of the APOC1 gene, separated by 7.5 kb. The most upstream APOC1 gene codes for the known apoC1 protein, whereas the downstream APOC1 gene is believed to be a pseudogene (APOC1P1) [32].
Various genes, encoding enzymes and receptors important in lipid metabolism have also been localized (see table 4).

1.3.2 *The apolipoprotein multigene family*

The genes encoding APOE, APOC1, APOC2, APOC3, APOA1 and APOA2 have a similar structure, consisting of four exons separated by three introns (see figure 2). The introns are located in similar positions in these genes. The first intron is located in the 5' untranslated region of the corresponding mRNA, the second intron is located in the signal peptide of the protein coding region and the third intron interrupts the part of the gene encoding the mature plasma protein (at positions between residue 39 and 61, depending on the gene concerned). The APOA4 gene differs from the other genes by having only three exons (figure 2). It lacks the first intron in the 5'- untranslated region. The two other introns in this gene are present at the same positions as in the other six structurally related genes.
Characterization, including nucleotide sequence analysis of these seven genes revealed that they are relatively small with an overall length of 1337 bp (APOA2) to 4641 bp (APOC1). The length of the first three exons (the first two exons for APOA4) are very similar for all genes. The differences in the total length of the genes are mainly accounted for by the differences in the length of exon 4 (exon 3 for APOA4). These seven genes have been regarded as members of an apolipoprotein gene family because of their structural similarities [for a review see ref 61].

The genes coding for APOB and APOD show significant differences compared to the other seven apolipoprotein genes. The human APOB gene has a length of 43 kb, containing 29 exons and 28 introns. The APOD gene is about 12 kb long. It lacks, in contrast to the other seven APO genes, an intron in the signal peptide region. Furthermore, the APOD gene contains 3 introns (compared to one in the other genes) in the mature peptide coding region.
The different structures of the APOB and APOD genes suggest that they do not belong to the apolipoprotein multigene family [61].

Figure 2. Structure of the apolipoprotein genes A1, A2, A4, C1, C2, C3 and E. Wide bars represent the exons. Numbers indicate distances in nucleotides.



1.3.3 *Evolution of the apolipoprotein genes*

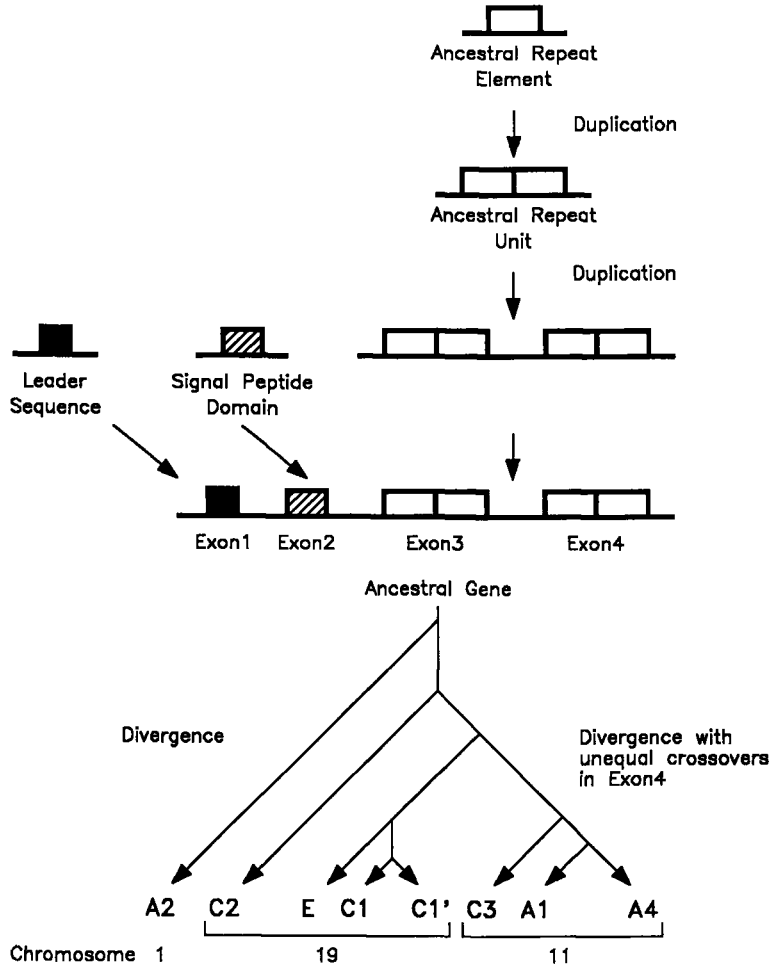
In 1977, Barker and Dayhoff [62], Fitch [63] and McLahlan [64] independently observed that the apoA1 protein contains multiple repeats of 22 amino acids (22-mers), made up of two 11-mers. Based on amino acid sequence data of several apolipoproteins, Barker and Dayhoff suggested, that they are derived from a common evolutionary precursor [62].

In the last few years, the existence of a 22-mer periodicity has been established in the apolipoproteins A1, A2, A4, C2, C3 and E [52,65-72]. In APOC1 one 11-mer was found [32,65]. Luo *et al.* [65], examined the nucleotide and amino acid sequence data in detail with computer analysis and discovered that the mature peptide regions of the genes are almost completely made up of tandem repeats of 11 codons. Exon 3 contains a common block of 33 codons, whereas exon 4 contains a variable number of 22-mers, made up of 11-mers.

Based on the close sequence homologies and the tandem associations of the repeat units, as well as the close similarities in gene structures, Luo *et al.* [65] have suggested that the genes for the apolipoproteins A1, A2, A4, C1, C2, C3 and E evolved from a common ancestral gene. These authors have proposed an evolutionary scenario for this multigene family in which the genes may have evolved from an ancestral gene through multiple internal duplications of the repeat blocks as well as duplications of entire genes. They believe that the ancestral gene had 3 introns at the same locations as the present APO genes and contained a block of 33 codons in the third exon and a repeat

unit of 11 codons in exon 4. In length and structure the ancestral gene is believed to be very similar to the APOC1 gene.
Taylor and co-workers [32,73] suggested a somewhat different evolutionary scheme, which takes into account not only the internal multiple repeats found in the apolipoprotein genes, but also their clustering on the specific chromosomes (figure 3).

Figure 3. Hypothetical scheme for the evolution of the apolipoprotein genes according to Taylor *et al.* [73,32].



1.3.4 *Apolipoprotein gene RFLPs and their possible relevance in predicting susceptibility to atherosclerosis*

Once cDNA or genomic probes for the apolipoproteins became readily available, much time and effort has been and still is being invested in identifying Restriction Fragment Length Polymorphisms (RFLPs) with these probes in an attempt to find DNA markers, that can be used as predictors for atherosclerosis.
Restriction site fragment length polymorphism is due to (minor) genetic variation among individuals within a population. The size of DNA fragments produced by restriction enzyme digestion changes when DNA has been inserted or deleted, or when point mutations have affected restriction cleavage sites. The latter type of RFLP is most commonly encountered in the kind of studies dealt with in this thesis. An RFLP is generally detected by hybridization of Southern blots [74]: genomic DNA of several individuals is digested with the appropriate restriction enzyme, the fragments are separated on an agarose gel and transferred to a membrane; subsequently, the fragments of interest are revealed by hybridization with a probe homologous to a region adjacent to the so-called polymorphic site [75].
So far, most studies have used RFLPs in the study of genetic hyperlipidemia to search for population associations between the neutral genetic variations detected by the RFLP and functionally significant variation in the relevant (nearby) gene. The generally applied approach is to determine the frequency of particular RFLP genotypes in a group of patients with a particular form of hyperlipidemia ("clinical phenotype") and in a random population sample. The relationship between an RFLP and disease susceptibility should be interpreted in terms of linkage disequilibrium. Linkage disequilibrium is the concomitant occurrence (association) of certain alleles on the same chromosome with a frequency different from the one expected on the basis of the frequencies of the individual alleles. Such a difference in frequency indicates that variation at or near this locus is involved in the development of the clinical phenotype under study.
When an association is observed, it has still to be determined whether a defect in an apolipoprotein gene or another as yet undetermined linked gene, is involved.
Many RFLPs have been reported for the apolipoprotein genes during the past 5 years. Some have been found to be associated with clinical phenotypes (tabel 5).
In this section, the most important RFLPs for each apolipoprotein gene or apolipoprotein gene cluster will be discussed.

At the APOA2 gene locus on chromosome 1, an RFLP with the enzyme MspI has been reported [76]. In this study, homozygosity for the minor allele was associated with significant higher plasma levels of apoA2 [76]. This association was not found in two other independent studies [13,77]. Ferns *et al.* [78] demonstrated that hypertriglyceridemic patients had a decreased frequency of the minor allele compared with normal controls (0.09 vs 0.19). The study of Deeb *et al.* [79], confirmed the association of the common allele with high levels of triglyceride and in addition they found a

significant association of the common allele with lower HDL levels.
On chromosome 2, the APOB RFLP with the restriction enzyme XbaI has been shown to be associated with altered lipid- and apolipoprotein levels and myocardial infarction. Law *et al.* found, in an English population sample, that the smaller XbaI allele (5 kb) was strongly associated with increased levels of total cholesterol and triglycerides [80]. The strong association with elevated cholesterol levels was confirmed by Berg *et al.* [81] and Talmud *et al.* [82]. Berg *et al.* [81] also found an association with increased apoB levels. Deeb *et al.* [79] found a significant association of the large 8.5 kb allele with higher triglyceride levels. A significant difference in allele frequency for the XbaI RFLP between normolipidemic individuals and FD patients has been described by Talmud *et al.* [82]. Hegele *et al.* [83] have examined the XbaI RFLP in patients with myocardial infarction (MI) and in matched controls. They found that the frequency of the large XbaI allele (8.6 kb) was significantly increased in the MI patients (0.64 vs 0.50). In this study, the APOB alleles were not significantly associated with elevated levels of LDL-cholesterol or apoB protein.

The chromosome 11 apolipoprotein gene cluster (APOA1-C3-A4) is the most extensively studied to date. Several studies suggest that polymorphisms at this locus are associated with various hyperlipidemias, although the results are not fully in agreement with each other.
The SstI (SacI) polymorphism, detectable with both APOA1 and APOC3 probes is the most extensively studied RFLP in this gene cluster. The frequency of the minor allele varies considerably between different racial groups: *e.g.* 0.01-0.17 in Caucasians [86-93], 0.27 in Negroes [93], 0.38 in American Indians [89] and 0.33-0.35 in Japanese [93,94]. Rees *et al.* [86] found a significant association between this SstI polymorphism and hypertriglyceridemia (Fredrickson type IV and V) in an English population. Other studies confirmed this finding [97] and additionally revealed that patients with coronary artery disease (CAD) and myocardial infarction (MI) also had an increased minor allele frequency when compared to normal controls [79,90]. Hegele *et al.*, however, found no significant increase in minor allele frequency in MI patients (0.04 vs 0.02) [13]. Kessling *et al.* [88] found an increase in minor allele frequency only in patients with type V hyperlipoproteinemia.

In the chromosome 19 (APOE-C1-C2) gene cluster, 8 RFLPs have been detected by various probes.
The HpaI/APOE RFLP has an increased frequency of the minor allele in type III HLP patients when compared with normal controls [103].
Assmann *et al.* [99] and Humphries *et al.* [100] could not detect significant associations for the TaqI/APOC2 RFLP with either serum lipid levels or atherosclerosis in European populations. Deeb *et al.* however, found significantly higher frequencies of the 3.8 kb (common) TaqI allele in individuals with elevated levels of total cholesterol [79].
For the RFLPs with the APOC1 gene on chromosome 19 no clinical association studies have been reported yet.

Table 5. Apolipoprotein RFLPs and their associations with clinical abnormalities

gene	enzyme	fragment sizes (kb)	allele frequencies	population	significant association with	reference
APOA2	MspI	3.7 / 3.0	0.19 / 0.81	white Eur Cauc	apoA2 level	[76]
				Germans	-	[77]
				Caucasians	triglyceride level	[78]
			0.13 / 0.87	Caucasians	triglyceride level and HDL level	[79]
APOB	XbaI	8.6 / 5.0	?	Europeans	triglyceride level	[80]
			0.48 / 0.52	Europeans	apoB level and cholesterol level	[81]
			0.50 / 0.50	Europeans	cholesterol level	[82]
			0.50 / 0.50	white Americans	MI	[83]
			0.60 / 0.40	white Europeans	-	[84]
			0.51 / 0.49	Europeans	-	[85]
			0.50 / 0.50	Caucasians	triglyceride level	[79]
APOA1/C3	SstI	4.2 / 3.2	0.95 / 0.05	Europeans	triglyceride level	[86]
			0.83 / 0.17	Norwegians	-	[87]
			0.94 / 0.06	Britons	type V HLP	[88]
			0.62 / 0.38	American Indians	-	[89]
			0.94 / 0.06	Europeans	-	[89]
			0.96 / 0.04	Blacks	-	[89]
			0.98 / 0.02	Caucasians	MI	[90]
			0.96 / 0.04	Britons	-	[91]
			0.94 / 0.06	Blacks	-	[92]
			0.90 / 0.10	Coloureds	-	[92]
			0.99 / 0.01	Cauc (not Afr speaking)	-	[92]
			0.88 / 0.12	Cauc (Afr speaking)	-	[92]
			0.99 / 0.01	Caucasians	-	[93]
			0.73 / 0.27	Negroes	-	[93]
			0.81 / 0.29	Indian Asians	-	[93]
			0.65 / 0.35	Japanese	-	[93]
			0.67 / 0.33	Japanese	-	[94]

			0.94 / 0.06	Caucasians	CAD	[79]
			0.95 / 0.05	Canadians	-	[95]
			0.52 / 0.48	Chinese	-	[96]
			0.81 / 0.19	Japanese	-	[96]
			0.85 / 0.15	Africans	-	[96]
			0.82 / 0.18	Indian Asians	-	[96]
			1.00 / 0.00	Caucasians	triglyceride level	[96]
			?	Caucasians	triglyceride level	[97]
APOC2	TaqI	3.8 / 3.5	0.59 / 0.41	Europeans	-	[98]
			0.56 / 0.44	Europeans	-	[99]
			0.56 / 0.44	Caucasians	-	[100]
			0.56 / 0.44	Europeans	-	[101]
			0.60 / 0.40	Caucasian	-	[102]
			0.44 / 0.56	Japanese	-	[102]
			0.42 / 0.58	Chinese	-	[102]
			0.50 / 0.50	Asian Indians	-	[102]
			0.52 / 0.48	African	-	[102]
			0.48 / 0.52	West Indians	-	[102]
			0.65 / 0.35	Caucasians	cholesterol level	[79]
APOE	HpaI	60 / 20	0.62 / 0.38	Europeans	type III HLP	[103]

CAD: coronary artery disease; MI: myocardial infarction; Afr: Afrikaans

It is obvious that there are large discrepancies between the results of the various groups. These discrepancies can be due to the small sample sizes involved and to differences in criteria used to define patients. However, the most important reason for the differences is probably the variation in allele frequencies between races or within genetic subgroups of races (see table 5 or example SstI/A1).
This variation hampers the interpretation of possible asssociations. Differences between cases and controls will only be meaningful, when both groups have the same genetic background.
To improve the diagnostic power of the RFLPs, haplotype analysis should be carried out, since haplotypes may be more specific for particular mutations than single RFLPs. Ferns *et al.*, applied haplotype analysis to a group of Caucasian survivors of myocardial infarction with the MspI, SstI and PstI RFLPs of the APOA1 gene. He was unable to find a haplotype that gave a better discrimination than the use of single RFLPs [90]. In a Japanese population, Rees *et al.* [94] found a haplotype M2-S1 (MspI/A1 + SstI/A1) which was significantly increased in hypertriglyceridic individuals, whereas there was no difference in the allele frequencies of the individual RFLPs between normolipidemic and hyperlipidemic Japanese.
At present, the published data suggest that variation within the apolipoprotein genes contributes to atherosclerotic risk but the associations are weak and may not be the same in different populations. RFLPs of the apolipoprotein genes are not currently useful as predictive markers for the identification of individuals at risk, but have only indicated possible candidate genes [104]. Therefore, it is important to perform family studies, *i.e.* to study the co-segregation between a candidate gene or gene cluster and the clinical phenotype of atherosclerosis. If co-segregation occurs, the genes of patients and healthy controls should be isolated and analyzed in detail.

1.4 **Apolipoprotein E and familial dysbetalipoproteinemia**

1.4.1 *Physiological role of apoE*

In normal individuals, the chylomicron- and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver [105]. The apolipoprotein (apo) E, which is present on the lipoprotein remnants, plays an important role as ligand for binding to the high affinity hepatic lipoprotein receptors [106].
Apolipoprotein E (apoE) was first identified by Shore and Shore [107]. It is a protein with a molecular mass of 34 kD and is one of the major protein constituents of chylomicrons and VLDL.
In humans, apoE is synthesized primarily by the liver but it is also produced by a variety of non-hepatic tissues such as brain, adrenal and kidney [108,109].
In addition to the major physiological role of apoE in mediating the binding of the remnants to the hepatic lipoprotein receptors, apoE is also involved in the reverse

cholesterol transport from cells with excess cholesterol to cells requiring cholesterol [for a review see ref 110]. Other possible functions of apoE in the immune system and in the nervous system are more speculative [for a review see ref 110].

1.4.2. *Polymorphism of apoE*

With isoelectric focusing, apoE can be separated into three common isoforms: E2, E3 and E4, encoded by three different codominant APOE alleles (designated E*2, E*3 and E*4, according to the International System for Human Gene Nomenclature 1979 [111]) at a single gene locus on chromosome 19 [112-116].
ApoE4 is the most basic isoform, whereas apoE2 is the most acid variant. There are three homozygous phenotypes (E4E4, E3E3 and E2E2) and three heterozygous phenotypes (E4E3, E4E2 and E3E2). In Caucasians the APOE allele frequencies are: E*4: 14-15%; E*3: 74-78% and E*2: 8-12% [117-120], suggesting that the APOE*3 allele represents the wild type allele.

Recently a new method for determining the apoE phenotype has been developed [121]. It involves isoelectric focusing of delipidated plasma and detection of apoE by immunoblotting with an anti-apoE antibody. The advantages of this new method are its speed (ultracentrifugation is not necessary) and its sensitivity (only 10-20 μl plasma is needed).

The amino acid sequence of the three common isoforms has been determined [122]. The three isoforms differ by substitutions of one or two amino acid residues (at positions 112 and 158) on the 299 amino acid chain of the mature apoE protein. ApoE4 has arginine residues at both sites, whereas E2 usually (see below) has cysteine residues at both sites. E3 (the wild type isoform) has a cysteine residue at position 112 and an arginine residue at position 158. Therefore E4 has one more positive charge than E3, while E2 has one less. The E4 and E2 variants are designated E4(cys112→arg) and E2(arg158→cys), respectively.
ApoE2 is genetically heterogeneous; in addition to the common E2(arg158→cys) variant, three rare E2 variants have been described, each differing from apoE3 at a single site: E2(lys146→gln), E2(arg145→cys) and E2(arg136→ser) [123-125] (table 6).
The number of cysteine residues per molecule of apoE can be determined by a cysteamine modification technique [126]. On cysteamine treatment, the cysteine residues are converted to a positively charged lysine analogue, thereby introducing a positive charge for each cysteine residue modified. Therefore, E3, E2(lys146→gln) and E2(arg136→ser), all containing one cysteine residue, are shifted one charge position on isoelectric focusing, thereby giving bands at the E4 or E3 position. E2(arg158→cys) and E2(arg145→cys) contain two cysteine residues and are shifted two charge units on isoelectric focusing, giving a band at the E4 position. ApoE4, which lacks cysteine residues is not affected by cysteamine modification.

The amino acid substitutions resulting in the different variants are due to point mutations in the APOE gene. The APOE gene has been isolated and sequenced by two different groups [52,66]. Knowledge of the sequence of the APOE gene, enables the detection of the variants on the DNA level by means of hybridization with variant-specific oligonucleotide probes [127,128].

Table 6. Characteristics of various apolipoprotein E variants [125]

variant	charge relative to E3	mutation	receptor binding activity (%)	reference
E3	0	wild type	100	122
E1	-2	gly27→asp;arg158→cys	4	129
E1-Harrisburg	-2	lys→glu	?	130
E1-Bethesda	-2	? (2 cys residues)	?	131,132
E2	-1	arg158→cys	< 2	122
E2*	-1	lys146→gln	40	123
E2**	-1	arg145→cys	45	124
E2-Christchurch	-1	arg136→ser	41	125
E3*	0	ala99→thr;ala152→pro	?	133
E3**	0	cys112→arg;arg142→cys	<20	134
E3-Leiden	0	? (no cys residues)	30	135,136
E4	+1	cys112→arg	100	124
E5	+2	glu3→lys	?	137,138
E7-Suita	+4	glu244→lys;glu245→lys	?	139,140

1.4.3 *Receptor binding of apoE*

In vitro binding studies revealed that E3 and E4 bind efficiently to the LDL receptors [124,141]. *In vivo*, E4 even has an enhanced catabolic rate compared to E3 [142,143]. On the contrary, the other apoE variants are defective in binding to the lipoprotein receptors. These variants possess binding activities ranging from < 2% to 45% of normal (see table 6). The most severely defective mutant (< 2% of normal binding) is the E2(arg158→cys) mutant. E2(arg145→cys), E2(lys146→gln) and E2(arg136→ser) display binding activities of 45%, 40% and 41% respectively compared to the binding of E3 [124,125,144,145]. In all of these binding-defective mutants, basic amino acid residues (arginine, lysine) are substituted by neutral amino acids.
These latter studies have shown that the single amino acid substitutions in the binding defective mutants are clustered near residues 140-160. It has therefore been suggested that this region of apoE is the receptor binding domain.
Many investigations into this receptor binding domain of apoE have been performed.

In one such study, apoE was cleaved into smaller fragments by thrombin or cyanogen bromide. Thrombin cleavage produced two major fragments: the amino-terminal residues 1 to 191 (22 kD) and the carboxyl-terminal residues 216 to 299 (10 kD). The amino-terminal fragment possessed full receptor binding activity, whereas the carboxyl-terminal fragment had no binding activity. Cyanogen bromide digestion produced eight fragments. Only the largest fragment, spanning the residues 126 to 218 possessed receptor binding activity [146].

An additional approach to define the receptor binding domain of apoE, was the mapping of epitopes of apoE monoclonal antibodies. The epitope of a monoclonal anti-apoE antibody, which blocked the receptor binding activity, was localized between the residues 139 to 169, most probably to the immediate vicinity of residues 140 to 150 [147]. Two other studies demonstrated that the arginine, present at residue 158 does not interact directly with the receptor, but plays a role in the stabilization of the conformation of the binding domain, which spans residues 140 to 150. Substitution of a cysteine for this arginine would alter the conformation of the binding domain, resulting in a poor binding activity (<2%). Weisgraber *et al.* [144] showed that the binding activity of this E2(arg158→cys) variant could be increased by treatment with cysteamine. Fully restored binding activity of the E2(arg→cys) variant could be obtained by the removal of the carboxyl-terminal one-third of the molecule (a.a. 216-299) by thrombin, followed by cysteamine modification [134].

Recently, apoE mutants have been made with recombinant DNA techniques, which possess altered receptor binding activity [148,149] (table 7). The role of specific amino acid residues in the putative receptor binding domain has been elucidated by site-directed mutagenesis [148]. The substitution of basic amino acids by neutral amino acids gave a reduced receptor binding activity (10 to 50% of normal), comparable to that of the naturally occurring variants. In contrast, a double mutant (ser139→arg; leu149→ala) displayed an increased binding capacity. In this study no single substitution resulted in a complete absence of binding, suggesting that there are multiple interactions between apoE and the receptor and that the basic residues in this region may cooperate in binding to the receptor [148].

Table 7. Receptor binding activities of apoE variants made by site-directed mutagenesis [148].

variant	percentage of normal receptor binding activity
arg136→ser	40
his140→ala	42
lys143→ala	9
leu144→pro	13
arg150→ala	24
ala152→pro	27
ser139→arg;leu149→ala	154

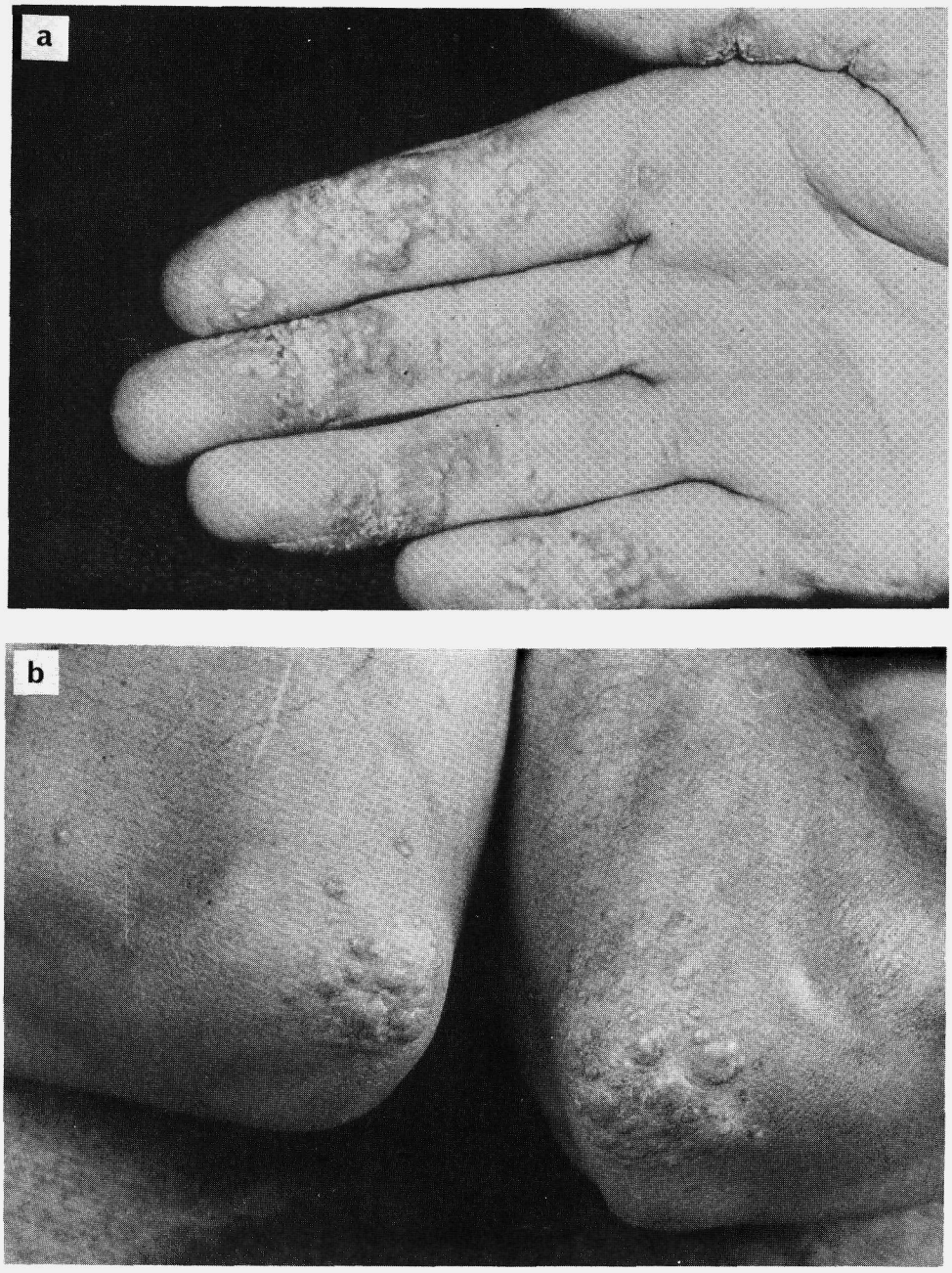


Figure 4. Xanthomas, pathognomonic for familial dysbetalipoproteinemia.
a: xanthomata palmaria, **b**: tuberous xanthomas on the elbows

1.4.4 *Clinical features of familial dysbetalipoproteinemia*

Familial dysbetalipoproteinemia (FD), or type III hyperlipoproteinemia, is a genetic disorder of lipoprotein metabolism with a frequency of about 1-4 per 10000 individuals [150,151]. The disease does not usually manifest itself before the third decade in males. In females, FD rarely develops prior to the menopause, possibly due to beneficial hormonal effects [152,153]. FD is associated with a strong predisposition to premature coronary and peripheral vascular disease.
The presence of xanthomas (unusual lipid deposits) is a major clinical feature of FD. Approximately 50% of the FD patients have xanthomas at the time of diagnosis. Palmar xanthomas (xanthomata palmaria, figure 4a) are characteristic for FD. Occasionally, xanthomas occur as yellowish-brown linear elevations along the palmar creases (xanthomata striata palmaria) or as yellowish discoloration of the palmar creases (xanthochromia striata palmaris). Although the palmar xanthomas are not always present, when found they are virtually pathognomonic for FD [154]. Furthermore, tuberous xanthomas on the elbows frequently occur in FD patients (figure 4b). All xanthomas may appear over a few weeks and resolve in a few months depending on the plasma lipid level.
In FD patients both cholesterol and triglyceride levels are elevated (cholesterol > 7.5 mmol/l; triglycerides > 2.0 mmol/l) due to the accumulation of chylomicron and VLDL remnants. The VLDL cholesterol/ total plasma triglyceride ratio is increased in patients compared to normal persons (>0.69 on a mmolar basis) [155,156]. In most FD patients LDL-cholesterol is significantly decreased. A characteristic feature is the extreme intra-individual variability of cholesterol and triglyceride concentrations in FD patients. In general, the lipid levels are extremely sensitive to changes in calory and fat intake, probably due to an effect on chylomicron- and VLDL synthesis [157,158]. This variability in plasma lipid levels will lead to an underdiagnosis of FD patients.
In contrast to several other forms of hyperlipoproteinemia, FD shows a high responsiveness to therapy. Dietary treatment and weight reduction often results in a dramatic reducing effect on the plasma lipid levels. However, additional treatment with drugs, *e.g.* clofibrate, may be necessary to normalize the lipid levels [152].

1.4.5 *The genetics of FD*

The genetic polymorphism of apoE plays an important role in the predisposition to familial dysbetalipoproteinemia (FD). Most FD patients (>90%) have the apo E2E2 phenotype, whereas FD patients with heterozygosity for E2 are very rare. This suggests a recessive inheritance pattern [159,160].
Furthermore, some FD patients heterozygous for rare apoE variants have been found [125,132,133,135-138,161] (tabel 6) and FD has also been described in one family with apoE deficiency [162].
The elevated cholesterol and triglyceride level in the plasma of the FD patients is due

to an accumulation of chylomicron- and VLDL remnants. It is commonly assumed that this accumulation of remnants is due to an impaired clearance of these particles by hepatic lipoprotein receptors. As apoE acts as a ligand for both the apoE- and LDL receptor, on both chylomicron- and VLDL remnants, it can be assumed that the presence of receptor binding defective apoE variants are involved in the development of FD (table 6). However, the defective binding cannot fully explain the pathogenesis. In the literature [150;151], it is suggested that the frequency of FD in Caucasians is about 1-4 per 10000 individuals, whereas only 1 per 100 of the general population has the E2E2 phenotype. This implies that only 1-4% of all E2E2 individuals develop FD, *i.e.* an example of reduced penetrance [159]. The reduced penetrance could be due to the existence of additional genetic factors, required for the development of FD, which might differ from patient to patient.
Utermann *et al.* [159] suggested that E2E2 homozygotes, inheriting a gene predisposing to hyperlipidemia, will be prone to develop FD. This putative gene has been suggested as being involved in the chylomicron- and VLDL synthesis [163].
Alternatively, it is possible that the discrepancy between healthy and diseased E2E2 individuals is caused by additional microheterogeneity in the APOE gene, *i.e.* that in patients another E2 variant is present than in healthy E2E2 individuals. Notably, four different E2 mutations have been described at present: E2(arg158→cys), E2(lys146→gln), E2(arg145→cys) and E2(arg136→ser) [122-125].
In addition, various (environmental) factors, like sex, age, hormones, nutrition and alcohol consumption, may influence the expression of FD.

1.5 *Outline of this thesis*

The aim of this study was to elucidate the genetic background of FD in order to develop preclinical diagnostic tests for FD. Early identification of individuals with a high risk for FD is important, since a simple diet and/or medication can prevent the development of hyperlipidemia and, consequently, reduce the risk for peripheral and coronary atherosclerosis in middle age.
The present study was based on an FD patient population sample from the Lipid Clinics in Leiden and Nijmegen. A general control population sample and a sample of control E2E2 homozygotes, was obtained by screening a large population of 2018, 35 years old Dutch males (chapter 2). Such a large scale screening was necessary because of the low frequency of the E2E2 phenotype in the general population.
This population screening also resulted in the finding of a healthy individual with a rare apoE variant (chapter 3).
To investigate whether microheterogeneity in the APOE gene is involved in the predisposition to FD, we determined the APOE genotype of FD patients with the E2E2 and with the E3E2 phenotype and of healthy individuals with the E2E2 phenotype. Therefore we used the technique of isoelectric focusing of cysteamine modified samples (chapter 4) as well as hybridization of *in vitro* amplified genomic DNA with variant-

specific oligonucleotide probes (chapter 5).
The hypothesis that an extra gene is co-segregating with the E2E2 phenotype in FD patients was tested (chapter 6). Several RFLPs were analyzed in FD patients, in control E2E2 individuals and in a random population sample.
As the APOE gene is located in a cluster of apolipoprotein genes on chromosome 19, it was important to elucidate the exact location of this gene relative to these other genes (chapters 7 and 8).

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Chapter 2

Apolipoprotein E polymorphism in the Netherlands and its effect on plasma lipid and apolipoprotein levels

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Apolipoprotein E polymorphism in the Netherlands and its effect on plasma lipid and apolipoprotein levels

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Summary. By isoelectric focusing of delipidated sera followed by immunoblotting we studied the apolipoprotein (apo) E polymorphism in 2018 randomly selected 35-years-old males from three different areas in the Netherlands. Comparison of the APOE allele (E*2, E*3, and E*4) frequencies estimated in this study with those reported for several other population samples showed that there are marked differences between the Dutch population and the populations of Japan, New Zealand, Finland, and the United States. These differences in APOE allele frequencies appeared to be mainly due to differences in frequencies of the E*2 allele (decreased in Japan and Finland; increased in New Zealand) and the E*4 allele (increased in Finland; decreased in Japan and the United States). No difference in APOE allele frequencies was found between the Dutch population and the populations of West Germany and Scotland. Measurements of plasma cholesterol and apo B and E concentrations showed that the E*4 allele is associated with elevated plasma cholesterol and apo B levels and with decreased apo E concentrations, whereas the opposite is true for the E*2 allele. In the Dutch population, the sum of average allelic effects of the common APOE alleles on plasma cholesterol and apo B levels is 6.8% and 14.2%, respectively, of the total population mean. The total average allelic effect on plasma apo E concentrations was more pronounced (50.1%), suggesting that the APOE alleles primarily affect apo E concentrations rather than plasma cholesterol and apo B levels. This hypothesis is sustained by the observation that for plasma apo E levels the genetic variance associated with the APOE gene locus contributed about 18% to the total phenotypic variance. For plasma cholesterol and apo B this contribution was only 1.4% and 2.3% and is relatively low as compared with that reported for other population samples.

Introduction

The apolipoprotein E (apo E) present on chylomicron and very low density lipoprotein (VLDL) remnants plays a central role in the hepatic metabolism of these particles, as this apolipoprotein is recognized with high affinity by hepatic lipoprotein receptors (Sherrill et al. 1980; Weisgraber et al. 1982).

Human apo E can be separated by isoelectric focusing into three major isoforms, E2, E3, and E4, which differ in pI by a single charge unit, apo E4 being the most basic and E2, the most acidic isoform. This heterogeneity is the result of three different APOE alleles, E*4, E*3, and E*2, at one single genetic locus (Zannis and Breslow 1981; Utermann et al. 1982).

Apo E3 is the most commonly occurring, or wild-type, form. Apo E4 is derived from E3 by a Cys → Arg substitution at position 112 and is designated E4(Cys_{112} → Arg). Apo E2 is derived from E3 by an Arg → Cys substitution at position 158 and is designated E2(Arg_{158} → Cys). Up till now a number of very rare mutants of apo E have been described. Some variants are either more basic than apo E4 or more acidic than apo E2 while others have the same electric charge as E2 or E3 (Rall et al. 1982, 1983; Innerarity et al. 1984; Yamamura et al. 1984a, b; Havel et al. 1983; Ghiselli et al. 1984; Havekes et al. 1986).

Several population studies on apo E polymorphism have been reported (Utermann et al. 1979, 1984a; Menzel et al. 1983; Wardell et al. 1982; Robertson and Cumming 1985; Ehnholm et al. 1986; Utermann 1987; Eto et al. 1986b; Ordovas et al. 1987). Although the APOE gene frequencies in some European and the North American populations seem quite similar, differences in APOE allele frequencies between different populations have also been reported (Eto et al. 1986a; Ehnholm et al. 1986; Utermann 1987; Boerwinkle et al. 1987).

From these population studies it has been firmly established that the apo E polymorphism affects plasma lipid levels. The E*2 allele appeared to be associated with subnormal plasma and LDL cholesterol levels whereas the E*4 allele is associated with elevated plasma cholesterol levels (Utermann et al. 1979, 1984a; Ehnholm et al. 1986; Utermann 1987; Ordovas et al. 1987).

In this paper we report the APOE phenotype and gene frequencies together with plasma levels of cholesterol, triglyceride, apo B, and apo E in 2018 randomly selected 35-years-old male individuals from the Dutch population. From this population study we calculated the average effects of APOE allelic substitution on plasma lipid and apolipoprotein levels as well as the contribution of the genetic variance associated with the APOE gene locus to the total phenotypic variance of these lipoprotein parameters.

Materials and methods

Collection of samples

Two thousand eighteen 35-year-old males were randomly selected from three different areas in the Netherlands. EDTA

plasmas were obtained by venipuncture and stored at −20°C until the assays were performed.

Apo E phenotyping

Apo E phenotyping was performed using a recently developed rapid micromethod, which is based on isoelectric focusing of delipidated plasma samples followed by immunoblotting (Havekes et al. 1987) using a polyclonal anti-apo E antiserum as first antibodies. This method is especially suitable for large-scale screening.

Determination of plasma cholesterol, triglyceride, apo B, and apo E levels

Plasma cholesterol and triglycerides were measured enzymatically using Boehringer test-kits (cholesterol CHOD-PAP and triglyceride GPO-PAP, respectively). Apo B concentrations were measured by immunonephelometric assay (INA) as described by Rosseneu et al. (1981). Plasma apo E levels were measured by enzyme-linked immunosorbent assay (ELISA) as described by Bury et al. (1986).

Statistical analyses

Allele frequencies were estimated using the gene-counting method. Differences in APOE phenotype distribution between different population samples were determined by chi-square analysis. Differences in mean lipid and apolipoprotein levels between APOE phenotypic groups were evaluated by parametric (one-way analysis of variance) and nonparametric (Kruskal-Wallis) tests, In-pairs differences between APOE phenotypic groups were estimated using the procedure of Scheffe (parametric test) as well as the Mann-Whitney U-Wilcoxon rank sum (nonparametric) test. The average effects of the APOE alleles on the plasma cholesterol apo B and apo E concentrations and the variance of these parameters attributable to genotypic differences were estimated exactly according to the method of Sing and Davignon (1985).

Results

APOE phenotype distribution and allele frequencies

The sample of 2018 35-year-old males was randomly selected from three different geographic areas in the Netherlands. The APOE phenotype distribution and the APOE allele frequencies are presented in Table 1. The distribution of the different APOE phenotypes was in Hardy-Weinberg equilibrium ($\chi^2 = 2.82$; $P < 0.05$ at $\chi^2 < 11.0$; $df = 5$).

In Table 2 the apo E allele frequencies obtained in the present study are compared with those observed in other populations. In this table, only populations with more than 300 subjects are considered. A chi-square test of heterogeneity indicates statistically significant differences in the APOE allele frequency distribution between the different populations ($df = 16$; $\chi^2 = 149$; $P < 0.001$). Two-sample χ^2 analysis showed that the allele frequencies of the Dutch population differ highly significantly from those of the two Japanese populations and the populations of Finland, New Zealand ($P < 0.001$), and the United States ($P < 0.005$). No significant differences were found with the German populations and the

Table 1. APOE phenotype and allele frequencies in randomly selected 35-years-old males. χ^2 Hardy-Weinberg distribution is 2.82 ($df = 5$)

Phenotype	No. observed	Relative frequency (%)
E4/E4	59	2.9
E4/E3	512	25.4
E4/E2	45	2.2
E3/E3	1128	55.9
E3/E2	261	12.9
E2/E2	13	0.7
Total	2018	100
	Gene frequencies	
E*4	0.167	
E*3	0.750	
E*2	0.082	

population of Scotland. From the tables generated from the respective two-sample χ^2 analyses (tables not shown), we were able to calculate the separate contribution of the χ^2 data for each allele frequency to the total χ^2 value (Table 3).

From the data presented in Table 3, it is obvious that 50% of the differences in APOE allele frequencies, measurd as χ^2 values, between the Dutch and Japanese population from Asahikawa (Eto et al. 1986a) is due to the relatively low E*2 allele frequency in this Japanese population. However, in the other Japanese population (Utermann 1987) more than 80% of the difference between the Dutch population in apo E allele frequencies is due to a low E*4 allele frequency. From these results we calculated that the two Japanese populations differ significantly from each other regarding APOE allele frequencies ($df = 2$; $\chi^2 = 22.0$; $P < 0.001$). The APOE allele frequencies of the New Zealand population (Wardell et al. 1982) differ from that of the Dutch population mainly (78%) because of its high E*2 allele frequency. The Finnish population (Ehnholm et al. 1986) differs from the Dutch population in APOE allele frequencies both by a decreased E*2 allele and an increased E*4 allele frequency. For the American population (Ordovas et al. 1987) the E*4 allele fequency is the major contributor (72%) to the difference in APOE allele frequencies.

Effect of allele substitution at the APOE gene locus on plasma lipid and apolipoprotein levels

To evaluate whether the allelic variation at the APOE locus significantly affects the serum lipid and apolipoprotein levels, plasma cholesterol, triglycerides, and apo B and E were assayed. Table 4 presents the mean plasma cholesterol, triglyceride, apo B, and apo E levels in the different APOE phenotype groups. We used the one-way analysis of variance for testing the equality of the mean values among APOE phenotypes. As we could not find homogeneity of the variance among phenotypes and within phenotypes, we also estimated levels of significance using the nonparametric test of Kruskal-Wallis. Both statistical analyses showed that plasma cholesterol, apo B, and apo E levels differ among APOE phenotype groups. The triglyceride level was not affected by the APOE phenotype. Using the procedure of Scheffe (parametric test) as well as the Mann-Whitney U-Wilcoxon rank sum test (non-

Table 2. APOE gene frequencies in several random population samples. χ^2 values at $df = 2$ and P values of 0.01 and 0.001 are 9.21 and 13.95, respectively

Population sample	No. of subjects	APOE allele frequency			Hardy-Weinberg distribution		Difference from the Dutch population		Reference
		E*2	E*3	E*4	χ^2 ($df = 5$)	P	χ^2 ($df = 2$)	P	
The Netherlands	2018	0.082	0.751	0.167	2.83	N.S.	–	–	This study
Scotland	400	0.083	0.770	0.145	3.70	N.S.	2.31	N.S.	Cumming and Robertson (1984)
FRG (Münster)	1000	0.078	0.783	0.139	7.15	N.S.	8.71	N.S.	Menzel et al. (1983)
FRG (Marburg)	1031	0.077	0.773	0.150	7.24	N.S.	3.74	N.S.	Utermann et al. (1984)
USA	1204	0.075	0.786	0.135	15.30	<0.01	13.34	<0.005	Ordovas et al. (1987)
Finland	615	0.041	0.733	0.227	7.09	N.S.	41.02	<0.001	Ehnholm et al. (1986)
New Zealand	426	0.119	0.739	0.141	14.28	<0.05	13.96	<0.001	Wardell et al. (1982)
Japan (Asahikawa)	576	0.037	0.846	0.117	2.70	N.S.	50.05	<0.001	Eto et al. (1986a)
Japan	319	0.081	0.849	0.067	3.51	N.S.	37.41	<0.001	Utermann (1987)

Table 3. Relative contribution of the different APOE alleles to the total χ^2 value as estimated for the difference in allele frequencies between the Dutch and other populations. ↑, ↓, The contribution to the total χ^2 is due to an increased or decreased allele frequency, respectively

Apo E allele	Population (reference)				
	Japan[a]		New Zealand (Wardell et al. 1982)	Finland (Ehnholm et al. 1986)	USA (Ordovas et al. 1987)
	Eto et al. (1986)	Uterman (1987)			
E*2	0.50 ↓	0.01 ↓	0.78 ↑	0.54 ↓	0.06 ↓
E*3	0.21 ↑	0.17 ↑	0.01 ↓	0.01 ↓	0.22 ↑
E*4	0.29 ↓	0.82 ↓	0.21 ↓	0.45 ↑	0.72 ↓

[a] Two-sample χ^2 analysis showed a significant difference in APOE allele frequencies between both Japanese population samples ($df = 2$; $\chi^2 = 22.0$; $P < 0.001$)

Table 4. Mean plasma cholesterol, triglyceride, apo B, and apo E levels (in mg/dl) among different APOE phenotypes

Trait	Pooled	Phenotype						Significance	
		E2/E2	E3/E2	E4/E2	E3/E3	E4/E3	E4/E4	P^a	P^b
Cholesterol	215.5	209.0	202.4	207.4	216.8	219.0	225.2	<0.001	<0.001
	(41.7)[c]	(65.0)	(48.8)	(41.0)	(39.5)	(40.7)	(40.7)		
Triglyceride	154.2	155.8	158.4	168.7	149.7	161.1	152.0	0.186	0.580
	(90.9)	(71.8)	(98.2)	(110.6)	(85.0)	(98.9)	(75.1)		
Apo B	117.4	80.0	107.8	111.9	117.3	122.9	127.0	<0.001	<0.001
	(37.4)	(26.5)	(42.5)	(35.7)	(35.2)	(38.6)	(34.6)		
Apo E	5.6	13.8	7.3	6.7	5.5	5.0	4.4	<0.001	<0.001
	(2.4)	(3.8)	(2.9)	(2.1)	(2.0)	(2.0)	(2.0)		

[a] Level of significance estimated by one-way analysis of variance
[b] Level of significance estimated by the nonparametric test of Kruskal-Wallis
[c] Values in parentheses represent standard deviations

parametric test), we estimated the significance of the differences in mean lipid and apolipoprotein levels between the phenotypic groups in pairs (Table 5). Again, gross differences between the parametric and nonparametric test were not found. From the results presented in Table 5 it is obvious that there are statistically significant differences in mean levels of plasma cholesterol, apo B, and apo E among several APOE phenotype groups. Compared with the most common E*3 allele, the E*4 allele leads to elevated plasma cholesterol levels, whereas the E*2 allele is associated with a decreased plasma cholesterol concentration. The mean plasma cholesterol level of the E2/E2 homozygotes did not differ significantly from that of the other phenotypes, as 3 of the 13 E2/E2 homozygotes were hyperlipidemic.

For plasma apo B levels the statistically significant differences between the APOE phenotypes was more pronounced than for plasma cholesterol. The effect of allelic substitution at the APOE gene locus on apo E levels is the opposite to that on plasma cholesterol and apo B. The E*4 allele leads to decreased apo E levels, whereas the E*2 allele is strongly associated with an increased apo E concentration. When the mean apo B and apo E levels calculated for each phenotype group

Table 5. In pairs statistical analyses[a] of the differences in mean cholesterol, triglyceride, apo B, and apo E levels between the different APOE phenotype groups

Different from	Cholesterol						Apo B						Apo E					
	2/2	3/2	4/2	3/3	4/3	4/4	2/2	3/2	4/2	3/3	4/3	4/4	2/2	3/2	4/2	3/3	4/3	4/4
	Mean (mg/dl)						Mean (mg/dl)						Mean (mg/dl)					
	209.0	202.4	216.7	207.4	219.0	225.2	80.0	107.8	117.3	111.9	122.9	127.0	13.8	7.26	6.68	5.45	5.00	4.37
2/2								0	0	*0	*0	*0		*0	*0	*0	*0	*0
3/2				0*	0*	0*				*0	*0	*0				*0	*0	*0
4/2																*0	*0	*0
3/3																	*0	*0
4/3																		
4/4																		

[a] In pairs statistical analysis was performed by a parametric test (procedure Scheffe, $P<0.05$;*) and a nonparametric test (Mann-Whitney U-Wilcoxon Rank sum; $P<0.01$;0)

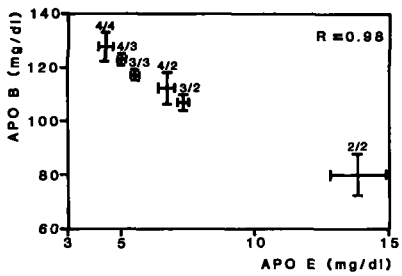

Fig. 1. Inverse relationship between mean apo B and apo E levels calculated in each phenotype group. *Vertical* and *horizontal bars* represent ± SEM for apo B and apo E, respectively

Table 6. Average effects (in mg/dl) of the common APOE alleles on plasma cholesterol, apo B, and apo E levels

Apo E allele	Average effect[a]					
	Cholesterol		Apo B		Apo E	
E*2	−9.7	(−4.5)	−11.4	(−9.7)	2.1	(37.6)
E*3	0.6	(0.3)	0.0	(0.0)	−0.1	(−1.8)
E*4	4.3	(2.0)	5.3	(4.5)	−0.6	(−10.7)

[a] Values in parentheses represent the average effects expressed as percentages of the respective population means

Table 7. Relative contribution of the genetic variance associated with the APOE locus to the total phenotypic variance

Lipoprotein parameter	Total genetic variance associated with the APOE locus (% of total phenotypic variance)	Total phenotypic variance $(mg/dl)^2$
Cholesterol	1.4	1739
Apo B	2.3	1399
Apo E	18.1	5.7

were considered separately, a strong inverse relationship was observed between apo B and apo E (Fig. 1).

We calculated the average effects of the three APOE alleles on the lipid and apolipoprotein levels (Table 6) according to the formula of Sing and Davignon (1985). The average effect of the E*2 allele is to reduce plasma cholesterol (−9.7 mg/dl) and apo B (−11.4 mg/dl) and to raise apo E levels (+2.1 mg/dl), whereas the E*4 allele induces an opposite effect (+4.3, +5.3, and −0.6 mg/dl, respectively). In comparison with the average effects of E*2 and E*4 alleles, the E*3 allele does not seem to influence these lipoprotein parameters. Table 6 demonstrates that the relative effect of the allelic substitution at the APOE locus on plasma apo B is more pronounced than the relative effect on total plasma cholesterol levels, whereas the effect on plasma apo E levels is most dramatic and the opposite to that on plasma cholesterol and apo B.

Relative contribution of the genetic variance associated with the APOE locus to the total phenotype variance

Table 7 presents the estimates of the relative contribution of the APOE gene to the total phenotypic variation of the measured lipoprotein parameters. The total genetic variance associated with the APOE locus contributed more than 18% to the total population variability in apo E levels. The effects of the genetic variation associated with the APOE gene on the variability of total cholesterol and plasma apo B levels are much less pronounced (1.4% and 2.3%, respectively).

Discussion

In this study we present the APOE phenotype distribution and allele frequencies for the Dutch population. The blood samples were randomly selected, from 35-year-old males living in three different geographical areas (Amsterdam, Leiden, and Doetinchem). The Amsterdam and Leiden samples represent urban populations, while the Doetinchem sample represents a more or less rural community.

Statistically significant differences in APOE phenotype distribution among the three areas were not found, indicating that the combined population sample (2018 individuals) is rep-

resentative for the whole Dutch population. This close similarity in phenotpye distribution between the three areas was not surprising since a genetic drift maintained by national-geographic or social-cultural isolation is highly unlikely in the densly populated Netherlands.

Comparison of the APOE allele frequencies estimated in this study with those reported for other population samples (Table 2) showed that there are marked differences between the Dutch population and that of Japan, New Zealand, Finland and the United States. As presented in Table 3, these differences are mainly due to differences in frequencies of the E*2 alleles (decreased in Japan and Finland; increased in New Zealand) and the E*4 allele (increased in Finland; decreased in Japan and the United States), whereas the frequencies of the E*3 allele appear to be rather similar for all population samples considered.

The differences in APOE allele frequencies among the Dutch, the Finnish, and the Japanese populations may be due to differences in ethnic background and geographical isolation and are similar to the differences between the German and Finnish populations described by Ehnholm et al. (1986) and the differences between the Caucasian and Japanese populations reported by Eto et al. (1986a). The statistically significant differences observed in APOE allele frequency among the Dutch population, a community in New Zealand (Wardell et al. 1982), and a U.S. population (Ordovas et al. 1987) (Table 2) might be due to a combination of population admixture and genetic drift.

It should, however, be noted that for both the New Zealand and U.S. population samples the observed APOE phenotype distributions differ significantly from the expected Hardy-Weinberg distributions (Table 2). We calculated that more than 90% of these differences can be attributed to differences between the observed and expected numbers of phenotypes exhibiting the E*4 allele (estimated data not presented). In particular, for the U.S. population this deviation from the expected Hardy-Weinberg distribution contributes to the observed difference in APOE allele frequency compared with that of the Dutch population sample (see also Table 3).

Boerwinkle et al. (1987) also observed statistically significant differences in APOE allele frequencies among different ethnically and/or geographically distinct populations. In contrast to the present study, they also considered relatively small population samples in this respect.

Several studies (Utermann et al. 1979, 1984a; Sing and Davignon 1985; Ehnholm et al. 1986; Eto et al. 1986b; Utermann 1987) have shown a association of the E*4 allele with elevated plasma cholesterol and apo B levels, whereas the E*2 allele appeared to be associated with decreased levels of plasma cholesterol and apo B. Reciprocally the E*4 allele is associated with a reduced plasma apo E level, whereas the E*2 allele leads to a highly significant increase in plasma apo E concentration. This effect of allelic substitution at the APOE locus on plasma cholesterol, apo B, and apo E levels has been confirmed in the present study of the Dutch population. The mechanisms underlying these associations are at present assumed to be the result of (I) a more efficient catabolism of chylomicron and VLDL remnants by the liver in individuals with the E*4 allele and (II) a less efficient catabolism of these lipoprotein particles in subjects exhibiting the E*2 allele due to a defect in binding of apo E2 to hepatic lipoprotein receptors. An enhanced uptake by the liver of chylomicron and VLDL remnants will supply the liver with extra cholesterol, thereby reducing the hepatic LDL receptor activity and thus elevating plasma LDL levels. Reciprocally, a diminished uptake of lipoprotein remnants will lead to an enhanced hepatic LDL receptor activity and eventually to a lower plasma LDL concentration. A more detailed description of this suggested mechanism was presented by Utermann (1985, 1987), who suggested that the APOE gene primarily affects apo E concentrations and thus that the metabolism of apo E-containing lipoproteins thereby regulates the LDL cholesterol and apo B concentrations in plasma. This hypothesis is clearly sustained by our data (Tables 6, 7) and the data published by Eto et al. (1986b) and Boerwinkle and Utermann (1988).

In the Dutch population, the contribtuion of the genetic variance associated with the APOE locus to the total phenotypic variance of plasma cholesterol and apo B levels (Table 7) is low compared with the results of Sing and Davignon (1985) and Boerwinkle and Utermann (1988). This difference can be ascribed almost exclusively to the pronounced total phenotypic variance of these parameters in the Dutch population compared with the Canadian and German population samples. The relatively low total phenotypic variance of the Canadian population sample is most probably due to a truncation of this population by selecting subjects whose plasma cholesterol and triglyceride had been normal on a previous visit. Such a preselection of subjects was not made for our population study. Truncation of the Dutch population sample (35-year-old men) afterwards, by excluding subjects with cholesterol and apo B and apo E values outside the range of the respective mean $\pm$ 2 SD, slightly reduced the total phenotypic variance but did not result in a marked increase in the relative contribution of the genetic variance to the total phenotypic variance of plasma cholesterol and apo B.

Consequently, we concluded that the total genetic variance of cholesterol and apo B associated with APOE locus is almost negligible in the Netherlands as compared with the Canadian and German population samples. The reason for these interpopulation differences in the contribution of genetic variance associated with the APOE locus to the total phenotypic variance is at present subject to speculation.

Irrespective of the convincing data concerning the effect of allelic substitutions at the APOE locus on plasma cholesterol, apo B, and apo E levels, a simple relationship between apo E phenotype and atherosclerotic risk has not yet been established. In some reports a lower frequency of the E*4 allele was found in patients with myocardial infarction (Utermann et al. 1984b), whereas an increased frequency was also reported (Menzel et al. 1983; Cumming and Robertson 1984). The elevated plasma (LDL) cholesterol levels in individuals with the E*4 allele is due to a more efficient catabolism of chylomicrons and VLDL in these subjects (Gregg et al. 1986), thereby preventing the accumulation of atherogenic chylomicron and VLDL remnants. If the LDL concentrations are only moderately elevated, E*4-bearing individuals will be at lower risk. In individuals with the E*2 allele, the LDL cholesterol levels are low due to an impaired VLDL and chylomicron remnant catabolism. These individuals are at lower risk as long as the levels of the atherogenic remnant particles remain below the level at which atherosclerotic risk increases. This relationship between the efficiency of chylomicron and VLDL remnant catabolism on the one hand and the level of LDL cholesterol on the other hand might be responsible for the lack of a general relationship between APOE phenotype and atherosclerotic risk notwithstanding the, for some populations, firm con-

tribution of the polymorphic APOE gene locus to the plasma cholesterol, apo B, and apo E levels.

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Chapter 3

Rare apolipoprotein E variant cosegregating with a unique APOE-C1-C2 haplotype in a normolipidemic family

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Rare Apolipoprotein E Variant Cosegregating with a Unique APOE-C1-C2 Haplotype in a Normolipidemic Family

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Abstract. The genes coding for the apolipoproteins E, C1 and C2 are clustered on the long arm of chromosome 19 in a region of approximately 45 kilobases (kb). In a normolipidemic individual, we detected a new apoE variant with an isoelectric point between that of E3 and E4. As this variant lacks cysteine residues and has probably arisen from an E*4 allele, it is designated E4*. To gain further insight into the origin of the mutation, the haplotypes of the propositus were extended by restriction fragment length polymorphism (RFLP) analysis of the family. The apoE variant cosegregates with the H2 allele of the HpaI polymorphism visualized with an APOE probe and with a new rare 4.5-kb fragment (T3) of the TaqI RFLP detectable with an APOC2 probe. As the propositus and the first-degree relatives with this unique haplotype are normolipidemic, this apoE variant does not seem to be associated with disturbances in the lipoprotein metabolism.

Introduction

The genes coding for the apolipoproteins (apo for the protein; APO for the gene) E, C1 and C2 are closely linked on the long arm of chromosome 19 (cen-q13.2) [Shaw et al., 1986]. Analysis of overlapping cosmid clones allowed us to construct a detailed restriction map of the E-C1-C2 gene cluster [Smit et al., 1988]. The distances between the E, C1, C1′ (pseudogene) and C2 genes are approximately 5, 7.5 and 15 kilobases (kb), respectively, the whole gene cluster spanning about 45 kb. This general picture is in agreement with existing data [Davison et al., 1986; Myklebost and Rogne, 1986; Lauer et al., 1988].

Only a few restriction fragment length polymorphisms (RFLP) have been reported in this gene cluster. Recently, we detected an HpaI RFLP with an APOE probe being strongly associated with familial dysbetalipoproteinemia [Klasen et al., 1987b]. In addition, a common RFLP with TaqI using an APOC2 probe has been de-

scribed [Humphries et al., 1983; Wallis et al., 1984].

With isoelectric focusing (IEF), human apoE can be separated into three major isoforms, i.e. E2, E3 and E4 [Utermann et al., 1977; Zannis and Breslow, 1981] encoded by three different alleles, E*2, E*3 and E*4 [Zannis and Breslow, 1981; Utermann et al., 1982; Zannis et al., 1982]. ApoE3 is the most commonly occurring or wild type form. ApoE4 is supposed to be derived from E3 by a Cys → Arg substitution at position 112 and is designated E4(Cys112 → Arg), whereas the E2 variant is derived from E3 by an Arg → Cys substitution at position 158, E2(Arg158 → Cys). Besides these common isoforms, a number of rare apoE variants have been described. Some of these variants are more basic than apoE4 or more acidic than apoE2, while some of them have the same electric charge as E2 or E3 [for an overview, see Klasen et al., 1987a].

ApoE4 contains no, apoE3 contains 1, and apoE2(Arg158 → Cys) contains 2 cysteine residues. This means that after cysteamine treatment, converting cysteine residues to a positively charged analogue of lysine, both E3 and E2 will focus at the position of E4 [Weisgraber et al., 1982].

Recently, we determined the apoE phenotype distribution in 2,000 35-year-old males randomly selected from the Dutch population [Klasen et al., 1987a]. One subject exhibited heterozygosity for a new rare apoE variant with an isoelectric point (pI value) intermediate to that of E3 and E4.

In the present study, we have characterized this variant on the protein level and defined the haplotype carrying the mutant allele.

Material and Methods

EDTA plasma was obtained by venipuncture and stored at -20 °C until use. ApoE phenotyping was performed using a rapid micromethod [Havekes et al., 1987] which is based on IEF of plasma followed by immunoblotting using anti-apoE antiserum at first antibody. Cysteamine treatment of plasma prior to IEF was performed as described by Weisgraber et al. [1982].

DNA was isolated from leukocytes in whole blood as described by Hofker et al. [1985]. DNA was digested with TaqI and BglI (Pharmacia) under the conditions recommended by the manufacturer. The fragments were separated by electrophoresis on a 0.7% agarose gel at 1.5 V/cm for 16 h and transferred to GeneScreen Plus filters by Southern blotting.

The APOE cDNA clone pE-368 [Zannis et al., 1984] and an APOC2 cDNA clone [Myklebost et al., 1984] were used as probes. The probes (100 ng) were labeled by nicktranslation using a kit and α-^{32}P-dCTP (3,000 Ci/mmol; Amersham, UK). Hybridization was performed as described by Van Ommen et al. [1983]. The filters were exposed to an X-ray film for 1-3 days. The size of the hybridizing fragments was estimated by comparison with HindIII digested phage λ DNA markers run in parallel tracks and co-hybridized with 1 ng of labeled λ DNA.

Results and Discussion

The apoE phenotype was determined [Klasen et al., 1987a] by a rapid micromethod [Havekes et al., 1987] based on IEF of plasma followed by immunoblotting in 2,000 35-year-old males, randomly selected from three different areas in the Netherlands. One of these individuals was heterozygous for apoE3 and a new apoE variant with an isoelectric point intermediate between that of apoE3 and apoE4 (fig. 1). Upon cysteamine modification, a normal E4/E3 phenotype changes into an E4/E4 phenotype as expected (lanes 10 and 9). By comparing e.g. lanes 4 and 3, it

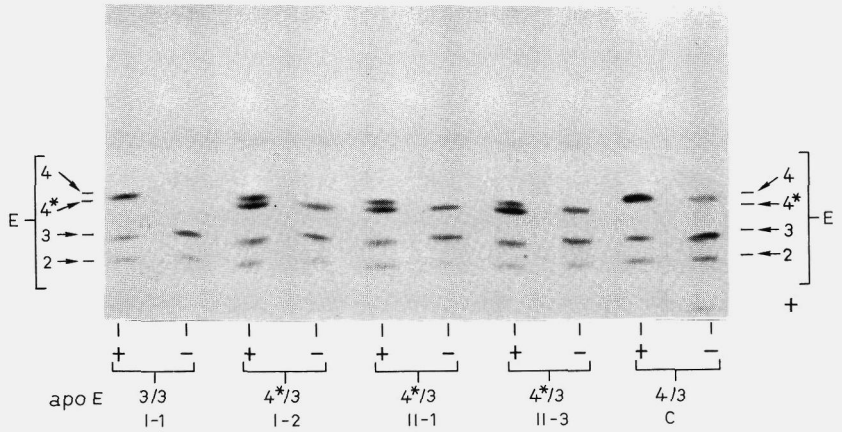

Fig. 1. apoE phenotyping of part of the family (see fig. 2) and of a healthy control (C). Each plasma sample was analyzed with (+) or without (–) cysteamine treatment.

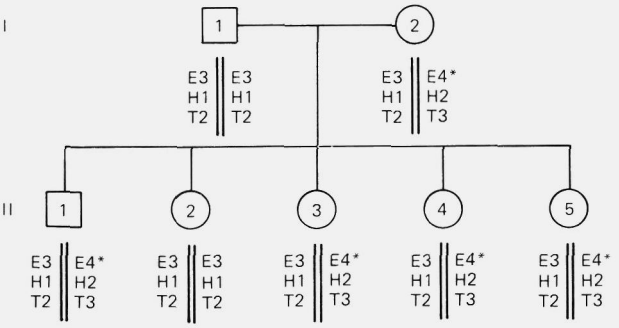

Fig. 2. Pedigree of the family, showing the segregation of the haplotypes. E4*, E3: apoE protein polymorphism; H1, H2: APOE/HpaI polymorphism; T1, T2, T3: APOC2/TaqI polymorphism.

appears that the variant is not modified by cysteamine treatment, indicating a lack of cysteine residues. Since apoE4 is also devoid of cysteine residues, these results strongly suggest that the new variant arose by a mutation in an E*4 allele. Consequently, we tentatively designated the variant E4*. Menzel and Utermann [1986] have also described an apoE variant that focuses between E3 and E4 and that could not be modified by cysteamine treatment. Whether their mutant is identical with the present mutant still has to be evaluated.

The inheritance of E4* was studied in the family of the propositus (fig. 2). The mother is heterozygous for E3 and E4*, while the father is homozygous for E3. E4* is transmitted to 4 of the 5 children in accordance with a codominant Mendelian segregation pattern. To further define the chromosome haplotype where the mutation had taken place, the known RFLPs of

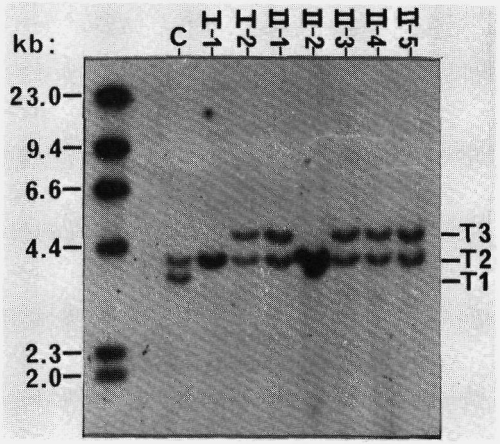

Fig. 3. Southern blot analysis of the APOC2/TaqI polymorphism in the family (see fig. 2). T1: 3.5 kb; T2: 3.8 kb; T3: 4.5 kb. C: a healthy T1T2 control.

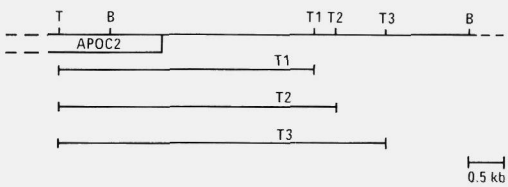

Fig. 4. Restriction map of the 3′-end of the APOC2 gene with the TaqI (T) and BglI (B) sites. The polymorphic TaqI fragments are shown (T1: 3.5 kb; T2: 3.8 kb; T3: 4.5 kb).

the APOE-C1-C2 gene cluster were studied. The HpaI polymorphism, detectable with an APOE cDNA probe reveals two common alleles, H1 (60 kb) and H2 (20 kb) with gene frequencies of 0.6 and 0.4, respectively [Klasen et al., 1987b]. As the father is homozygote H1H1 and the mother heterozygote H1H2 (fig. 2), and the propositus and 3 sibs have inherited the H2 allele as well as the E*4* allele from their mother, the E4* mutation has arisen in an H2 haplotype. For the TaqI polymorphism with an APOC2 cDNA probe, two common alleles have been reported: T1 (3.5 kb) and T2 (3.8 kb) with gene frequencies of 0.4 and 0.6, respectively [Humphries et al., 1983; Wallis et al., 1984]. Surprisingly the propositus and the 3 sibs with E4* have inherited a 4.5-kb fragment (T3) from their mother (fig. 2, 3). The family study (fig. 2) shows that the mother has transmitted a unique haplotype, E4*-H2-T3, containing both rare mutations to 4 of the 5 children.

We have screened more than 200 unrelated individuals for the TaqI RFLP. Only the family described in this paper exhibits the new T3 TaqI allele of 4.5 kb. In order to localize the TaqI site responsible for the 4.5-kb hybridizing fragment, we digested DNA from the father (T2T2), the mother (T3T2), individual II.1 (T3T2) and a T1T1 homozygote with the restriction enzyme BglI and with BglI + TaqI (data not shown). In all 4 individuals, a 6.6-kb hybridizing BglI fragment was revealed. Therefore, the new TaqI fragment is most likely not due to an insertion or a deletion of a fragment larger than 0.1 kb. The BglI/TaqI double digestion resulted in different hybridizing fragments; a band of 2.8 kb for T1, 3.1 kb for T2 and 3.8 kb for T3. Consequently the TaqI site responsible for the 4.5 kb (T3) fragment is located 0.7 kb downstream of the TaqI site responsible for the T2 fragment (fig. 4).

All family members with the E4* variant had normal plasma cholesterol and triglyceride levels. In contrast to patients with familial dysbetalipoproteinemia, they did not show the presence in their

plasma of VLDL particles with β-electrophoretic mobility (β-VLDL) or increased cholesterol/triglyceride ratios. Consequently, heterozygosity for this rare E4*H2-T3 haplotype is not associated with disturbances in lipoprotein metabolism.

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Chapter 4

Familial dysbetalipoproteinemic subjects with the E3/E2 phenotype exhibit an E2 isoform with only one cysteine residue

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Familial dysbetalipoproteinemic subjects with the E3/E2 phenotype exhibit an E2 isoform with only one cysteine residue

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Most familial dysbetalipoproteinemic patients are E2/E2 homozygotes for the apolipoprotein E (apoE) polymorphism, whereas patients with the E4/E2 or E3/E2 phenotype are very rare. Three out of 41 dysbetalipoproteinemic patients from our lipid clinic appeared to be E3/E2 heterozygotes. ApoE protein phenotyping and DNA oligonucleotide hybridization techniques showed that all three patients exhibit an uncommon E2 variant that contains only one cysteine residue. These results suggest that, in contrast to the by far most frequently occurring E2($Arg_{158} \rightarrow Cys$) allele, heterozygosity for this uncommon E2 allele may cause familial dysbetalipoproteinemia. Preliminary family studies suggest that this uncommon E2 allele cosegregates with familial dysbetalipoproteinemia.

Key words: apolipoprotein E phenotype; cysteamine modification; familial dysbetalipoproteinemia; synthetic oligonucleotides.

Familial dysbetalipoproteinemia or Type III hyperlipoproteinemia is characterized by the accumulation in the plasma of chylomicron and very low density lipoprotein (VLDL) remnants. The accumulation of these remnants often results in premature coronary artery and peripheral vascular disease and in xanthomatous lesions (Fredrickson et al. 1980). In normal subjects the remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver or conversion into low density lipoprotein (LDL) (Brown et al. 1981). The apolipoprotein E (apoE) present on lipoprotein remnants plays a central role in the hepatic metabolism of remnant particles, as this apolipoprotein is recognized with high affinity by the hepatic receptors involved in remnant uptake (Sherill et al. 1980).

With isoelectric focusing, human apoE can be separated into three major isoforms, i.e., E2, E3 and E4 (Utermann et al. 1977, Zannis & Breslow 1981) encoded by three different apoE alleles ($\varepsilon 4$, $\varepsilon 3$ and $\varepsilon 2$) at a single genetic locus (Zannis & Breslow 1981, Utermann et al. 1982, Zannis et al. 1982).

ApoE3 is the most commonly occurring (or wild) type form. ApoE4 is supposed to be derived from E3 by a $Cys \rightarrow Arg$ substitution at position 112 and is designated as E4($Cys_{112} \rightarrow Arg$). At present three different mutations have been described as giving rise to apoE2: E2($Arg_{158} \rightarrow Cys$), E2($Arg_{145} \rightarrow Cys$) and E2($Lys_{146} \rightarrow Gln$) (Rall et al. 1982a). E2($Arg_{158} \rightarrow Cys$) is by far the most frequently occurring form of apoE2. ApoE3 and E2($Lys_{146} \rightarrow Gln$) contain one cysteine residue, whereas E2($Arg_{158} \rightarrow Cys$) and

E2(Arg_{145} → Cys) contain two cysteine residues. This means that after cysteamine treatment, converting cysteine residues to a positively charged analogue of lysine, E3, E2(Arg_{158} → Cys) and E2(Arg_{145} → Cys) will focus at the E4 position, whereas the E2(Lys_{146} → Gln) variant will focus at the E3 position (Weisgraber et al. 1982).

Homozygosity for apoE2 has been shown to be the major metabolic defect in familial dysbetalipoproteinemia due to a defect of apoE2 in binding to the hepatic lipoprotein receptors (Schneider et al. 1981, Rall et al. 1982b). About 4% of the E2/E2 homozygotes display dysbetalipoproteinemia, whereas E3/E2 or E4/E2 heterozygotes very rarely develop this disease.

In this paper, we describe three genetically unrelated familial dysbetalipoproteinemic patients exhibiting the E3/E2 phenotype. In all three patients the E2 isoform appeared to be an uncommon E2 variant that contains only one cysteine residue.

Material and Methods

Patients with familial dysbetalipoproteinemia were diagnosed on the basis of the presence of hyperlipidemia, concomitant with floating beta lipoproteins and an elevated cholesterol/triglyceride ratio in VLDL (> 0.6 by weight). Furthermore, a typical symptom of the disease, xanthochromia striata palmaris (yellowish discoloration of the palmar and digital creases) was present in all patients in this study.

EDTA plasma was obtained by venipuncture and stored at −20°C until use. ApoE phenotyping was performed using a recently developed rapid micro-method (Havekes et al. 1987) based on isoelectric focusing of delipidated plasma followed by immunoblotting using polyclonal anti-apoE antiserum as first antibody. ApoE immunoreactive material acidic to the major isoform(s) represents sialylated and/or deamidated derivatives of the major apoE isoforms and is comparable to the banding pattern obtained with the conventional method based on isoelectric focusing of delipidated VLDL followed by protein staining. Because of partial sialylation and/or deamidation of the major isoforms, the phenotyping was based on the following criterion: the banding pattern was classified as homozygous when the most basic band was clearly stronger than the more acidic bands. Conversely, when the most basic band was equal to or weaker than a more acidic band the phenotype was scored as heterozygote. For apoE phenotyping with the conventional method, we used the method described by Warnick et al. (1979). Cysteamine treatment of plasma prior to isoelectric focusing was performed as described by Weisgraber et al. (1982).

DNA was isolated from leukocytes in whole blood by standard methods (Hofker et al. 1985). DNA was digested with TaqI (Pharmacia) under conditions as recommended by the manufacturer. The fragments were separated by electrophoresis on a 0.5% agarose gel at 1.2 V/cm for 16 h. "In gel" hybridization with synthetic oligonucleotides was performed according to Bos et al. (1984), except that the oligonucleotides were labeled with γ ^{32}P dATP and T4 polynucleotide kinase (Pharmacia) to a specific activity of 5.10^8 dpm/μg.

Two synthetic oligonucleotides (19-mer) were used: one complementary to the E2(Arg_{158} → Cys) allele and designated as 158Cys-probe and the other complementary to the wild type (E3) allele designated as 158Arg-probe.

The (pre-)hybridization was performed in 5 × SSPE, 1% SDS, and 100 μg/ml denatured salmon sperm DNA at 53°C for 2 h and 16 h, respectively. After hybridization the gels were washed twice for 30 min in 2 × SSPE, 0.1% SDS at 20°C, followed by washing in 5 × SSPE, 0.1% SDS at 53°C for

15 min and a final stringent wash in 5 × SSPE, 0.1% SDS at 63 °C for 10 min for both oligomer probes.

Results and Discussion

Three out of 41 consecutive patients with familial dysbetalipoproteinemia appeared to be heterozygous E3/E2, while the rest showed the expected E2/E2 phenotype.

Fig. 1 represents an apoE immunoblot of an isoelectric focusing slab gel (pH range 5 to 7) applied with delipidated plasma samples from a normolipidemic E3/E2 heterozygote and a normolipidemic E3/E3 homozygote, as indicated. After cysteamine treatment of their plasma both apoE isoelectric focusing patterns changed into E4/E4. This indicates that in these plasma samples E2 contains two cysteine residues, whereas E3 contains one cysteine residue, as expected.

The same technique of cysteamine treatment was used for our familial dysbetalipoproteinemic patients. Fig. 1 shows the effect of cysteamine treatment on the apoE isoelectric focusing pattern of one patient with the E2/E2 phenotype and one patient with the uncommon E3/E2 phenotype (as far as the familial dysbetalipoproteinemia is concerned). After cysteamine treatment, the E2/E2 pattern changed into E4/E4, indicating that also in this sample E2 contains two

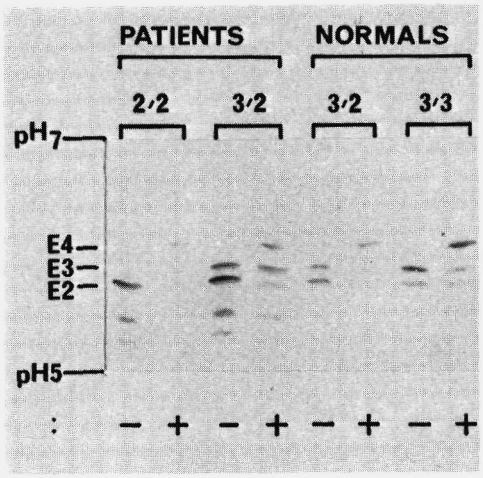

Fig. 1. Apolipoprotein E phenotyping using the immunoblotting technique. Plasmas from two normolipidemic and two familial dysbetalipoproteinemic subjects were applied as indicated. Each plasma sample was treated with (+) or without (−) cysteamine.

cysteine residues. Strikingly, after cysteamine treatment the apoE isoelectric focusing pattern of the patient with the E3/E2 phenotype changed into E4/E3 instead of the E4/E4 pattern.

The results presented in Table 1 show that all three genetically unrelated familial dysbetalipoproteinemic patients with the E3/E2 phenotype displayed the unexpected E4/E3 pattern after cysteamine treatment of their plasma. The cysteamine treatment technique was also used for 11 familial dys-

Table 1

Apolipoprotein E phenotyping and synthetic oligonucleotide hybridization in patients with familial dysbetalipoproteinemia and in controls

Subjects	Number	Phenotype cysteamine treatment		Hybridization with synthetic oligonucleotide probe*	
		Without	With	158Cys	158Arg
Familial dysbetalipoproteinemia	3	3/2	4/3	−	+
	11	2/2	4/4	+	−
Controls	50	3/2	4/4	n.d.	n.d.

* 158Cys- and 158Arg-probes are synthetic oligonucleotides (19-mer) complementary to the E2($Arg_{158} \rightarrow Cys$) allele and the wild type (E3) allele, respectively.

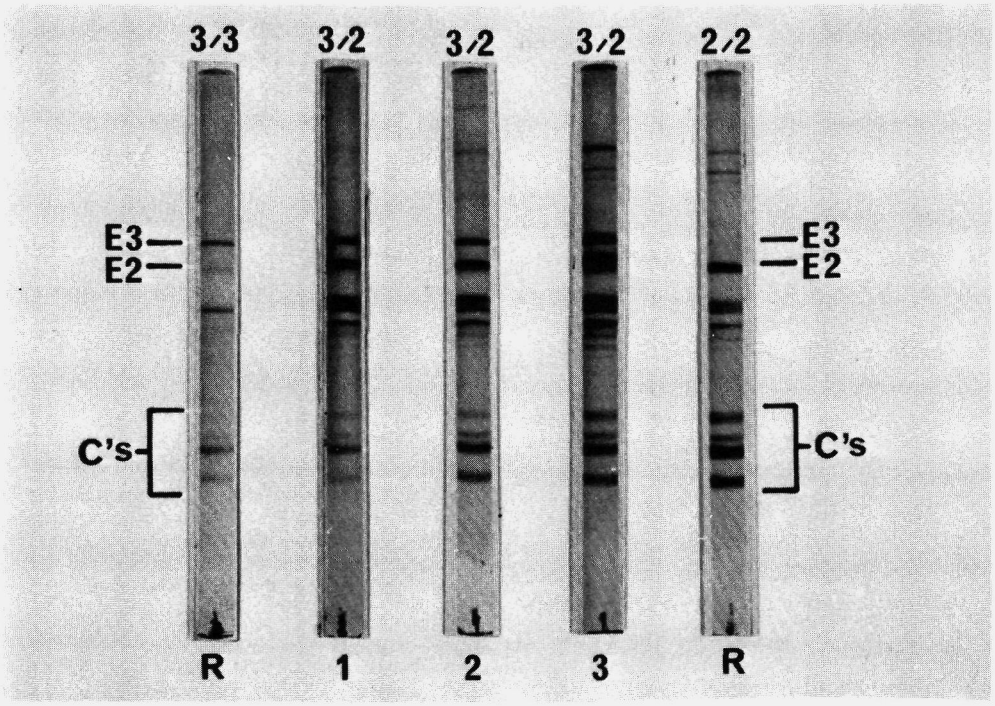

Fig. 2. Apoliprotein E phenotyping with the conventional method using isolated VLDL. Numbers 1, 2 and 3 represent the three familial dysbetalipoproteinemia patients with the E3/E2 phenotype as determined by the immunoblotting technique (Table 1). R stands for reference samples, i.e. subject with E3/E3 homozygosity (left) and E2/E2 homozygosity (right).

betalipoproteinemic patients with the E2/E2 phenotype and for 50 apparently healthy subjects with the E3/E2 phenotype. All these subjects displayed the E4/E4 phenotype after cysteamine treatment of their plasma (Table 1).

It is known that degradation products of apoE may focus in the E3 position. This problem is particularly relevant when the apoE phenotyping is performed with the present immunoblotting technique using total plasma (Menzel & Utermann 1986). Therefore, for the three patients with the uncommon E3/E2 phenotype the apoE phenotyping was also carried out by the conventional method using delipidated VLDL. From the results shown in Fig. 2 it is obvious that these three patients also exhibited the E3/E2 phenotype when isolated VLDL was used. Consequently, in these patients the presence of apoE3 as determined by the immunoblotting method is not due to the presence of an apoE degradation product in their plasma.

Our results indicate that the three genetically unrelated familial dysbetalipoproteinemic patients with the E3/E2 phenotype either contain an uncommon E2 isoform with only one cysteine residue or an uncommon E3 isoform that contains no cysteine residues. To discriminate between these two possibilities, we studied family members of these patients. In all three families the subjects with the E3/E3 phenotype displayed the E4/E4 phenotype after cysteamine treatment, as shown for one of these families in

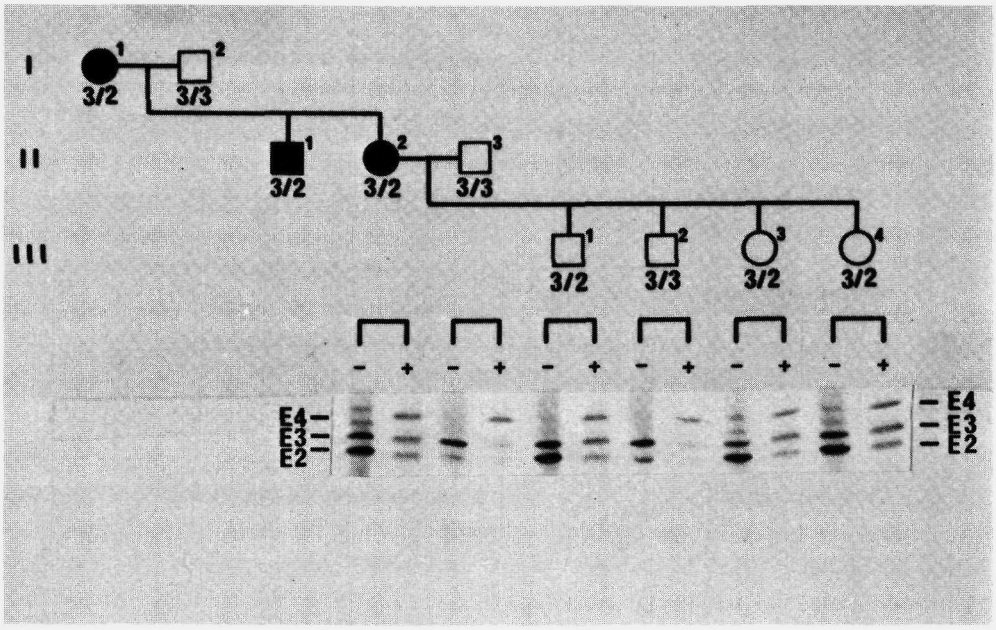

Fig. 3. Pedigree and corresponding apoE phenotyping of part of the family of a familial dysbetalipoproteinemic patient with a E3/E2 phenotype. ApoE phenotyping was performed using the immunoblotting technique. Each plasma sample was treated with (+) or without (−) cysteamine. The additional minor bands at positions between E3 and E4 and basic to E4 in subjects II-2, III-3 and III-4 without cysteamine treatment are obtained since relatively high amounts of old serum were applied to the gel. These minor bands occur only when old serum samples are used and are most probably derived from proteolysis during storage (Havekes et al., 1987). ●, ■ = patients with clinical symptoms of familial dysbetalipoproteinemia.

Fig. 3. This indicates that in the patients the E3 isoform normally contains one cysteine residue. Consequently, they exhibit an uncommon E2 isoform that contains only one cysteine residue.

In addition, the pedigree presented in Fig. 3 shows that in this family the uncommon E2 allele cosegregates with familial dysbetalipoproteinemia in the first and second generation. In the third generation the subjects are still too young to express familial dysbetalipoproteinemia, which usually develops after the third decade.

In SDS polyacrylamide gel electrophoresis, E2(Arg_{158} → Cys) displays a slightly slower electrophoretic mobility than E3 (Utermann et al. 1984). Using this technique, in VLDL from the patients with the E3/2 phenotype, slow-moving E2 could not be detected (kindly performed by Dr. Utermann, results not shown). This sustains the hypothesis that these patients exhibit an E2 allele different from the common E2(Arg_{158} → Cys) allele. The absence of the E2(Arg_{158} → Cys) allele in these patients has been confirmed by DNA hybridization techniques using synthetic oligomer (19-mer) DNA probes complementary to the E2(Arg_{158} → Cys) mutation (designated as 158 Cys-probe) and to the wild type allele (158 Arg-probe). The 158 Cys-probe did not hybridize with DNA from the patients with the E3/E2 phenotype, whereas the 158 Arg-probe did. Reciprocally, DNA from the patients with the E2/E2 phenotype hybridized with the 158 Cys-probe but not with the 158 Arg-probe (Table 1).

At present, it is unknown whether these

patients with the E3/E2 phenotype exhibit the same uncommon E2 allele. Two rare E2 alleles have been described so far, E2($Arg_{145} \rightarrow Cys$) and E2($Lys_{146} \rightarrow Gln$) (Rall et al. 1982a). The presence in these E3/E2 patients of the E2($Arg_{145} \rightarrow Cys$) allele can be excluded, as the E2($Arg_{145} \rightarrow Cys$) variant contains two cysteine residues and would therefore focus at the E4 position after cysteamine treatment. Rall et al. (1983) reported the existence of two dysbetalipoproteinemic patients with the E3/E2($Lys_{146} \rightarrow Gln$) phenotype. Whether the E2($Lys_{146} \rightarrow Gln$) variant that contains one cysteine residue is also present in our E3/E2 patients, is currently under investigation.

Because of the fact that familial dysbetalipoproteinemic patients with the E3/E2 phenotype are very rare and the fact that our three E3/E2 patients all exhibit an uncommon E2 allele, instead of the by far most frequently occurring E2($Arg_{158} \rightarrow Cys$) allele, we would like to suggest that this uncommon E2 allele behaves like a dominant trait in the expression of familial dysbetalipoproteinemia. This hypothesis is sustained by the observation that the uncommon E2 allele cosegregates with this disease, at least in the family presented in Fig. 3. At present, further family studies are being performed in order to evaluate this "dominance hypothesis".

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Chapter 5

Genetic heterogeneity in familial dysbetalipoproteinemia revealed by apolipoprotein E mutation-specific oligonucleotide probes

Submitted for publication

Genetic heterogeneity in familial dysbetalipoproteinemia revealed by apolipoprotein E mutation-specific oligonucleotide probes

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Summary

As determined by isoelectric focusing, most patients with familial dysbetalipoproteinemia (FD) exhibit the homozygous apolipoprotein (apo) E2E2 phenotype, whereas FD patients with the heterozygous phenotypes E3E2 or E4E2 are very rare.
Since isoelectric focusing cannot discriminate between the different E2 mutations described in the literature, we developed a procedure to study the genetic heterogeneity of the APOE gene based on hybridization of enzymatically amplified genomic DNA with mutation-specific oligonucleotide probes.
All FD patients (n=40) with the E2E2 phenotype proved 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. The absence of this mutation among individuals with the E2E2 (n=13) and E3E2 phenotype (n= 120) from a random population suggests that, in contrast to the common E2(arg158→cys) variant, the E2(lys146→gln) variant behaves like a dominant trait in the expression of FD.
This study demonstrates the power of mutation-specific oligonucleotide probes in combination with amplified DNA for the rapid and reliable identification of genetic microheterogeneity in the APOE gene. This is of primary importance for the elucidation of the relationship between the APOE polymorphism and susceptibility to familial dysbetalipoproteinemia.

Introduction

In normal individuals the chylomicron- and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver [1]. The apolipoprotein (apo) E, which is present on the lipoprotein remnants plays an important role as ligand for the high affinity hepatic lipoprotein receptors [2]. With isoelectric focusing apoE can be separated into three common isoforms, *i.e.* E2,

E3 and E4 [3,4] encoded by codominant alleles at a single APOE gene locus on chromosome 19 [5].
The apoE3 isoform is the most frequently occurring (or wild type) isoform. 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 E2 position with isoelectric focusing have been described. These are designated E2(arg158→cys), being the most common E2 mutation, E2(lys146→gln), E2(arg145→cys) and E2-Christchurch (arg136→ser) [6,7,8,9]. In addition, also double mutations have been described: E3(cys112→arg; arg142→cys) [10], E3(ala99→thr; ala152→pro) [11] and E1(gly127→asp; arg 158→cys) [12].
Familial dysbetalipoproteinemia (FD) or type III hyperlipoproteinemia is a genetic disorder of lipid metabolism, characterized by increased plasma cholesterol and triglyceride levels, 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 [13].
The vast majority of all FD patients exhibits the E2E2 phenotype, as defined by isoelectric focusing [14,15]. It has been shown that the different E2 isoforms display defective binding to the hepatic lipoprotein receptors [6,16] and delayed clearance from plasma [17]. However, only a small percentage (1-4%) of the E2E2 homozygotes, develops familial dysbetalipoproteinemia, suggesting that additional genetic and/or environmental factors are needed to express this disease. FD patients with the phenotypes E3E2 or E4E2 are very rare.
Recently, we encountered three unrelated FD patients with the E3E2 phenotype [18]. These patients exhibit a rare E2 variant containing only one cysteine residue. This E2 variant co-segregates with FD in their families, suggesting it to be 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, by using the technique of genomic DNA amplification (polymerase chain reaction, PCR) followed by hybridization with mutation-specific synthetic oligonucleotides.

Materials and methods

Patients
Patients with familial dysbetalipoproteinemia (FD) were diagnosed on the basis of the presence of hyperlipidemia, concomitant with floating beta lipoproteins and an elevated VLDL cholesterol/ total plasma triglyceride ratio (>0.69 on a mmolar basis). Furthermore, palmar and tuberous elbow xanthomas were frequently present in the patients. All patients with the E3E2 phenotype have the complete chemical and clinical picture.

ApoE phenotyping
EDTA plasma was obtained by 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 [19].

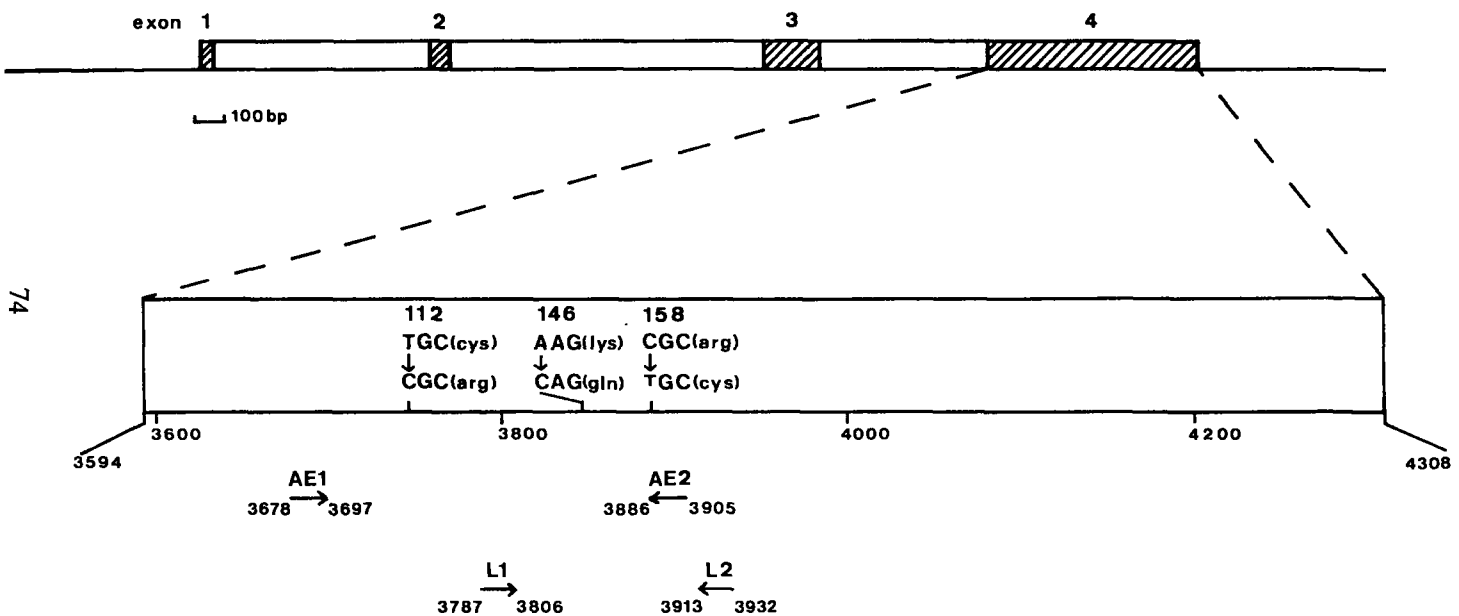
Genomic DNA isolation
DNA was isolated from leukocytes of whole blood by standard methods [20].

Polymerase Chain Reaction (PCR)
The procedure used for DNA amplification *in vitro* was a modification of the original procedure described by Saiki *et al.* [21].
The reaction mixture contained 1 μg of genomic DNA, 1 μM of each amplimer (oligonucleotides used for the chain elongation initiation) 0.1 mM dNTP's, 67 mM Tris.HCl pH 8.8, 6.7 mM $MgCl_2$, 6.7 mM EDTA, 10 mM ß-mercaptoethanol, 6.7 mM $(NH_4)_2SO_4$, 170 μg/ml BSA and 10 % v/v dimethylsulphoxide (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) was added to each sample and the chain elongation was performed at 65° C for 2 minutes. 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 unit at round 30.

Variant specific oligonucleotide probes
The oligonucleotides were synthesized by the solid phase triester method as described [22].
The oligonucleotides were end-labeled using ^{32}P ATP and T4 polynucleotide kinase in a 10 μl reaction mixture containing 10 pmol of oligonucleotide, 50 mM Tris.HCl pH7.6, 10 mM MgCl2, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA and 10 μg/ml BSA for 30 min at 37° C.
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. The labeled oligonucleotide was visualized by autoradiography and the excised band was eluted in 600 μl 1mM EDTA for 90 min at 65° C. The eluate was used directly for hybridization. The ^{32}P labeled oligonucleotides routinely have a specific activity between 10^8-10^9 cpm/μg.

Agarose gel electrophoresis/ Southern blotting and dot-blots
5 μl of the amplified DNA was separated by electrophoresis on a 1.5% agarose gel for 1 hour, followed by Southern blotting to GeneScreen Plus filters (New England Nuclear) in 0.4 M NaOH/ 0.6 M NaCl.

Figure 1. Schematic representation of the APOE gene; exon 4 enlarged. Localization of the 2 sets of amplimers (AE1/AE2 and L1/L2) used for the amplification of a part of exon 4 of the APOE gene. The most common mutations are indicated. Numbering of nucleotides is according to Paik et al. [33].



For dot-blots, GeneScreen Plus filters were pretreated by successive incubations in distilled water and 10 x SSC and were dried at 60° C. 3 μl of the amplified (denatured) DNA was spotted onto the filters [23].

Hybridization

The filters used for hybridization with the 158-Cys and 158-Arg oligonucleotides (for nomenclature of the oligonucleotides see table 3) were prehybridized (6 hr) and hybridized (16 hr) in 0.5 M Na_3PO_4, 7% SDS, 1 mM EDTA ('NaPi'). The filters used for hybridization with all other oligonucleotides were prehybridized (6 hr) and hybridized (16 hr) in 5 x SSPE (1 x SSPE=10 mM sodiumphosphate pH 7.0, 0.18 M NaCl and 1 mM EDTA), 0.3% SDS and 10 μg/ml denatured salmon sperm DNA. The temperature at which the (pre)hybridization took place was dependent on the set of oligonucleotides used. The filters hybridized with the 158-Cys and 158-Arg oligonucleotides were washed in 1.5 x SSC, 0.1% SDS, followed by washing in 3 x SSC, 0.1% SDS. The filters hybridized with the other oligonucleotide probes were washed in 5 x SSPE, 0.3% SDS followed by washing in 1 x SSPE, 0.3% SDS (see table 4 for all hybridization and washing conditions).

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

Results

Experimental

To improve the sensitivity and specificity of the oligonucleotide hybridization method for the detection of APOE variants, an *in vitro* amplification step was introduced. In the amplification procedure synthetic oligodeoxynucleotides (amplimers) complementary to sequences 5' and (in opposite orientation) 3' of the region of the APOE gene to be amplified are annealed to denatured genomic DNA and elongated using heat-stable Taq polymerase. Two sets of amplimers were used (AE1/AE2 and L1/L2, see table 2) with slightly different locations in the fourth exon of the APOE gene (figure 1) producing amplified bands of 188 bp and 106 bp respectively. With both sets of amplimers a single DNA band was visible after 35 cycles of amplification as evaluated by 1.5% agarose gel electrophoresis of 5 μl of the amplified DNA sample followed by ethidium bromide staining. These bands hybridize specifically with the APOE oligonucleotide probes (see figure 2).

In table 1 the amino acid and deduced nucleotide substitutions for a number of known apoE variants are given. For a number of these apoE variants, we designed sets of synthetic oligonucleotides against the mutant as well as the normal (E3) sequence (table 2), differing by a single nucleotide substitution.

For each set of oligonucleotides the optimal hybridization and specific washing conditions (temperature and buffer) were experimentally determined (table 3).

Figure 2. Hybridization signals after hybridization with the 158-arg oligonucleotide probe. The samples in lane A, D, E and G are amplified using the AE1 and AE2 amplimers (producing a single band of 188 bp), whereas the samples in lane B, C, F and H are amplified with the L1 and L2 amplimers (giving a band of 106 bp). Arrows indicate the positions of the wells.

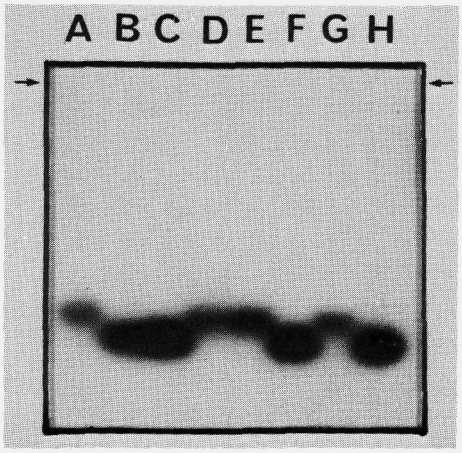


Table 1. APOE variants with known amino acid and deduced nucleotide substitutions [33]

variant	amino acid position									ref.
	99	112	127	136	142	145	146	152	158	
E3	GCG ala	TGC cys	GGC gly	CGC arg	CGC arg	CGT arg	AAG lys	GCC ala	CGC arg	
E4(cys112→arg)		CGC arg								[6]
E3(cys112→arg;arg142→cys)		CGC arg			TGC cys					[10]
E3(ala99→thr;ala152→pro)	ACG thr							CCC pro		[11]
E2(arg145→cys)						TGT cys				[6]
E2(lys146→gln)							CAG gln			[8]
E2(arg158→cys)									TGC cys	[7]
E2(arg136→ser)				AGC ser						[9]
E1(gly127→asp;arg158→cys)			GAC asp						TGC cys	[12]

Table 2. The sequences of the synthetic oligonucleotides used as amplimers and of the mutation-specific oligonucleotides used as hybridization probes. The AE1/AE2 amplimers are identical to those described by Smeets *et al.* [30].

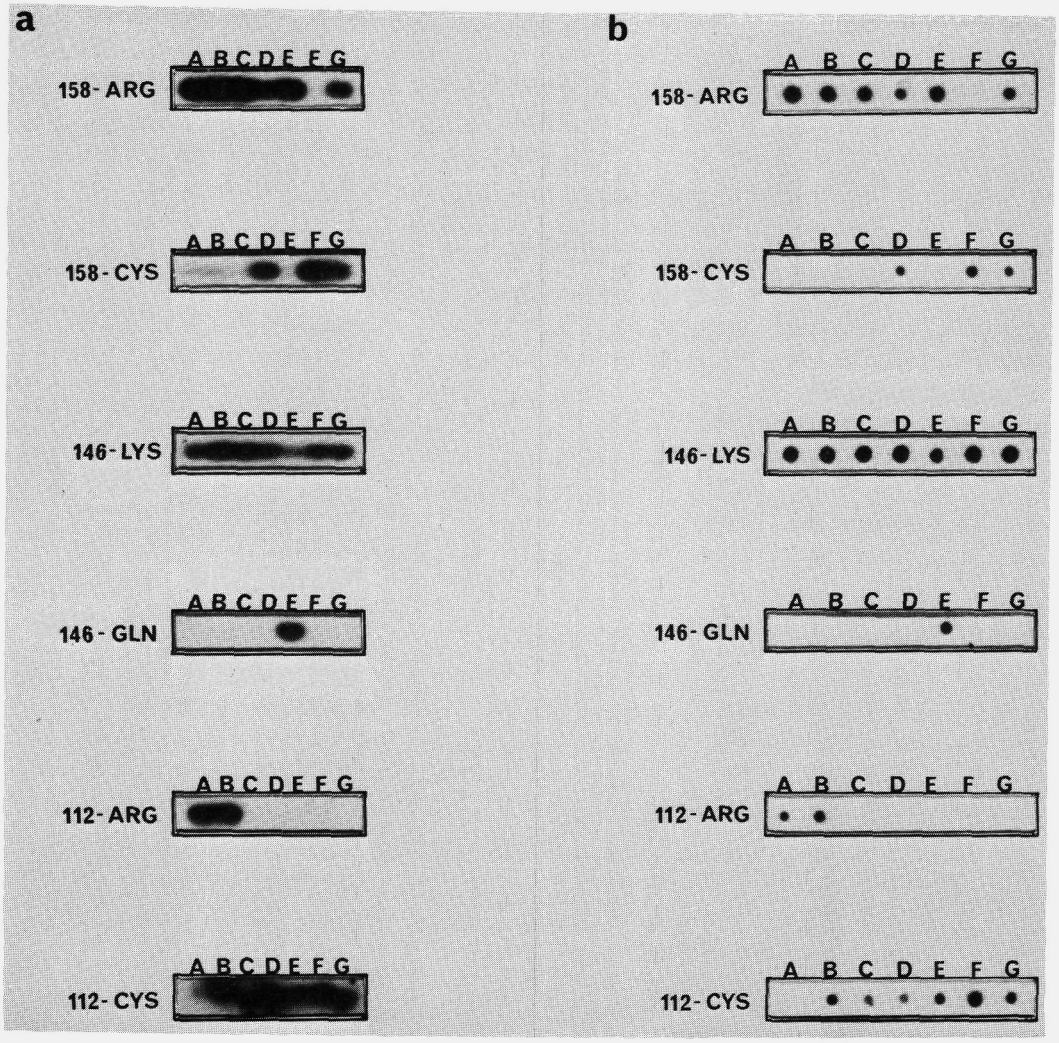
amplimer	**sequence**
AE1	5'-GCGGGCACGGCTGTCCAAGG- 3'
AE2	5'-CCGGCCTGGTACACTGCCAG- 3'
L1	5'-CTCGGCCAGAGCACCGAGGA- 3'
L2	5'-AGGCCGCGCTCGGCGCCCTC- 3'
mutation-specific oligonucleotide	**sequence**
112-arg	5'-AGGCGGCCGCGCACGTCCTCC-3'
112-cys	5'-AGGCGGCCGCACACGTCCTCC-3'
146-gln	5'-GGAGCCGCTGACGCAGCTT- 3'
146-lys	5'-GGAGCCGCTTACGCAGCTT- 3'
158-cys	5'-CTGCCAGGCACTTCTGCAG- 3'
158-arg	5'-CTGCCAGGCGCTTCTGCAG- 3'
145-cys	5'-AGCCGCTTACACAGCTTGCGC-3'
142-cys	5'-TCCCACCTGTGCAAGCTGC- 3'

Table 3. Hybridization and washing conditions of the different oligonucleotide probes (see also materials and methods)

oligo-nucleotide	hybr. temp.(°C)	hybr. buffer	washing procedure *	specific washtemp.(°C)
112-arg	64	SSPE	1	73.5
112-cys	64	SSPE	1	73.5
146-gln	53	SSPE	1	63.5
146-lys	53	SSPE	1	63.5
158-cys	53	NaPi	2	63.5
158-arg	53	NaPi	2	63.5
145-cys	57	SSPE	1	63.5
142-cys	53	SSPE	1	61

* Washing procedures:
procedure 1: 2 x 5' at room temperature in 5 x SSPE, 0.3% SDS
10' at hybridization temp. in 1 x SSPE, 0.3% SDS
10' at specific wash temp. in 1 x SSPE, 0.3% SDS
procedure 2: 2 x 30' at room temp. in 1.5 x SSC, 0.1% SDS
15' at hybridization temp. in 3 x SSC, 0.1% SDS
10' at specific wash temp. in 3 x SSC, 0.1% SDS

Figure 3. Detection of the APOE mutants using amplification and hybridization with mutant-specific oligonucleotide probes. **a**: 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 (see Materials and methods). **b**: 3 μl of the amplified DNA was spotted on pretreated GeneScreen Plus filters (see Materials and methods). The hybridization and washing conditions in 3a and 3b were identical and are described in table 3. In both kinds of experiments the same samples were investigated: A: E4E4; B: E4E3; C: E3E3; D: E3E2(arg158→cys); E: E3E2(lys146→gln); F: E2(arg158→cys)E2(arg158→cys); G: E2(arg158→cys)E2(?) 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. A schematic representation of these results is given in table 4.



The hybridization temperature was chosen 10-12 degrees below the melting temperature (Tm), which was calculated according to the formula Tm (° C)= 4 (G+C) + 2 (A+T), where G, C, A and T indicate the number of the corresponding nucleotides in the oligomer [24].
For a routine screening of the various mutant alleles, 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. After autoradiography for 1 to 3 hours strong signals were obtained (see figure 3a for examples). As the hybridization signals correspond with the amplified fragments visible on the ethidium bromide stained gel (figure 2), the electrophoresis step can be substituted by a direct dot blot procedure (figure 3b). The results presented in figure 3 are schematically presented in table 4. Two rare variants have been found. Sample E represents a subject heterozygous for 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.

Table 4. Schematic representation of the results of mutant-specific oligonucleotide hybridization shown in figure 3.

		phenotype by isoelectric focusing						
	oligo-nucleotide	A 4/4	B 4/3	C 3/3	D 3/2	E 2/2	F 2/2	G 2/2
	158-cys	-	-	-	+	-	+	+
	158-arg	+	+	+	+	+	-	+
	146-gln	-	-	-	-	+	-	-
	146-lys	+	+	+	+	+	+	+
	112-arg	+	+	-	-	-	-	-
	112-cys	-	+	+	+	+	+	+
	145-cys*	nd	nd	-	-	nd	-	-
	142-cys*	nd	nd	-	-	nd	-	-
deduced genotype	allele 1:	arg112	arg112	wt	wt	wt	cys158	cys158
	allele 2:	arg112	wt	wt	cys158	gln146	cys158	?

* The hybridization results using the 145-cys and 142-cys oligonucleotides are not shown in figure 3. nd: not determined

Screening of patients and healthy controls

The PCR method for DNA amplification followed by hybridization with mutant-specific oligonucleotides (figure 3) was applied for screening a sample of 40 FD patients with the E2E2 phenotype and 3 FD patients with the E3E2 phenotype. The amplified DNA

was hybridized with the mutation-specific oligonucleotides presented in table 2.
The results of the screening are presented in table 5. All FD patients with the E2E2 phenotype appeared to be homozygous for the common E2(arg158→cys) mutation. All three patients with the E3E2 phenotype were shown to carry the E2(lys146→gln) mutation.

Recently we performed apoE phenotyping by IEF in a population sample of 2000 35 years old males [25,26]. In this population study, 13 E2E2 homozygotes were found. 11 out of these 13 E2E2 individuals appeared to be homozygous for the E2(arg158→cys) mutation, whereas two of them appeared to be heterozygous for this common E2(arg158→cys) mutation and another so far unknown E2 mutation. The E2(lys146→gln) variant was not present in these individuals.
In addition to the 13 E2E2 homozygotes, 50 unrelated normolipidemic individuals with the E3E2 phenotype were screened by hybridization with the variant-specific oligonucleotide probes and appeared to contain the E2(arg158→cys) mutation.

Table 5. APOE genotypes, determined by hybridization with variant-specific oligonucleotides to amplified genomic DNA

subjects	**phenotype (IEF)**	**genotype (PCR-oligonucleotides)**
FD patients	E2E2 (n=40)	E2(arg158→cys) / E2(arg158→cys)
	E3E2 (n= 3)	E3 / E2(lys146→gln)
controls	E2E2 (n=11)	E2(arg158→cys) / E2(arg158→cys)
	E2E2 (n= 2)	E2(arg158→cys) / E2(?)
	E3E2 (n=50)	E3 / E2(arg158→cys)

Discussion

With isoelectric focusing, three common isoforms of apoE can be identified: E2, E3 and E4, differing one charge unit from each other.
ApoE2 is strongly associated with FD, since most patients have the E2E2 phenotype. However, only 1-4% of all individuals with the E2E2 phenotype develops this disease. Utermann *et al.* [27] suggested that either another gene and/or environmental factors are necessary in addition to the E2E2 phenotype to express FD. Furthermore, it is also possible that microheterogeneity in APOE2 is responsible for this reduced penetrance, *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(arg136→ser) [6,7,8,9]. Unequivocal detection of each of the

different known mutations can be obtained by DNA hybridization using mutation-specific oligonucleotides.

For mutation-specific oligonucleotide hybridization we previously used an 'in gel' hybridization procedure of restriction enzyme digested genomic DNA with variant-specific synthetic oligonucleotides [28]. Detection of an apoE variant with this procedure has also been described by Funke *et al.* [29]. In our hands this method is less suitable for screening purposes since relatively large amounts of genomic DNA (10 μg) and long exposure times are needed and additional (non-specific) bands are visible.
To improve the sensitivity, speed and specificity of the method for the detection of mutations in the APOE gene, an *in vitro* amplification step applying the heat stable Taq polymerase was included. This procedure, starting with 1 μg of genomic DNA produced strong, specific signals within 1 to 3 hours of exposure, making the procedure suitable for routine screening. Recently Smeets *et al.* [30] published an amplification technique using Klenow polymerase for the detection of the E2(arg158→cys) and E4(cys112→arg) mutations.
In the present paper the presence of the following variants was evaluated: E4(cys112→arg), E2(arg158→cys), E2(lys146→gln), E2(arg145→cys) and the E3(cys112→arg;arg142→cys). In particular, the unequivocal detection of the various E2 mutations is very useful, since they can not be distinguished from each other by isoelectric focusing.
We were able to demonstrate homozygosity for the E2(arg158→cys) mutation in 40 FD patients with the E2E2 phenotype and heterozygosity for the E2(lys146→gln) mutations in 3 FD patients with the E3E2 phenotype.

In a previous paper [18] 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 it to be the previously described E2(lys146→gln) variant [8], as the other known E2 variants contain two cysteine residues. 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.
Apparently, heterozygosity for this E2(lys146→gln) mutation is sufficient for the expression of FD. In our previous paper we described one family, in which the E2(lys146→gln) mutation co-segregates with the disease. Later we found that the same is true for the other two families of the FD probands with the E3E2 phenotype. Therefore we conclude that, in contrast to the common E2(arg158→cys) mutation, the E2(lys146→gln) mutation is dominant in the predisposition of an individual to FD.
To estimate the frequency of the E2(lys146→gln) variant in the Dutch population, 50 E3E2 unrelated normolipidemic individuals [25,26] were screened by hybridization with the variant specific oligonucleotide probes. They all appeared to contain the E2(arg158→cys) mutation (table 5). Additionally, another 70 E3E2 individuals from the same population were studied by the cysteamine modification followed by isoelectric

focusing [18]. Two cysteine residues were found in all E2 isoforms tested. Thus in a total of 120 E3E2 subjects from a random population the presence of the E2(lys146→gln) mutation could be excluded. In addition none of the 53 individuals with the E2E2 phenotype tested in the present study appeared to carry the E2(lys146→gln) mutation (see table 5). Based on these results, we conclude that the E2(lys146→gln) gene is very rare with a frequency of less than 0.001 and is so far exclusively found in FD patients with the E3E2 phenotype.
To investigate whether microheterogeneity in the APOE gene is involved in the reduced penetrance of FD in E2E2 homozygotes, we determined, in addition to the APOE genotype of 40 FD patients, also the APOE genotype of 13 clinically normal individuals with the apo E2E2 phenotype. All 40 patients and 11 out of the 13 control individuals proved to be homozygous for the E2(arg158→cys) mutation (table 5). The remaining 2 normolipidemic individuals were heterozygous for this common E2(arg158→cys) mutation and another so far unknown E2 mutation. These two individuals do not exhibit the known mutations at the positions 145 (arg→cys), 146 (lys→gln) and 142 (arg→cys) (see table 4). The nature of the E2 variants in these subjects is presently under investigation. As mentioned above, 50 individuals with the E3E2 phenotype also appeared to have the E2(arg158→cys) mutation. Since these heterozygotes do not express FD, we conclude that the E2(arg158→cys) mutation behaves like a recessive trait in the expression of FD.

From our present study, we conclude, that the reduced penetrance of FD in individuals with E2E2 homozygosity can not be explained by the presence of one of the known APOE point mutations in FD patients, since both patients and controls with the E2E2 phenotype appeared to be homozygous for the common E2(arg158→cys) variant. Still it can not be excluded that an additional mutation within the APOE gene, not detectable by presently available techniques, is responsible for the expression of FD in E2E2 homozygotes.

The hypothesis that, at least in some cases, a mutation in only one of the APOE genes (heterozygosity) is responsible for the expression of FD, is strongly supported by the presence of the E2(lys146→gln) variant in the three FD patients with the E3E2 phenotype (described in this paper). This variant alone seems to be sufficient to predispose an individual to develop FD. Previously, we described the E3-Leiden mutation which also behaves like a dominant mutation in the expression of FD [31]. Recently a second family has been discovered in which the E3-Leiden variant co-segregates with familial dysbetalipoproteinemia [Havekes, unpublished data]. Mann *et al.* [32] recently described a kindred in which an apoE variant, E1-Harrisburg, results in a dominant expression of FD.
The finding of different apoE variants predisposing to FD in a dominant manner should caution against the use of the E2E2 phenotype as an obligatory diagnostic criterium for FD.

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Chapter 6

DNA polymorphisms in the APOE-C1-C2 gene cluster and the APOB gene in relation to familial dysbetalipoproteinemia

Submitted for publication

DNA polymorphisms in the APOE-C1-C2 gene cluster and the APOB gene in relation to familial dysbetalipoproteinemia

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Summary

Most patients with familial dysbetalipoproteinemia (FD) are homozygous for the apo E2E2 phenotype, suggesting a recessive inheritance pattern. FD shows a reduced penetrance among E2E2 individuals, as only about 1-4% of all E2E2 homozygotes will develop the disease.

The present study aims at explaining the reduced penetrance considering two possibilities: microheterogeneity in the APOE gene and interaction with other hyperlipidemia genes. For this purpose we investigated a number of Restriction Fragment Length Polymorphisms (RFLPs) in the APOE-C1-C2 gene cluster on chromosome 19 and the APOB gene on chromosome 2.

Significant differences in allele frequencies were found between the control population sample and the group of FD patients for the HpaI/APOC1 ($p<0.0005$) and the TaqI/APOC2 RFLP ($p<0.005$) on chromosome 19. It appeared that the HpaI/APOC1 RFLP is strongly associated with the apoE polymorphism. ApoE3 is linked to the H1 allele, whereas apoE2 and E4 are associated with the H2 allele. The increased T1 frequency of the TaqI/APOC2 RFLP among FD patients could also be due to linkage disequilibrium with E2.

For the polymorphisms on chromosome 2 only the XbaI/APOB RFLP showed a significant difference in allele frequencies between the control population sample and the group of FD patients ($p<0.01$). Although the present study sustains the hypothesis that the APOB gene is involved in the predisposition to FD, extensive family studies are indispensable to obtain further support for this candidate gene hypothesis.

Introduction

Familial dysbetalipoproteinemia (FD, or type III hyperlipoproteinemia) is an autosomally inherited disorder with a prevalence of about 1-4 per 10000 individuals [1,2]. This disease is characterized by high plasma levels of cholesterol and triglycerides due to the accumulation of chylomicron- and very low density lipoprotein (VLDL) remnants. A high concentration of the remnants often results in premature coronary and peripheral atherosclerosis and xanthomatosis [3]. In healthy individuals the remnants are rapidly removed from the circulation by receptor-mediated uptake through the liver or conversion into low density lipoprotein (LDL) [4]. Apolipoprotein E (apoE) is the most important surface protein on the remnants and plays an important role in the hepatic uptake of the remnants, because it is recognized with high affinity by the lipoprotein receptors [5]. On the basis of isoelectric focusing (IEF) three major isoforms of apoE have been described, *i.e.* E2, E3 and E4, encoded by codominant alleles at a single APOE gene locus on chromosome 19 [6,7]. Six common apoE phenotypes can be distinguished in the general population: E2E2, E3E3, E4E4, E3E2, E4E2 and E4E3. Most FD patients exhibit the E2E2 phenotype. The accumulation of the lipoprotein remnants in FD patients is caused by a defective binding of apoE2 to the hepatic lipoprotein receptors [8,9] resulting in a delayed clearance of the remnants from plasma [10]. In the general population, the E2E2 phenotype has a frequency of about 1%. However, only 1-4% of all E2E2 individuals develop FD, suggesting a recessive inheritance pattern with a reduced penetrance. It is suggested that, in addition to the E2E2 phenotype, certain endogenous or exogenous factors like age, sex, hormones, nutrition and alcohol consumption are necessary for the expression of FD [3].

In addition, it has been suggested that other genetic factors are required for the expression of FD in the E2E2 homozygous individuals. In principle there are two, not mutually exclusive possibilities: microheterogeneity in the APOE gene and/or interaction with a gene (or genes) both predisposing to hyperlipidemia [11]. Genes involved in lipoprotein metabolism, especially in the metabolism of the chylomicrons and VLDL, are considered as candidate genes.
In this report, we present RFLP studies on the E-C1-C2 gene cluster on chromosome 19 and the APOB gene on chromosome 2 in a search for possible linkage disequilibria with FD. We studied the HpaI/APOC1 polymorphism because of a previously suggested association with FD [12]. The TaqI/APOC2 RFLP [13] was investigated because of the close linkage of the APOC2 gene with the APOE gene and because of the role of apoC2 as a co-factor for lipoprotein lipase (LPL). Furthermore, as the APOB gene has been suggested as another candidate gene [14], we examined the XbaI/APOB and EcoRI/APOB RFLPs [15] in our group of FD patients and in a random population sample.

Materials and Methods

Collection of the blood samples
The 45 FD patients were obtained from the Lipid Clinics in Leiden and Nijmegen. Patients with FD were diagnosed on the basis of the presence of hyperlipidemia (cholesterol > 7.5 mmol/l; triglycerides > 2.0mmol/l), concomitant with floating beta lipoproteins and an elevated VLDL cholesterol/ plasma triglycerides ratio (>0.69 on a mmolar basis). Furthermore, palmar and tuberous xanthomas were frequently present in the patients. Three of the FD patients exhibited the E3E2 phenotype, whereas the other patients appeared to be E2E2 homozygotes.
Recently we performed a population study involving 2018 35-year old males, randomly selected from three different areas in the Netherlands [16,17]. In this population sample we found 13 E2E2 individuals. From this general population we sampled randomly a subgroup of 130 individuals. The frequency of the APOE alleles in this small population sample was not significantly different from the frequency in the whole population.

ApoE phenotyping
ApoE phenotyping was performed using a micromethod which is based on isoelectric focusing of delipidated plasma samples followed by immunoblotting using anti-apoE antiserum [18].

DNA analysis
Genomic DNA was isolated from leukocytes in whole blood by standard methods [19]. The DNA was digested with the restriction enzymes EcoRI, XbaI, TaqI and HpaI, under conditions as recommended by the manufacturers (Pharmacia, Beckman). For the HpaI polymorphism, a HpaI-HindIII double digestion was performed. The fragments were separated by electrophoresis on 0.7% agarose gels at 1.5 V/cm for 16 hr. The gels were denatured for 2 x 15 min in 0.4 M NaOH/ 0.6 M NaCl and transferred in the same solution to GeneScreen Plus filters (New England Nuclear).
The following probes were used: a SacI fragment containing a part of the APOC1 gene [20], an APOC2 cDNA probe [21] and the APOB cDNA probe pAB1 [22]. All probes were labeled with [α^{32}P] dCTP using a multiprime kit (Amersham). Hybridization was performed as described by van Ommen *et al.* [23].

Statistical analysis
The Hardy-Weinberg equilibrium was evaluated by X^2 analysis.
We used the X^2 analysis of "2 x 2" tables based on gene counting to compare the genotype frequencies of the various RFLPs between the population sample and the patients group. The level of 0.05 was considered as statistically significant.
Haplotypes (chromosome 2: XbaI/APOB and EcoRI/APOB; chromosome 19: HpaI/APOC1 and TaqI/APOC2) were assigned for all unrelated individuals of the random control population except for those individuals heterozygous for both polymorphisms.

The pairwise linkage disequilibrium correlation coefficients (r) were estimated as described by Chakravarti *et al.* [24] and Litt and Jorde [25]. This r value is defined as

$$r = \frac{p_{11} - p_{10}p_{01}}{(p_{10}q_{10}p_{01}q_{01})^{1/2}}$$

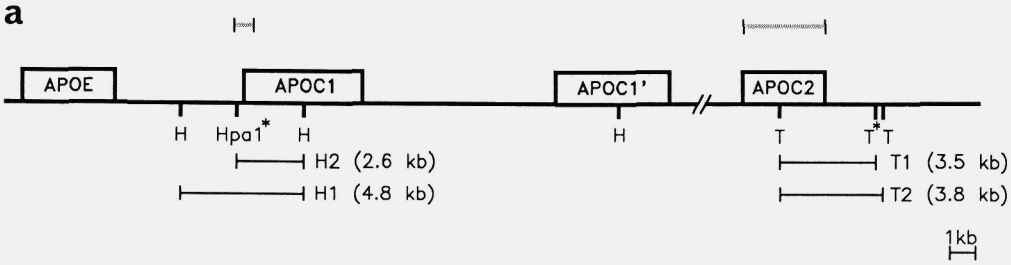
where p_{11}, p_{12}, p_{21} and p_{22} are the frequencies of the ++, +-, -+ and -- haplotypes (*e.g.* R1X1, R1X2, R2X1 and R2X2) and $p_{10} = p_{11} + p_{12}$, $q_{10} = 1 - p_{10}$, $p_{01} = p_{11} + p_{21}$ and $q_{01} = 1 - p_{01}$. This correlation coefficient is zero when there is linkage equilibrium. The (significant) differences from zero are calculated using the formula: $N (r)^2$ with df=1.

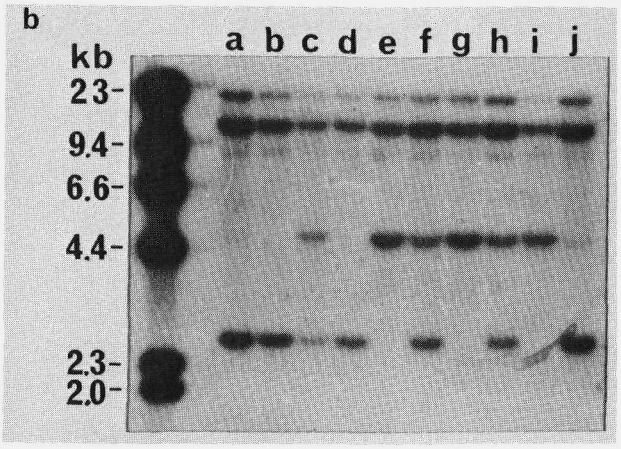
Results

Polymorphisms

HpaI/APOC1: Double digestion with HindIII and HpaI and hybridization with a genomic APOC1 fragment revealed a constant band of 12.2 kb and two polymorphic bands of 4.8 kb (H1) or 2.6 kb (H2) (see figure 1b). The polymorphic HpaI site maps to the 5' flanking region of the APOC1 gene [19] (figure 1a). This HpaI polymorphism was originally detected by hybridizing an APOE cDNA probe to HpaI digested DNA, giving large fragments of 60 kb (H1) and 20 kb (H2) [12]. The HindIII-HpaI double digestion and the use of a genomic APOC1 probe gave smaller fragments and stronger hybridization signals, making this method more reliable than the previously described procedure.
TaqI/APOC2: Digestion with TaqI and hybridization with an APOC2 probe detected a two allele polymorphism with bands of 3.5 kb (T1) and 3.8 kb (T2) (see figure 1c). The polymorphism is located 3' of the APOC2 gene [13] (figure 1a).
XbaI/APOB: XbaI digestion and hybridization with an APOB probe (pAB1) showed a two allele polymorphism with fragments of 8.6 kb (X1) or 5.0 kb (X2) and a constant band of 3 kb (figure 2b). The polymorphism is due to a single base change in the coding region of the gene without altering the amino acid sequence of the protein [26] (figure 2a).
EcoRI/APOB: Digestion with EcoRI and hybridization with an APOB probe (pAB1) detected a two allele polymorphism with bands of 10.5+2.0 kb (R1) and 12.5 kb (R2) and constant bands of 1.6 and 1.1 kb (figure 2c). This polymorphism is also due to a single base change in the coding region of the APOB gene, substituting a lysine residue for a glutamic acid [27] (figure 2a).

Figure 1. HpaI/APOC1 and TaqI/APOC2 RFLPs.
a: Physical map of the APOE-C1-C2 gene cluster on chromosome 19. Bars at the bottom indicate the polymorphic fragments. The extent of the probes is shown above the map. *: polymorphic sites, H: HindIII, T: TaqI. **b**: Southern blot analysis of the HpaI/APOC1 RFLP after a double digestion with HindIII and HpaI. Lanes a,b,d and j: H2H2; lanes c,f and h: H1H2; lanes e,g and i: H1H1. **c**: Southern blot analysis of the TaqI/APOC2 RFLP. Lanes a,d,g,i and j: T2T2; lanes b,c and e: T1T2; lanes f and h: T1T1.





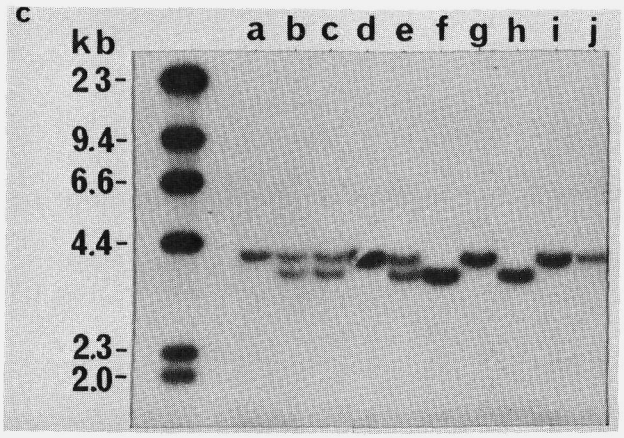
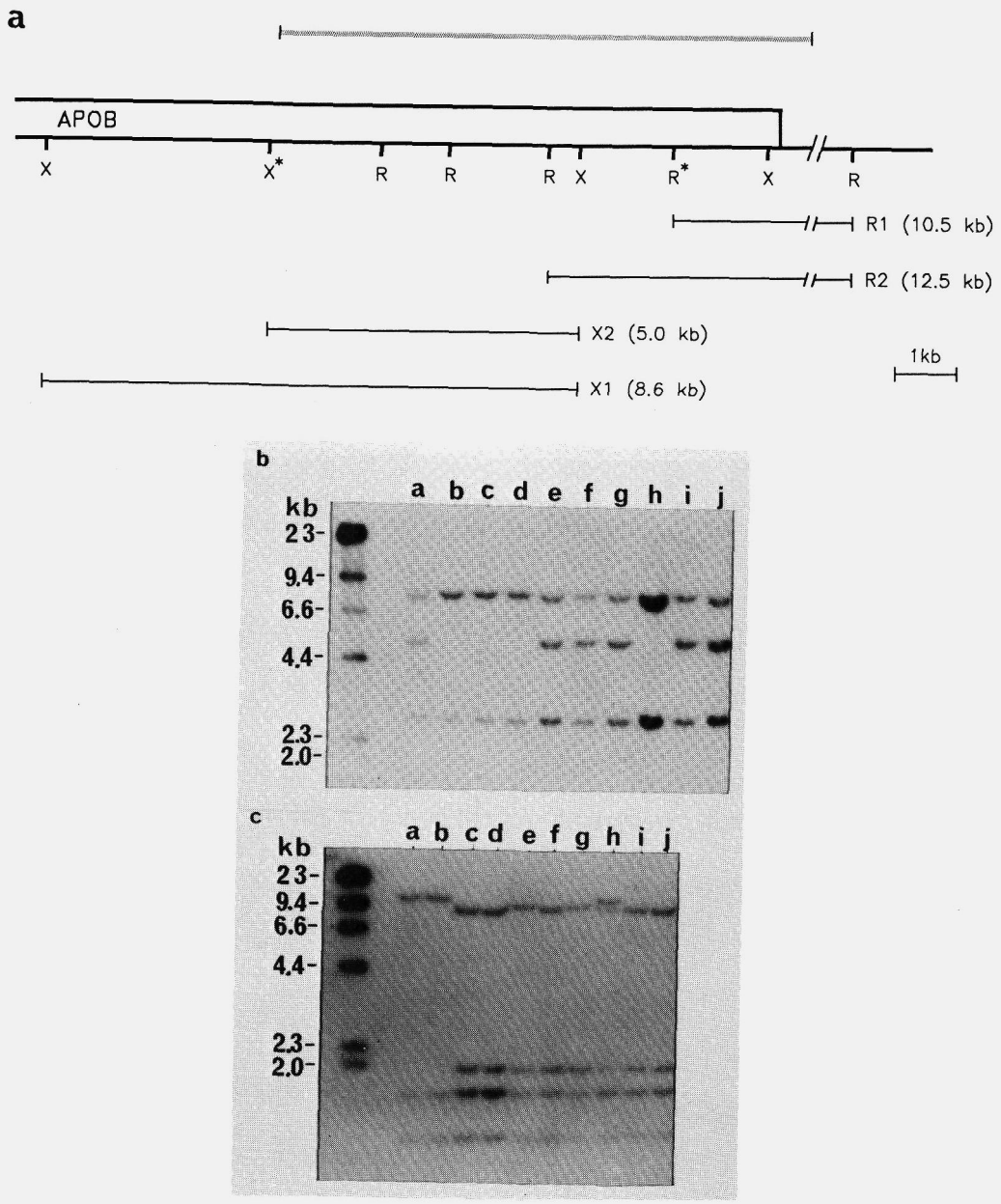


Figure 2. XbaI/APOB and EcoRI/APOB RFLPs.
a: Physical map of the carboxyl-terminal part of the APOB gene. Bars at the bottom indicate the polymorphic fragments. The extent of the probe is shown above the map. *: polymorphic sites, X: XbaI, R: EcoRI. **b**: Southern blot analysis of the XbaI/APOB RFLP. Lanes a,e,f,g,i and j: X1X2; lanes b,c,d, and h: X1X1. **c**: Southern blot analysis of the EcoRI/APOB RFLP. Lanes a and b: R2R2; lanes c,d,e,f,g,i and j: R1R1 and lane H: R1R2.



Chromosome 19: the apoE, HpaI/APOC1 and TaqI/APOC2 polymorphisms

Table 1 represents the genotype- and allele frequencies of the HpaI/APOC1 and TaqI/APOC2 RFLPs on chromosome 19 determined in a group of FD patients and in a random control population sample. In the random population sample the genotype distributions were in Hardy-Weinberg equilibrium. In the sample of FD patients, the genotype distribution for the TaqI/APOC2 RFLP was in Hardy-Weinberg equilibrium, whereas the distribution for the HpaI/APOC1 RFLP was not ($p<0.001$).
Comparing the allele frequencies between the two different groups we found significant differences in allele frequencies for both the HpaI/APOC1 and TaqI/APOC2 RFLPs ($p<0.0005$ and $p<0.005$, respectively).
For the HpaI/APOC1 RFLP only homozygotes (H1H1 and H2H2) were found in the patient group. Taking the apoE phenotype in account, it appeared that all patients with the E2E2 phenotype exhibited the H2H2 genotype, whereas the three patients with the E3E2 phenotype were H1H1 homozygotes (table 2). It should be noted that these E3E2 FD patients exhibited the rare apoE2 variant E2(lys146→gln), which appears to be dominant in the expression of FD (Smit *et al.*, unpublished results).
To establish whether the strong association was due to a linkage disequilibrium between the apoE polymorphism and the HpaI RFLP, 13 clinically normal E2E2 individuals, obtained from our former population screening [16,17], were examined. Eleven of them were H2H2 homozygotes, whereas the other two individuals were heterozygous H1H2 (table 2). This obvious association between apoE2 and the H2 allele prompted us to examine the control population sample in more detail, by estimating the association of the apoE phenotypes with the HpaI genotypes. The numbers of the pairwise combinations of phenotypes and genotypes are presented in table 3. It appeared that in addition to the association of the E*2 allele with the H2 allele, the E*4 allele is also associated with the H2 allele, whereas the E*3 allele is linked to the H1 allele (table 3). X^2 analysis confirmed that the HpaI/APOC1 polymorphism is in strong linkage disequilibrium with the apoE polymorphism ($p<0.0005$).
When we determined the TaqI/APOC2 genotypes in 13 clinically normal E2E2 individuals, we observed a T1 frequency of 0.69, which is close to the T1 frequency in FD patients (0.60), suggesting that the association of the TaqI/APOC2 polymorphism with FD is due to linkage with apoE2 (table 2). In the random population sample, there appeared to be linkage equilibrium between the apoE polymorphism and the TaqI/APOC2 polymorphism, and between the HpaI/APOC1 and the TaqI/APOC2 RFLPs (table 3).
For the HpaI/APOC1 and TaqI/APOC2 RFLPs on chromosome 19, haplotypes were assigned for all individuals of the random population, except for the double heterozygous individuals (data not shown). For the HpaI-TaqI haplotypes the standard linkage disequilibrium correlation coefficient r was calculated to prove the equilibrium between both polymorphisms. The linkage disequilibrium correlation coefficient was 0.003, which is not significantly different from zero ($p>0.05$), demonstrating linkage equilibrium.

Table 1. *Genotypes and allele frequencies for the HpaI/APOC1 and TaqI/APOC2 RFLPs in a random population sample and in a group of FD patients.*

polymorphism	N	genotypes			allele frequencies		H-W distribution X^2 (df=2)	p	PIC value	FD vs control X^2(df=1)	p
HpaI/APOC1		**H1H1**	**H1H2**	**H2H2**	**H1**	**H2**					
random population	130	75 (70.89)	42 (50.22)	13 (08.89)	0.738	0.263	3.48	n.s.	0.31	87.0524	p<0.0005
FD patients	31	3 (0.29)	0 (5.42)	28 (25.29)	0.097	0.903	31.0	p<0.001			
TaqI/APOC2		**T1T1**	**T1T2**	**T2T2**	**T1**	**T2**					
random population	130	25 (23.27)	60 (63.46)	45 (43.27)	0.423	0.577	0.39	n.s.	0.37	8.4042	p<0.005
FD patients	45	17 (16.20)	20 (21.60)	8 (7.20)	0.600	0.400	0.25	n.s.			

n.s.: not significant (p>0.05); H-W: Hardy-Weinberg; PIC: polymorphism information content
The differences in allele frequencies between both groups were estimated using the X^2 analysis of "2 x 2" tables based on gene counting. The expected numbers of genotypes are shown in parenthesis.

Table 2. The distribution of the HpaI/APOC1 and TaqI/APOC2 genotypes in FD patients and in apparently healthy, unrelated, individuals with the E2E2 phenotype.

HpaI/APOC1	N	H1H1	H1H2	H2H2
control subjects with the E2E2 phenotype	13	0	2	11
FD patients with the E2E2 phenotype	28	0	0	28
FD patients with the E3E2 phenotype	3	3	0	0
TaqI/APOC2	**N**	**T1T1**	**T1T2**	**T2T2**
control subjects with the E2E2 phenotype	13	6	6	1
FD patients with the E2E2 phenotype	42	16	18	8
FD patients with the E3E2 phenotype	3	1	2	0

Table 3. Observed and expected numbers of individuals from the random control population (N=130) with different paired combinations of phenotypes/ genotypes for the three polymorphisms on chromosome 19: apoE, HpaI/APOC1 and TaqI/APOC2.

apoE-HpaI/APOC1

	N	H1H1	H1H2	H2H2	
E2E2	1	0 (0.58)	0 (0.32)	1 (0.1)	
E3E2	17	0 (9.81)	17 (5.49)	0 (1.7)	
E3E3	73	73 (42.12)	0 (23.58)	0 (7.3)	
E4E3	29	2 (17.73)	23 (9.37)	4 (2.9)	
E4E4	4	0 (2.31)	1 (1.29)	3 (0.4)	
E4E2	6	0 (3.46)	1 (1.94)	5 (0.6)	total X^2 = 186.8264; df=10; p<0.0005

apoE-TaqI/APOC2

	N	T1T1	T1T2	T2T2	
E2E2	1	0 (0.19)	1 (0.46)	0 (0.35)	
E3E2	17	1 (3.27)	9 (7.85)	7 (5.88)	
E3E3	73	16 (14.04)	32 (3.69)	25 (25.27)	
E4E3	29	7 (5.58)	11 (13.38)	11 (10.04)	
E4E4	4	0 (0.77)	4 (1.85)	0 (1.38)	
E4E2	6	1 (1.15)	3 (2.77)	2 (2.08)	total X^2 = 9.074159; df=10 ; n.s.

HpaI/APOC1-TaqI/APOC2

	N	T1T1	T1T2	T2T2	
H1H1	75	16 (14.42)	32 (34.62)	27 (25.96)	
H1H2	42	8 (8.08)	19 (19.38)	15 (14.54)	
H2H2	13	1 (2.50)	9 (6.00)	3 (4.50)	total X^2 = 3.334571; df=4 ; n.s.

The expected numbers are shown in parenthesis. The differences between the observed and expected numbers were calculated using X^2 analysis. n.s.: not significant (p>0.05)

Table 4. Genotypes and allele frequencies for the XbaI/APOB and EcoRI/APOB RFLPs in a random population sample and in a group of FD patients with the E2E2 phenotype.

polymorphism	N	genotypes			allele frequencies		H-W distribution X^2 (df=2) p		PIC value	FD vs control X^2 (df=1) p	
XbaI/APOB		**X1X1**	**X1X2**	**X2X2**	**X1**	**X2**					
random population	128	40 (38.28)	60 (63.44)	28 (26.28)	0.547	0.453	0.38	n.s.	0.37	7.0817	p<0.01
FD patients	41	4 (5.86)	23 (19.28)	14 (15.86)	0.378	0.622	1.53	n.s.			
EcoRI/APOB		**R1R1**	**R1R2**	**R2R2**	**R1**	**R2**					
random population	128	79 (78.91)	43 (43.18)	6 (5.91)	0.785	0.215	0.00	n.s.	0.28	0.4823	n.s.
FD patients	34	23 (23.06)	10 (9.88)	1 (1.06)	0.824	0.176	0.00	n.s.			

n.s.: not significant (p>0.05); H-W: Hardy-Weinberg; PIC: polymorphism information content
The differences in allele frequencies between both groups were estimated using the X^2 analysis of "2 x 2" tables based on gene counting. The expected numbers of genotypes are shown in parenthesis.

The informativeness of the HpaI-TaqI haplotypes was evaluated by estimating the pairwise polymorphism information content (PIC) [28]. This value was compared to the maximum PIC value (PIC-max) which was obtained based on the assumption of random association between both polymorphisms. The PIC value of the HpaI-TaqI haplotypes is 0.61, which is 97% of the PIC-max value (0.63), rendering these haplotypes informative.
Comparison of the frequencies of the HpaI-TaqI haplotypes between the FD patients group and the random control sample revealed a significant difference in frequencies (X^2=102, df=3, p<0.001; data not shown) as expected since both individual polymorphisms showed significant differences in allele frequencies between the two groups (table 1).

Chromosome 2: the XbaI/APOB and EcoRI/APOB RFLPs

Table 4 shows the genotype- and allele frequencies of the XbaI/APOB and EcoRI/APOB RFLPs in a group of FD patients and in a random control population sample.
In both the random population sample and the group of FD patients the genotype distributions were in Hardy-Weinberg equilibrium.
Using X^2 analysis we found significant differences in allele frequencies of the XbaI/APOB RFLP between the random population sample and the FD patients group (p<0.01). No significant difference was found in allele frequency between both groups for the EcoRI/APOB polymorphism.
For the two restriction fragment length polymorphisms on chromosome 2 (XbaI/APOB and EcoRI/APOB) the numbers of individuals with the various combinations of genotypes are presented in table 5. Differences between the expected and the observed number of haplotypes were found to be significant (p<0.001) implying that the XbaI and the EcoRI polymorphisms are in linkage disequilibrium.
This observation could be confirmed calculating the standard linkage disequilibrium correlation coefficient r. For the two RFLPs on chromosome 2 the value of r is -0.358, demonstrating a linkage disequilibrium between both RFLPs (p<0.001).
Haplotypes of both polymorphisms were established for all individuals, except for the double heterozygotes (data not shown).
The observed and maximum pairwise PIC-values for the XbaI-EcoRI haplotypes polymorphisms (0.53 and 0.60 respectively) revealed that using both polymorphisms gives only 88% of the maximum PIC-value, which could be expected because of the observed linkage disequilibrium between both polymorphisms.
Comparison of the XbaI-EcoRI haplotype frequencies between the patients group and the random control group showed a significant difference (X^2=11.9,df=3,p<0.01; data not shown) which was less significant than the difference between both groups in allele frequencies of the XbaI/APOB polymorphism alone as presented in table 4.

Table 5. Observed and expected numbers of individuals from the random control population sample (N=127) with different paired combinations of the XbaI/APOB and EcoRI/APOB genotypes. .

XbaI/APOB-EcoRI/APOB				
	N	X1X1	X1X2	X2X2
R1R1	78	17 (24.57)	34 (36.85)	27 (16.58)
R1R2	43	17 (13.54)	26 (20.31)	0 (9.14)
R2R2	6	6 (1.89)	0 (2.83)	0 (1.28)

total X^2 = 33.76032; df=4; p<0.001

The expected numbers are shown in parenthesis. The differences between the observed and expected numbers were calculated using X^2 analysis.

Discussion

Most FD patients are homozygous for the apo E2E2 phenotype. FD shows a reduced penetrance among E2E2 homozygotes, as only 1-4% of all individuals with the E2E2 phenotype develop FD. In addition, a number of rare apoE variants is known, predisposing to FD in a dominant manner [29-31].
The reduced penetrance of FD in E2E2 homozygotes might be due to microheterogeneity in the APOE gene and/ or the co-segregation of a second genetic factor, predisposing to other forms of hyperlipidemia, such as familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCH) or familial hypertriglyceridemia (FHT) [32-35]. Some large FD families provided evidence for the segregation of a gene for familial combined hyperlipidemia, independent from the APOE gene [11,34] whereas in general, relatives of FD patients often display hypertriglyceridemia [32]. These observations suggest that genes, predisposing to hyperlipidemia might be the additional factor necessary for the expression of FD in E2E2 homozygotes.
Using RFLP analysis, we investigated a number of candidate genes that might be involved in the development of FD along with the APOE gene: APOC1, APOC2 and APOB.
As microheterogeneity in the E-C1-C2 gene cluster can not be excluded as an explanation for the reduced penetrance of FD in E2E2 homozygotes, it is important to evaluate the allele frequencies of the HpaI/APOC1 and TaqI/APOC2 RFLPs in this respect. Furthermore, apoC1 is suggested to be an activator of lecithin cholesterol acyltransferase (LCAT) [36] whereas apoC2 acts as an activator of lipoprotein lipase (LPL) [37], an enzyme directly involved in the metabolism of chylomicrons and VLDL.

The APOB gene on chromosome 2 is a candidate gene, since apoB is involved in both the synthesis and catabolism of chylomicrons and VLDL. Previous RFLP studies suggested the possible involvement of the APOB gene in the development of FD [14].

Chromosome 19

In this study, we found a significant difference in allele frequencies of the HpaI/APOC1 RFLP between the group of FD patients and the random control population sample. In a previous paper Klasen *et al.* [12] also described the increased frequency of the H2 allele in a group of FD patients, suggesting a linkage disequilibrium between this polymorphism and a gene predisposing to FD, in addition to E2E2 homozygosity. In the present study we examined the HpaI RFLP in more detail by determining the allele frequencies in FD patients, in a random control population and in control E2E2 individuals. In contrast to the results described in the previous publication of Klasen *et al.* [12], we found that most healthy E2E2 individuals also appeared to be H2H2 homozygotes. This means that linkage disequilibrium between the H2 allele of this HpaI polymorphism and the APOE*2 allele is the explanation for the high frequency of the H2 allele in FD patients, since most FD patients are homozygous for the E*2 allele. The discrepancy of the present results with the results obtained by Klasen *et al.* [12], are probably due to previous problems in the determination of the HpaI/APOE genotype, involving detection of large fragments (H1 and H2, respectively 60 and 20 kb). The present method of HindIII-HpaI double digestion and the use of an APOC1 probe instead of the APOE cDNA probe improved the detection of this HpaI polymorphism.

As shown in table 3 our results indicate that the E*3 allele is associated with the H1 allele, whereas in addition to the E*2 allele, also the E*4 allele is associated with the H2 allele. A few exceptions in the E4E4 and E4E3 group have been found. The three FD patients with the E3E2 phenotype and the H1H1 genotype (table 1 and 2) appeared to be heterozygous E3E2(lys146→gln) whereas the two clinically normal E2E2 individuals with the H1H2 genotype exhibited heterozygosity for the common E2(arg158→cys) variant and an as yet unknown E2 mutation [Smit *et al.*, unpublished results].

From these unpublished apoE genotyping results and from the present HpaI/APOC1 RFLP results, we conclude that among all four apoE2 variants described thus far, only the common E2(arg158→cys) allele is linked to the H2 allele whereas all three rare E*2 alleles are, like the E*3 allele linked to the H1 allele. Thus, using the HpaI polymorphism together with apoE isoelectric focusing, it is possible to discriminate between the presence of the common E2(arg158→cys) variant or the presence of rare E2 variants.

From evolutionary point of view, the E2(arg158→cys) and E4 mutations could have occurred in an E3-H1 haplotype, since no E3-H2 haplotypes have been found. In that case both mutations were immediately followed by H1→H2 mutations, since the E2-H2 and E4-H2 haplotypes are the most common haplotypes for both variants.

In contrast to a previous report by Humphries *et al.* [13], we found a significant difference in allele frequencies of the TaqI/APOC2 RFLP between the FD patients and the random control population ($p<0.005$; table 1).

In the general population sample we could not find a significant linkage disequilibrium

between the apoE polymorphism and the TaqI/APOC2 RFLP. However, the observed T1 allele frequency of 0.69 in 13 clinically normal individuals with the E2E2 phenotype is close to the frequency of T1 in the group of FD patients with the E2E2 phenotype (0.60). This suggests an association between the TaqI/APOC2 T1 allele and the APOE*2 allele. This association was also previously described by Humphries *et al.* [38]. Additional studies are necessary to reveal whether the association of the TaqI/APOC2 RFLP with FD is due to a linkage disequilibrium with the apoE polymorphism rather than to an association with other genes in the chromosome 19 gene cluster.

Chromosome 2

Using the XbaI/APOB RFLP we detected a significant difference in allele frequencies between the random control population and the FD patients (X^2 =7.0817; $p<0.01$; table 4). With this finding we were able to confirm previous results, described by Talmud *et al.* [14], showing a significant difference in XbaI/APOB allele frequencies between healthy individuals and FD patients (X^2 =7.98; $p<0.01$).

In order to test the possible involvement of the APOB gene in the development of FD in E2E2 homozygotes extensive family studies should be performed. In our group family studies applying the highly polymorphic Variable Number of Tandem Repeats (VNTR) APOB probe [39] are in progress to increase the informativity in the families.

Several studies showing an association of the APOB gene with plasma cholesterol and triglyceride levels further support the possible involvement of apoB in the expression of FD [14,40,41].

In the present study we showed that the XbaI/APOB and EcoRI/APOB RFLPs are in linkage disequilibrium as could be expected because of their short distance of 5-6 kb [15]. Our data are in contradiction with the data recently published by Ferns *et al.* [42]. This difference can be due to a relative small sample size used in the studies by Ferns (n=22) or due to differences in population samples. From the present study it can be concluded that the APOB gene still is a candidate gene, involved in the predisposition to FD. Family studies are indispensable to obtain support for these observations.

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Chapter 7

Apolipoprotein gene cluster on chromosome 19

Definite localization of the APOC2 gene and the polymorphic HpaI site associated with type III hyperlipoproteinemia

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Short communications

Apolipoprotein gene cluster on chromosome 19

Definite localization of the APOC2 gene and the polymorphic HpaI site associated with type III hyperlipoproteinemia

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Summary. Recently, using an APOE cDNA probe, we discovered an HpaI restriction fragment length polymorphism (RFLP) that appeared to be strongly associated with the expression of type III hyperlipoproteinemia (Klasen et al. 1987). In the present report it is shown that the same HpaI RFLP can be revealed with both the APOC1 and APOC2 cDNA probes. This enabled us to localize the polymorphic HpaI site between the APOE and APOC1 genes and to localize the APOC2 gene approximately 22 kb 3′ of the APOC1 pseudogene on chromosome 19.

Introduction

The protein moiety of plasma lipoproteins consists of apolipoproteins, of which eight major classes have been described: A1, A2, A4, B, C1, C2, C3, and E. For most of these the role in plasma cholesterol and triglyceride transport is known. Comparison of the nucleotide sequence and gene structure of the apolipoproteins A1, A2, A4, C1, C2, C3, and E strongly suggests that they have evolved from a common ancestral gene (Boguski et al. 1986; Karathanasis et al. 1986; Luo et al. 1986). At present, two clusters of apolipoprotein genes have been reported. The genes coding for the apolipoprotein A1, C3, and A4 are closely linked on the long arm of chromosome 11, spanning no more than about 14 kb (Karathanasis 1985; Ferns and Galton 1986). The genes coding for the apolipoproteins C1, C2, and E are clustered on the long arm of chromosome 19 as demonstrated by linkage studies with chromosome 19 markers (Olaisen et al. 1982; Donald et al. 1985) and by somatic cell hybridization studies (Bruns et al. 1984; Das et al. 1985; Scott et al. 1985; Tata et al. 1985). Recently the APOC1 gene has been localized 3′ of the APOE gene at a distance of approximately 4.3 kb (Davison et al. 1986; Myklebost and Rogne 1986). A pseudogene for APOC1 (APOC1′) is located 3′ at approximately 6 kb from the APOC1 gene (Davison et al. 1986). Although close genetic linkage between the loci for APOE and APOC2 has been demonstrated (Humphries et al. 1984; Myklebost et al. 1984a), neither the exact distance between the APOE and

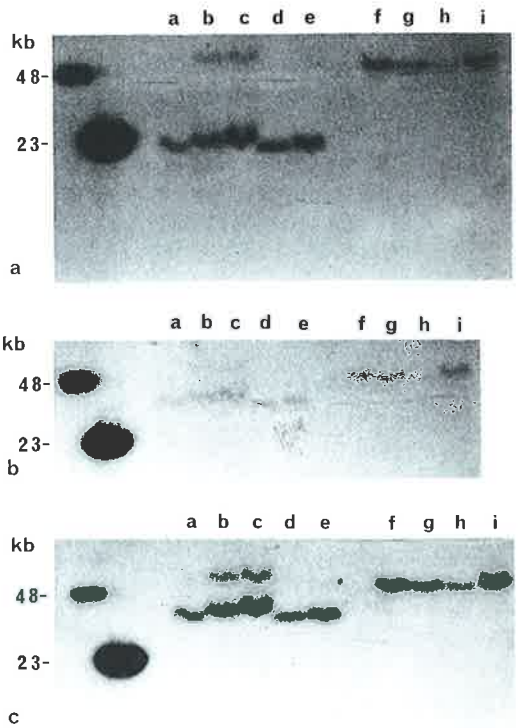

Fig. 1a–c. Southern blot analysis of the HpaI polymorphism detected with the APOE cDNA probe (**a**), the APOC1 cDNA probe (**b**) and the APOC2 cDNA probe (**c**). The same filter containing HpaI-digested DNA from nine unrelated individuals was sequentially hybridized with the APOE cDNA probe, the APOC1 cDNA probe, and the APOC2 cDNA probe. The previous probe was carefully removed before rehybridization (see Materials and methods). *Lanes a, d,* and *e* contain DNA from H2H2 individuals (polymorphic HpaI site present), *lanes f, g, h,* and *i* contain DNA from H1H1 individuals (missing the polymorphic HpaI site), and *lanes b* and *c* contain DNA from H1H2 heterozygotes

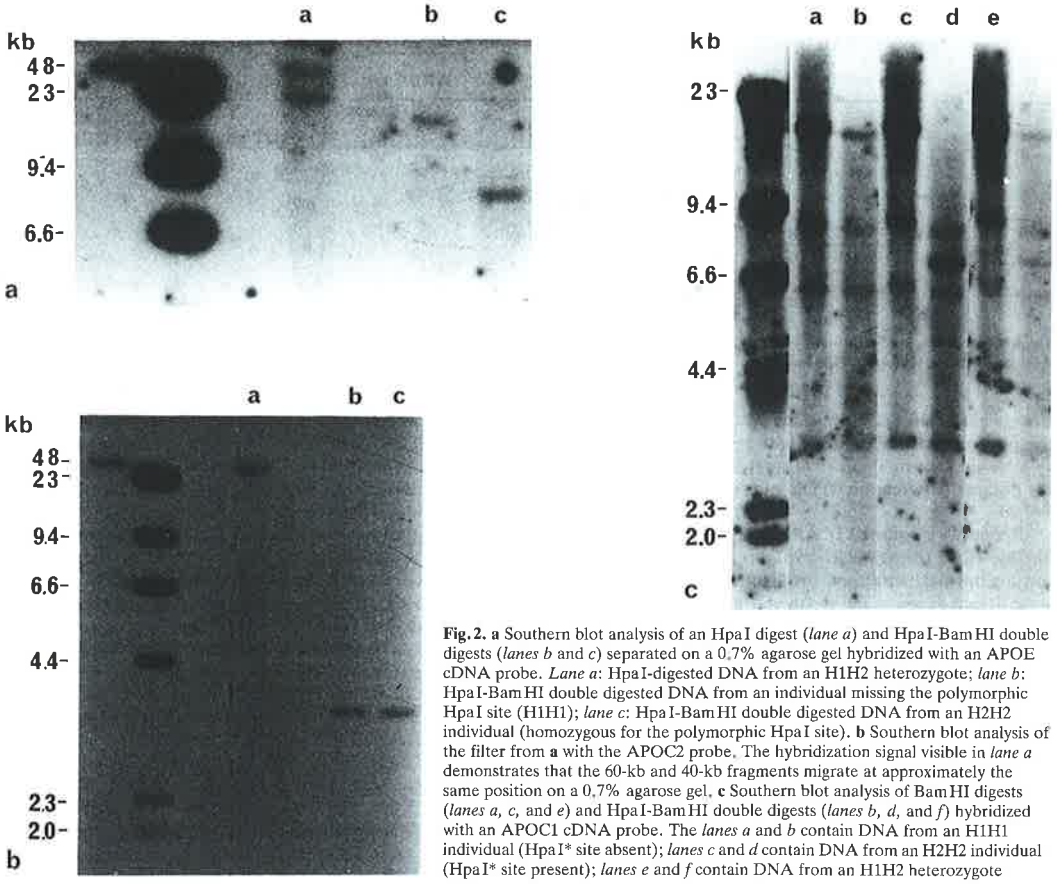

Fig. 2. **a** Southern blot analysis of an HpaI digest (*lane a*) and HpaI-BamHI double digests (*lanes b* and *c*) separated on a 0.7% agarose gel hybridized with an APOE cDNA probe. *Lane a*: HpaI-digested DNA from an H1H2 heterozygote; *lane b*: HpaI-BamHI double digested DNA from an individual missing the polymorphic HpaI site (H1H1); *lane c*: HpaI-BamHI double digested DNA from an H2H2 individual (homozygous for the polymorphic HpaI site). **b** Southern blot analysis of the filter from **a** with the APOC2 probe. The hybridization signal visible in *lane a* demonstrates that the 60-kb and 40-kb fragments migrate at approximately the same position on a 0.7% agarose gel. **c** Southern blot analysis of BamHI digests (*lanes a, c,* and *e*) and HpaI-BamHI double digests (*lanes b, d,* and *f*) hybridized with an APOC1 cDNA probe. The *lanes a* and *b* contain DNA from an H1H1 individual (HpaI* site absent); *lanes c* and *d* contain DNA from an H2H2 individual (HpaI* site present); *lanes e* and *f* contain DNA from an H1H2 heterozygote

APOC2 genes nor their relative order has been elucidated. Recently, we found an APOE RFLP with the restriction enzyme HpaI (Klasen et al. 1987). This RFLP appeared to be strongly associated with type III hyperlipoproteinemia. In this paper we demonstrate that the same HpaI RFLP can be detected with APOC1 and APOC2 cDNA probes. This enabled us to establish the definite localization of the APOC2 gene and the polymorphic HpaI site in the apolipoprotein gene cluster on chromosome 19.

Materials and methods

DNA was isolated from leukocytes in whole blood by standard methods (Hofker et al. 1985). DNA was digested with HpaI and BamHI (Pharmacia) under conditions recommended by the manufacturer. The HpaI fragments were separated by electrophoresis on a 0.4% agarose gel at 1.5 V/cm for 30 h. Before transfer to Gene Screen Plus filters by Southern blotting, the gel was treated for 40 s with 254 nm UV light. The BamHI digests and the HpaI-BamHI double digests were separated by electrophoresis on a 0.7% gel at 1.5 V/cm for 16 h. Hybridization was performed as described by van Ommen et al. (1983). The following probes were used: the APOE cDNA clone pE-368 (Zannis et al. 1984), the APOC2 cDNA probe (Myklebost et al. 1984b), and the APOC1 cDNA probe (Knott et al. 1984). Plasmid DNA (100 ng) was labeled by nick translation using a kit and α-^{32}P dCTP (800 Ci/mmole; Amersham). The filters were exposed to X-ray film for 1–4 days. Filters were rehybridized after "stripping" in 0.01 *N* NaOH (2 × 15 min) followed by a 2 × SSC wash (standard saline citrate: 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.0). The sizes of the hybridizing fragments were estimated by comparison with HindIII digested phage λ DNA markers run in parallel tracks and cohybridized with 1 ng of labeled λ DNA.

Results and discussion

It has previously been demonstrated that the genes coding for the apolipoproteins E, C1, and C2 are clustered on the long arm of chromosome 19 (19cen→19q13.2). Recently it has been established that the physical distance of the APOC1 gene and the APOC1 pseudogene 3′ of the APOE gene is 4.3 kb and 14 kb respectively (Davison et al. 1986; Myklebost and Rogne 1986). Although several studies have revealed

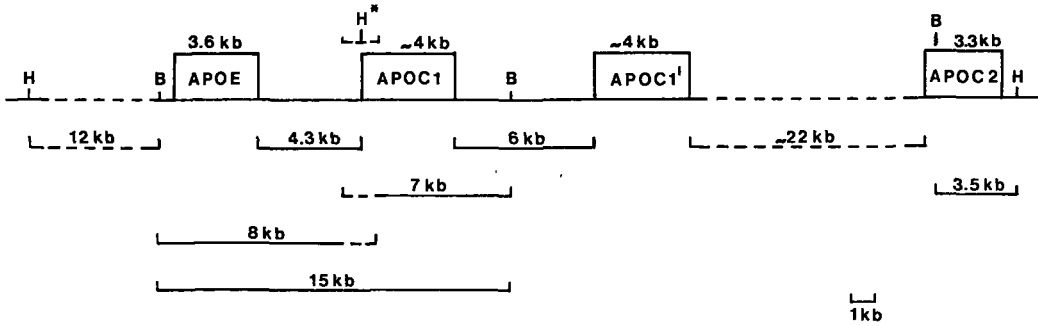

Fig. 3. Map of the apolipoprotein E-C1-C2 gene cluster on chromosome 19. The position of the polymorphic HpaI site is indicated with an asterisk. *B*, BamHI site; *H*, HpaI site

strong genetic linkage between the APOE and APOC2 genes, the physical distance between both genes and their relative order is not known. Studies by Das et al. (1985) indicated that the distance between the APOE and APOC2 genes is at least 5–7 kb.

Recently we reported an APOE RFLP with the restriction enzyme HpaI (Klasen et al. 1987). In individuals heterozygous for this polymorphism, two hybridizing DNA fragments were detected: one of 60[1] kb (the H1 allele) and one of 20 kb (the H2 allele; Fig. 1a). We found a very strong association between the H2H2 genotype and type III hyperlipoproteinemia in individuals exhibiting the APOE2/2 phenotype. As the polymorphic HpaI site could be located in another gene, which together with the APOE gene might be involved in the expression of type III hyperlipoproteinemia, it was important to try to localize this site. This was done by hybridizing the HpaI filters with other probes adjacent to the APOE gene (i.e., APOC1 and APOC2 cDNA probes). The same polymorphism was detected with an APOC1 cDNA probe revealing hybridizing fragments of 60 kb (H1) and 40 kb (H2; 20 kb with the APOE cDNA probe, see Fig. 1b). In our previous study (Klasen et al. 1987) the 40-kb and 60-kb fragments could not be separated on a 0.7% agarose gel; however with an extended run on a 0.4% agarose gel these large fragments were clearly separated. Rehybridization of the HpaI filters with an APOC2 cDNA probe resulted in a polymorphism identical to that found with an APOC1 cDNA probe with hybridizing fragments of 60 kb and 40 kb (Fig. 1c). Care was taken to ensure complete removal of the APOC1 cDNA probe.

Assuming one polymorphic HpaI site, the results presented above together with data from the literature suggest the position of the APOC2 gene to be 3′ of the APOC1 genes and that of the polymorphic HpaI site to be between the APOE gene and the APOC1 gene. To confirm the suggested structure of this apolipoprotein gene cluster, we took advantage of the BamHI sites in this area (Davison et al. 1986; Myklebost and Rogne 1986). In an individual missing the polymorphic HpaI site (H1H1) the APOE cDNA probe hybridized to a 15-kb BamHI-BamHI fragment (Fig. 2a, lane b). The same fragment was detected with the APOC1 cDNA probe (Fig. 2c, lane b). In an individual homozygous for the polymorphic HpaI site (H2H2) the 15-kb BamHI-BamHI fragment was split up into an 8-kb fragment (detectable with the APOE cDNA probe; Fig. 2a, lane c) and a 7-kb fragment (detectable with the APOC1 cDNA probe; Fig. 2c, lane d). When hybridized with the APOC1 cDNA probe, additional constant BamHI-BamHI fragments of approximately 8.5 kb, 6.3 kb, and 3 kb were visualized (Fig. 2c). These fragments can probably be explained by the presence of the APOC1 pseudogene, which is homologous to the APOC1 cDNA probe. The APOC2 cDNA probe detected a constant 3.5-kb band in both H1H1 and H2H2 individuals (Fig. 2b), which appeared to be a BamHI-HpaI fragment (data not shown).

With these BamHI-HpaI double digests we confirmed the localization of the polymorphic HpaI site between the APOE and APOC1 genes. Based upon known distances between the APOE and APOC1 genes (Davison et al. 1986; Myklebost and Rogne 1986) and upon the results from this study we suggest that the polymorphic HpaI site is located in, or 5′ nearby the APOC1 gene (Fig. 3). At present the sequencing of the region around the polymorphic HpaI site is in progress.

We can also definitely establish the localization of the APOC2 gene 3′ of the APOC1 genes at a distance of approximately 22 kb of the APOC1 pseudogene (Fig. 3). This means that the APOE-C1-C2 gene cluster on chromosome 19 spans approximately 48 kb.

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[1] When a marker different from that in the previous study (Klasen et al. 1987) was used, the size of the H1 fragment was more precisely estimated to be 60 kb, instead of 50 kb

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Chapter 8

Exact localization of the familial dysbetalipoproteinemia associated HpaI restriction site in the promoter region of the APOC1 gene

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EXACT LOCALIZATION OF THE FAMILIAL DYSBETALIPOPROTEINEMIA ASSOCIATED HPAI RESTRICTION SITE IN THE PROMOTER REGION OF THE APOC1 GENE

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An HpaI restriction fragment length polymorphism (RFLP) in the APOE-C1-C2 gene cluster on chromosome 19 is strongly associated with familial dysbetalipoproteinemia (type III hyperlipoproteinemia). Recently we localized this polymorphic HpaI site between the APOE and APOC1 genes. In the present paper we show by molecular cloning and sequencing that the polymorphic HpaI site is located 317 bp upstream of the transcription initiation site of the APOC1 gene. Overlapping cosmid clones allowed the construction of a detailed restriction map of the gene cluster, showing the APOC2 gene to be located 15 kb downstream of the APOC1 pseudogene.

Apolipoproteins play an essential role in lipoprotein metabolism. Eight major apolipoproteins have been described: A1, A2, A4, B, C1, C2, C3 and E. For most of these the role in lipid metabolism is known. Six of the apolipoprotein genes are linked in two clusters which are chromosomally dispersed. The genes coding for the apolipoproteins A1, C3 and A4 are clustered on the long arm of chromosome 11, spanning no more than about 14 kb (1,2). The genes coding for the apolipoproteins E, C1 and C2 are clustered on the long arm of chromosome 19 (cen-q13.2) (3). The APOC1 gene and the APOC1 pseudogene are located downstream of the APOE gene at distances of about 5 kb and 17 kb respectively (4,5,6). Recently, we mapped the APOC2 gene downstream of the APOC1 pseudogene (7). The APOE-C1-C1'-C2 gene cluster spans a region of 40-50 kb (7).

Familial dysbetalipoproteinemia (FD; type III hyperlipoproteinemia) has a frequency of 1 in 2500 and is characterized by elevated levels

of plasma cholesterol and triglycerides, xanthomatosis and premature coronary and peripheral atherosclerosis (8).

There is a strong association between FD and homozygosity for the APOE*2 allele (9,10,11). This is due to a defect of apoE2 in binding to the hepatic lipoprotein receptors, resulting in an accumulation of the atherogenic chylomicron- and VLDL-remnants in the plasma (12,13). Over 90% of patients with FD have the phenotype E2E2, but as about 1% of the population has this phenotype, other, probably genetic, factors are necessary for the development of familial dysbetalipoproteinemia. Recently, we reported a restriction fragment length polymorphism (RFLP) using an APOE cDNA probe with the restriction enzyme HpaI (14), displaying fragments of 60 kb (H1) and 20 kb (H2). This H2 allele appeared to be strongly associated with familial dysbetalipoproteinemia (14). In a previous paper (7), based on RFLP studies, we concluded that the polymorphic HpaI site is located in close proximity to the 5' end of the APOC1 gene.

In this study we present a detailed restriction map of the APOE-C1-C1'-C2 gene cluster on the basis of overlapping cosmid clones and the exact localization of the polymorphic HpaI site by DNA sequencing of the relevant region.

MATERIALS AND METHODS

Genomic DNA isolation, digestions, Southern blotting and hybridization

Genomic DNA was isolated from leukocytes in whole blood by standard methods (15). DNA was digested with various restriction enzymes under conditions as recommended by the manufacturers (Pharmacia, Beckman). The fragments were separated by electrophoresis on 0.7% agarose gels at 1.5 V/cm for 16 hr. Gels were denatured for 2 x 15 min in 0.4 M NaOH/0.6 M NaCl and transferred in the same solution to GeneScreen Plus filters (New England Nuclear). Probes were labeled with [α^{32}P] dCTP using a multiprime kit (Amersham). Hybridization was performed as described by van Ommen et al. (16).

Cosmid library

A cosmid library was constructed from DNA of a patient with familial dysbetalipoproteinemia by the ligation of size-fractionated partial MboI-digested DNA into the vector c2RB (17). In vitro packaging and transfection into E. coli 1046 was performed essentially as described by van Ommen et al. (16). An efficiency of 3.3 x 10^6 clones per µg DNA was obtained. The primary library was grown at a density of 45000 colonies per 90 mm dishes. Duplicate filters were prepared according to the method of Hanahan and Meselson (18) and hybridized with [α^{32}P] dCTP labeled cDNA inserts of the APOC1, APOC2 and APOE genes under standard conditions (16).

APOE, C1 and C2 probes

The following probes were used: the APOE cDNA clone pE-368 (19), an APOC2 cDNA probe (20), an APOC1 cDNA probe (21) and the genomic probe PSCII (22). For hybridizing the cosmid library filters the cDNA inserts were purified by electrophoresis on low-melting-temperature agarose.

Restriction map

The restriction map was obtained using a combination of single and double digestions and hybridization with different probes together with a procedure using partial digestions to determine the relative order of the restriction sites. According to this latter procedure, which is described in more detail by Hofker *et al.* (23), the cosmids were first linearized by SalI, followed by partial digestion with different enzymes (HindIII, EcoRI and BamHI). The samples were run on 0.35% agarose gels and transferred to duplicate GeneScreen filters. Each filter was hybridized with a probe flanking the insert, *viz.* either the SalI-AvaI or the SalI-BamHI fragment of pBR322.

Subcloning and DNA sequencing

A 0.7 kb SacI fragment from a positive cosmid clone was purified by electrophoresis on low-melting-temperature agarose and cloned in M13 mp18/19 (24). Additional SacI-SmaI subclones were made (figure 1B). DNA sequencing was performed using the dideoxynucleotide chain-termination procedure of Sanger (25). When necessary, sequence ladders were extended by a 3 minute incubation of the reaction mixtures with dNTP's (1 μM) only, prior to addition of ddNTP's.

RESULTS

Isolation and characterization of cosmid clones

A cosmid library was made from DNA of an FD patient, homozygous for the polymorphic HpaI site (H2H2). From 10^6 clones, we isolated 5 overlapping cosmids, containing the APOC1 and APOC2 genes and another 8 cosmids containing only the APOC2 gene. Restriction maps were constructed of 5 overlapping cosmids (see materials and methods). The map (figure 1A) is in agreement with genomic hybridization data, excluding gross rearrangements during cloning.

We conclude that cosmids 2 and 4 contain the HpaI site and that the APOC2 gene is located 15 kb downstream of the APOC1 pseudogene.

Cloning and sequencing of the region around the polymorphic HpaI site

Double digestions of cosmid 4 DNA with various restriction enzymes in combination with HpaI revealed that a 0.7 kb SacI fragment contains an HpaI site. This fragment was subcloned in M13 mp18/19 in order to determine its nucleotide sequence (figure 1B).

The exact size of the SacI fragment turned out to be 719 bp, with the HpaI site located 60 bp from the 5' SacI site (figure 2).

In order to confirm that the cloned HpaI site is the polymorphic site we used the SacI fragment as a probe to detect the polymorphism. Southern blots with HpaI-HindIII double digestions revealed a clear polymorphism with hybridizing fragments of 4.8 kb (H1) and 2.6 kb (H2) (figure 3). We have excluded the possibility of a HindIII polymorphism (data not shown).

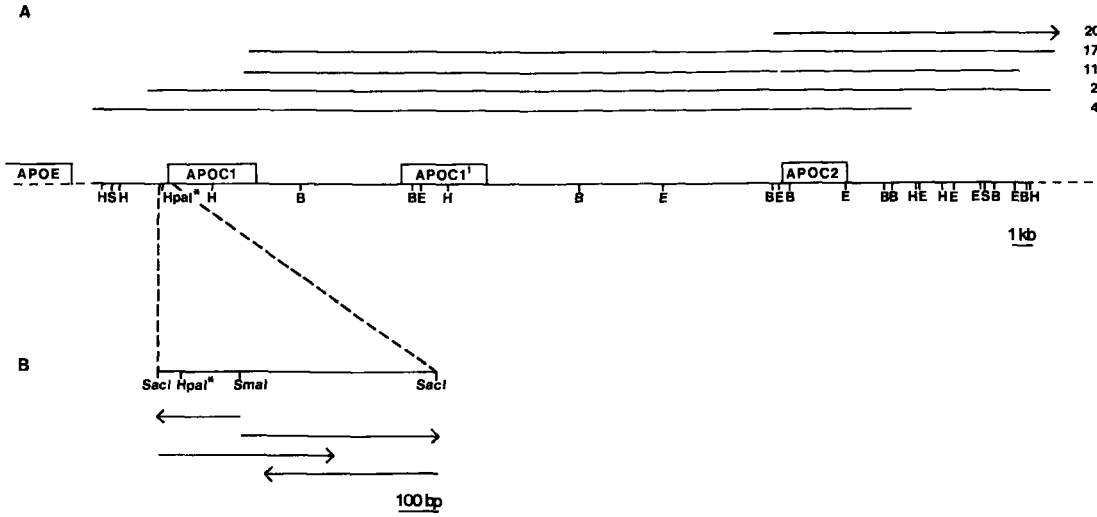

Figure 1A: Restriction map of the APOE-C1-C1'-C2 gene cluster for the enzymes EcoRI (E), BamHI (B), HindIII (H) and SalI (S). The position of the polymorphic HpaI site is indicated (HpaI*). The regions spanned by the individual cosmid clones (2,4,11,17 and 20) used to construct the map are indicated by lines.
Figure 1B: Sequence strategy for the SacI fragment, containing the polymorphic HpaI site. The arrows indicate direction and extent of sequencing. For details see Materials and Methods.

```
                                                                    -312
GAGCTCGGCTCTTGAGACAGGAATCTTGCCCATTCCCCGAACGAATAAACCCCTTCCTTCGTTAACTCA
 SacI                                                              HpaI
                                                                    -244
GCGTCTGAGGAATTTTGTCTGCGGCTCCTCCTGCTACATTCTGAGTGGGG-AAAGGGACTAAGGTGGTC
                                                        G
                                                                    -175
TGAGGACCCCACAGAGTCAGGAAGATTGAGAGGTGAGAGTGCTGAACGGGGAGGGGCTTTGGGGCTAAG

                                                                    -106
GGAAGTGCCCGGGACCCCACCTGACCCCAACGCTCACGGGACAGGGGCAGAGGAGAAAAACGTGGGTGG
        SmaI
                                                                    -37
ACAGAGGGAGGCAGGCGGTCAGGGGAAGGCTCAGGAGGAGGGAGATCAACATCAACCTGCCCCGCCCCC
                                                                    -
                                        1                           33
TCCCCAGCCTGATAAAGGTCCTGCGGGCAGGACAGGACCTCCCAACCAAGCCCTCCAGCAAGGATTCAG

                                                                    102
GTTGGTGCTGAGTGCCTGGGAGGGACACCCGCCTACACTCTGCAAGAAACTCAAAAAGGGAGATGAGGG

                                                                    171
GATCGTGGGAGGGAGGTAGGGAGGGAGGAGGGTGCCACTGATCCCCTGAACCCCTGCCTCTGCCTCCAG

                                                                    240
AGTGCCCCTCCGGCCTGGCCATGAGGCTCTTCCTGTCGCTCCCGGTCCTGGTGGTGGTTCTGTCGATCG
                        MetArgLeuPheLeuSerLeuProValLeuValValValLeuSerIleV
                                                                    308
TCTTGGAAGGTAAAAGTGGGATGGGAGAATTGCGGAGTT-GGAGATTTGGAAGAGTGAAGGTGGCTACA
alLeuGlu                                G       T

GGCCTGGGGTCCCGGCTTAGAGGACCTCTGAGAGCTC
                                    SacI
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Figure 2: Nucleotide sequence of the 719 bp SacI fragment (figure 1B) of cosmid 4, containing the first two exons (bold face) of the APOC1 gene. Differences with the APOC1 sequence according to Lauer et al. (6) at positions -262, -37, 273 and 279 are given.

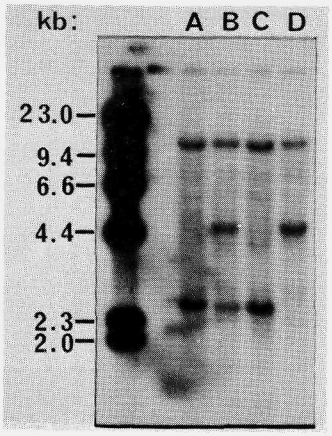

Figure 3: Demonstration of the HpaI polymorphism using the SacI fragment (figure 1B) as a probe. Genomic DNA was digested with HpaI and HindIII. The invariant band is a 12.2 kb HindIII-HindIII fragment containing the 3' part of the APOC1 gene and the 5' part of the APOC1' gene. Lanes A and C: H2H2, lane B: H1H2 and lane D: H1H1.

DISCUSSION

Previous studies have shown a strong association between familial dysbetalipoproteinemia and homozygosity for the E2 variant at the APOE locus. However, it has been suggested that less than 10% of the E2E2 homozygotes develop familial dysbetalipoproteinemia and premature atherosclerosis. Recently, we showed a significantly increased frequency of the H2 allele of the HpaI RFLP among FD patients with the E2E2 phenotype (14).

In the present study we cloned a SacI fragment (719 bp) containing the polymorphic HpaI site and determined its DNA sequence.
Comparison of the genomic sequence data with those of the APOC1 cDNA (21) showed that the SacI fragment carries an exon encoding the first 19 amino acids of the signal peptide of apoC1. Ten nucleotides upstream of the ATG translation initiation codon our genomic sequence deviates from the cDNA sequence published by Knott et al. (21), suggesting the presence of an intron at this position, although an intron-exon junction consensus sequence is absent.

Comparison with a genomic sequence of Lauer et al. (6) shows only minor (4/719) differences (figure 2). Their cDNA sequence data were in agreement with the genomic sequence and showed a presently unexplained discrepancy with the sequence published by Knott et al. (21).
We found that the HpaI site is located 508 nucleotides upstream of the ATG startcodon. Based on mapping data of Lauer et al. (6) we conclude

that the polymorphic HpaI site is located in the promoter region of the APOC1 gene at position -317 relative to the transcription initiation site.

At present we can only speculate on the relevance of the association of the HpaI RFLP with familial dysbetalipoproteinemia. Although being localized in the promoter region of the APOC1 gene, we have no evidence that this mutation influences the expression of the APOC1 gene. We found no differences in the plasma level of apoC1 between the different HpaI genotypes.

An alternative explanation is that the HpaI RFLP is in linkage disequilibrium with another gene defect, influencing the lipoprotein metabolism. In this respect it should be noted that the APOE, APOC1 and APOC2 genes are closely linked in one cluster.

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Summary and Discussion

Familial dysbetalipoproteinemia (FD), or type III hyperlipoproteinemia according to the Fredrickson nomenclature, is a disorder of lipoprotein metabolism with a strong genetic component. FD patients have elevated plasma cholesterol and triglyceride levels due to an accumulation in the plasma of chylomicron- and very low density lipoprotein (VLDL) remnants. The high concentration of remnants in the plasma often results in xanthomatosis and premature coronary and peripheral atherosclerosis.
The genetic polymorphism of apolipoprotein E (apoE) has been shown to play an important role in the predisposition to FD. ApoE is the ligand for the binding of the chylomicron- and VLDL remnants to the hepatic lipoprotein receptors. Using isoelectric focusing, three common apoE isoforms can be distinguished: E2, E3 and E4. These isoforms are encoded by different alleles at a single APOE locus on chromosome 19, giving rise to six different phenotypes: E2E2, E3E3, E4E4, E3E2, E4E2 and E4E3. ApoE3 is the most commonly occurring isoform. ApoE4 is supposed to be derived from E3 by a cys→arg substitution at position 112 and is designated E4(cys112→arg). At present four different E2 mutations have been described: E2(arg158→cys), E2(lys146→gln), E2(arg145→cys) and E2(arg136→ser). The most common E2 variant, E2(arg158→cys), exhibits a cysteine residue at position 158 instead of an arginine residue present in the common E3 variant.
Most FD patients (>90%) are homozygous for the E2E2 phenotype. FD among E2 heterozygotes is uncommon. Furthermore, FD patients with rare apoE mutations have been described.
The underlying metabolic defect of FD in the E2E2 homozygous individuals is a defective binding of apoE2 to the hepatic lipoprotein receptors. However, in the Caucasian population, the frequency of the E2E2 phenotype is about 1 per 100 whereas the frequency of FD is supposed to be 1-4 per 10000. This implies that only a small percentage of all E2E2 homozygous individuals (1-4%) develops FD.
The reduced penetrance of FD in E2E2 homozygotes and the occasional expression of FD in individuals heterozygous for apoE2 or rare apoE variants, prompted us to study the genetic background of FD in more detail.
Elucidation of the genetic background of FD will be important in order to identify individuals at risk for FD, since a simple diet or medication can prevent the development of hyperlipidemia.

The present study was based on a population sample of FD patients from the Lipid Clinics in Leiden and Nijmegen. A general control population and a group of 13 clinically normal E2E2 homozygotes were obtained by screening 2018 35 years old apparently healthy Dutch males. Such a large population sample was necessary because of the low frequency of the E2E2 phenotype (about 1%) in the general population.
The apoE phenotype was determined using isoelectric focusing of delipidated plasma

followed by immunoblotting (chapter 2). The APOE*2, E*3 and E*4 allele frequencies (0.082, 0.750 and 0.167 respectively) were in agreement with frequencies published for population samples of West Germany and Scotland. Marked differences were found between the APOE allele frequencies in the Dutch population and the populations in Japan, New Zealand, Finland and the United States.
In addition to the apoE phenotype, the plasma cholesterol, triglycerides, apoE and apoB levels were determined in the general population sample. The results showed that the E*2 allele is associated with decreased plasma cholesterol and apoB levels and with elevated apoE levels. The opposite is true for the E*4 allele. These results are in agreement with previously published results. In the Dutch population, the sum of average allelic effects of the three common APOE alleles on plasma cholesterol and apoB levels was 6.8% and 14.2%, respectively, while the total average allelic effect on plasma apoE concentrations was 50.1%. These data suggest that the APOE alleles primarily affect the apoE concentrations rather than plasma cholesterol and apoB levels. This hypothesis is substantiated by the observation that for plasma apoE levels, the genetic variance associated with the APOE gene locus contributed about 18% to the total phenotypic variance. For plasma cholesterol and apoB this contribution was only 1.4% and 2.3% and was relatively low as compared with that reported for other population samples.
The population study revealed two new apoE variants. One of them exhibits an isoelectric point between that of E3 and E4 and lacks cysteine residues (chapter 3). Since apoE4 is also devoid of cysteine residues, it is suggested that this new variant arose by a mutation in an E*4 allele. The new variant was designated $E4^*$. The inheritance of the new variant was studied in the family of the propositus. $E4^*$ appeared to co-segregate with a new, rare fragment of a TaqI restriction fragment length polymorphism, detectable with a probe derived from the APOC2 gene, which is clustered with the APOE gene on chromosome 19. The new $E4^*$ variant is not associated with disturbances in the lipoprotein metabolism.

In our total group of FD patients (n=45), three individuals exhibited the E3E2 phenotype, whereas all other patients appeared to be E2E2 homozygotes. Using a cysteine modification technique followed by isoelectric focusing we were able to show that all three unrelated patients with the E3E2 phenotype exhibited an uncommon E2 variant, containing only one cysteine residue (chapter 4). As the common E2(arg158→cys) variant and the rare E2(arg145→cys) variant both contain 2 cysteine residues, the presence of these two variants could be excluded. Rall *et al.* (1983) described the presence of another rare E2 variant, E2(lys146→gln) in two FD patients with the E3E2 phenotype. This E2 variant contains only one cysteine residue. Using *in vitro* amplification of genomic DNA followed by hybridization with synthetic oligonucleotide probes complementary to the different E2 mutations and to the wild type E3 sequence, we were able to demonstrate the presence of the E2(lys146→gln) mutation in the three patients with the E3E2 phenotype (chapter 5). Family studies of the three unrelated E3E2 patients showed that this E2(lys146→gln) variant co-segregates

with FD in the three families in a dominant manner. This E2 variant was not found among 13 E2E2 and 120 E3E2 individuals selected from the random population sample, which implies that the E2(lys146→gln) variant is very rare with a frequency of less than 0.001 and which has so far been found exclusively in FD patients. The results strongly suggest that the E2(lys146→gln) mutation is dominant in the predisposition of an individual to FD.
In order to investigate whether microheterogeneity in the APOE gene is involved in the reduced penetrance of FD in E2E2 homozygous individuals, we determined the APOE genotype in 40 FD patients with the E2E2 phenotype and in 13 clinically normal individuals with the E2E2 phenotype using *in vitro* DNA amplification and hybridization with synthetic mutation-specific oligonucleotides. All E2E2 FD patients and 11 out of the 13 control E2E2 individuals appeared to be homozygous for the common E2(arg158→cys) mutation. The remaining 2 (normolipidemic) individuals were heterozygous for the E2(arg158→cys) mutation and another so far unknown E2 mutation. In these individuals, the presence of known mutations at the positions 145 (arg→cys), 146 (lys→gln) and 142 (arg→cys) could be excluded.
This study was not able to discriminate between patients and apparently healthy E2E2 controls, since both groups are homozygous for the common (arg158→cys) mutation. It should be noted however, that the resolving power of the applied technique is limited to the specific mutations and does not exclude the possibility of additional heterogeneity in the APOE gene being responsible for the reduced penetrance of FD in E2E2 individuals. For a definite answer to this question it will be necessary to compare the complete APOE gene sequences of E2E2 patients and controls.

Instead of additional microheterogeneity in the APOE gene itself, another mechanism has been postulated to explain the reduced penetrance of FD in E2E2 homozygotes namely co-segreation of a second genetic factor, predisposing to other forms of hyperlipidemia, such as familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCH) or familial hypertriglyceridemia (FHT). This second gene (or genes), might be different from patient to patient. Genes involved in the metabolism of triglyceride-rich chylomicrons and VLDL, like the APOB gene, the APOC1 gene or the APOC2 gene are candidate genes.
To test this hypothesis, we studied some known restriction fragment length polymorphisms (RFLPs) of the APOE-C1-C2 gene cluster on chromosome 19 and of the APOB gene on chromosome 2 (chapter 6). A previously published paper by Klasen *et al.*, showed a significantly increased frequency of the H2 allele of the HpaI/APOE RFLP among FD patients with the E2E2 phenotype, suggesting a strong linkage disequilibrium between this HpaI polymorphism and a gene predisposing to FD. We studied this HpaI RFLP in more detail in FD patients, in 13 clinically normal E2E2 individuals and in a random control population. It appeared that the HpaI RFLP was in strong linkage disequilibrium with the apoE polymorphism. All FD patients with the E2E2 phenotype and 11 out of 13 control E2E2 individuals were homozygous for the H2 allele, having a frequency of 0.26 in the random population. In the random control

population the E*3 allele co-segregates with the H1 allele whereas the E*4 and E*2 alleles co-segregate with the H2 allele, with few exceptions. The association between the E*2 allele and the HpaI RFLP is dependent on the type of E2 mutation. The common E2(arg158→cys) allele co-segregates with the H2 allele, whereas the rare E2(lys146→gln) allele (chapter 5) is associated with the H1 allele. Two E2E2 control individuals heterozygous for the E2(arg158→cys) mutation and another, as yet unknown E2 mutation, are heterozygous for the HpaI polymorphism (H1H2), suggesting that this unknown E2 mutation also co-segregates with the H1 allele. The combination of isoelectric focusing with the HpaI polymorphism seems to be useful in predicting whether the common E2(arg158→cys) mutation or another E2 variant is present. Unfortunately, the HpaI RFLP gives no additional information on the reduced penetrance of FD in E2E2 individuals.
The TaqI/APOC2 polymorphism showed a significant difference in allele frequencies between the random population sample and the group of FD patients ($p<0.005$). This association might be due to a linkage disequilibrium of the T1 allele with the APOE*2 allele (chapter 6).
The results obtained by testing the two APOB RFLPs (XbaI/APOB and EcoRI/APOB) are also presented in chapter 6. We found a significant difference in allele frequencies of the XbaI/APOB RFLP between the random population sample and the group of FD patients ($p<0.01$). There was no significant difference in allele frequency between both groups for the EcoRI/APOB polymorphism. We have been able to demonstrate a linkage disequilibrium between the two APOB RFLPs.
More information about the possible involvement of the APOB gene in the development of FD can be obtained by performing family studies. In a preliminary study, we showed that in one family, an APOB haplotype cosegregated with the E2E2 phenotype and FD. Another family tested was not informative, since both parents were heterozygous for all APOB RFLPs tested (XbaI, EcoRI, PvuII, EcoRV). The collection of more families in which FD occurs in more than one generation is necessary to test the possible involvement of the APOB gene in the expression of FD.

In the course of this study, we were able to construct a detailed physical map of the apolipoprotein gene cluster on chromosome 19. Previously, the location of the APOE gene was published relative to the position of the APOC1 gene and APOC1 pseudogene. However, the location of the APOC2 gene, which was known to be linked to the APOE gene, was not exactly determined. As the HpaI polymorphism could be detected with an APOE probe as well as with APOC1 and APOC2 probes, we could roughly localize the APOC2 gene in the gene cluster, downstream of the APOC1 genes (chapter 7). Cosmid cloning of the gene cluster made it possible to construct a detailed restriction map of the APOE-C1-C2 gene cluster (chapter 8). The complete gene cluster spans a region of about 45 kb. A number of cosmid clones contained the polymorphic HpaI site, which allowed the determination of its exact location by sequencing a small genomic fragment. The polymorphic HpaI site is located in the promoter region of the APOC1 gene. We have no evidence that this mutation influences the expression of the

APOC1 gene, since no differences in the plasma level of apoC1 between the different HpaI genotypes could be detected.

Perspectives

The present study has clearly emphasized the phenomenon of genetic heterogeneity among FD patients. In the case of FD patients homozygous for the E2E2 phenotype, the inheritance pattern seems recessive, with a reduced penetrance, whereas in the case of the presence of rare apoE variants, like E2(lys146→gln), E3-Leiden and E1-Harrisburg, FD is inherited in a dominant manner as revealed by family studies.
Clearly, the existence of these apoE variants which predispose to FD in a dominant manner, should caution clinicians against the use of the E2E2 phenotype as an obligatory diagnostic criterium for FD.
Apart from the different mode of inheritance, there is an interesting discrepancy between the severity of the receptor binding defect of the apoE variants and their effect on the expression of FD. The *in vitro* binding activity of the E2(lys146→gln) and E3-Leiden variants is 30% and 40% respectively of the value for the wild type E3 isoform. It is striking that all of the individuals heterozygous for these rare apoE mutants develop FD. In contrast, individuals with the E2E2 phenotype, most likely homozygous for the common E2(arg158→cys) mutation with a binding activity of only 2% or less, rarely (1-4%) develop FD. This discrepancy suggests that apoE might have another, as yet unknown, important function besides its function as a ligand for the hepatic receptors.
As one of the explanations for the reduced penetrance of FD in E2E2 homozygotes one might consider the possible occurrence of different E2 variants among patients and clinically normal individuals. Although our results do not sustain this possibility, since patients as well as healthy controls with the E2E2 phenotype exhibit homozygosity for the E2(arg158→cys) mutation, an additional mutation in the APOE2 gene cannot be excluded. Comparison of complete APOE coding sequences from healthy and affected E2E2 individuals will give conclusive evidence.

Alternatively, the reduced penetrance of FD in E2E2 homozygotes could be due to an increased hepatic production of VLDL. In this respect, Utermann *et al.* proposed that besides E2E2 homozygosity an extra genetic factor leading to hyperlipidemia is necessary for the development of FD.
Although a number of genes could contribute to the development of hyperlipidemia in FD, the APOB gene is generally assumed to be one of the best candidate genes. ApoB is a major protein component of chylomicrons, VLDL and LDL. Furthermore, it is required for the assembly and secretion of chylomicrons and VLDL. RFLP studies have suggested the possible involvement of the APOB gene in the predisposition to FD. Significant differences have been observed comparing the allele frequencies of the XbaI/APOB RFLP between FD patients and a random control population sample (this

thesis). In addition it should be noted that several studies have shown a strong relationship between the XbaI/APOB polymorphism and plasma cholesterol and triglyceride levels. To obtain support for the hypothesis that the APOB gene is involved in the development of FD in E2E2 homozygotes, extensive family studies should be performed. However, it is very difficult to collect large, informative families with FD in more than one generation for a number of reasons: FD usually develops after the third decade in males and after the menopause in females. The expression of FD is strongly influenced by endogenous and exogenous factors, like diet and hormonal disturbances such as diabetes mellitus or hypothyroidism, making it difficult to unequivocally diagnose FD. The availability of a physiological diagnostic test (*e.g.* a carbohydrate and/or fat loading test) that pronounces the expression of FD would be helpful for an adequate diagnosis.
Furthermore, as conventional RFLPs have rather limited information content, highly polymorphic Variable Number of Tandem Repeats (VNTR) probes should be applied. Such VNTR probes will also facilitate family studies with other candidate genes, involved in lipid metabolism.

The aim of this study was to elucidate the genetic background of FD in order to develop preclinical diagnostic tests for FD. Such a presymptomatic diagnosis will be extremely beneficial to these individuals as the development of hyperlipidemia concomitant with an accelerated atherogenesis could easily be prevented by a simple diet and/or medication.
At this moment, even though we do not understand the molecular mechanism underlying the development of FD, the dominant mode of inheritance of FD in subjects bearing rare apoE variants (this thesis) provides the opportunity to perform early diagnosis in the probands families with respect to the development of FD after the second decade of life. Further genetic and metabolic studies will hopefully lead to presymptomatic tests in the near future to indicate all individuals at risk for FD.

Samenvatting

Familiaire dysbetalipoproteïnemie (FD, ook wel type III hyperlipoproteïnemie genoemd) is een erfelijke aandoening, welke bij ongeveer 1-4 op de 10000 individuen voorkomt. Bij FD is er sprake van een storing in het lipiden-metabolisme, hetgeen op jonge leeftijd tot hart- en vaatziekten kan leiden. Het in dit proefschrift beschreven onderzoek was er op gericht inzicht te verkrijgen in de erfelijke aspekten van de ziekte. Het uiteindelijke doel van dit onderzoek is een vroege opsporing mogelijk te maken van individuen met een verhoogd risiko voor het ontwikkelen van FD. Voor een goed begrip is het echter van belang eerst enige aandacht te besteden aan het lipiden-metabolisme.

Cholesterolesters en triglyceriden zijn hydrofobe lipiden, die in grote hoeveelheden in het bloedplasma voorkomen. Deze lipiden worden voor een deel in het lichaam zelf gesynthetiseerd (endogeen lipide) terwijl ook via de voeding elke dag een behoorlijke hoeveelheid van deze lipiden wordt opgenomen (exogeen lipide). De triglyceriden worden getransporteerd naar vet- en spierweefsel, waar ze - na hydrolyse - respektievelijk gebruikt worden voor opslag en energie. De cholesterolesters worden door de cel eerst gehydrolyseerd, waarna het onveresterde cholesterol gebruikt wordt als bouwsteen voor de plasmamembranen of als precursor voor de synthese van steroïdhormonen en galzuren.
Omdat hydrofobe lipiden onoplosbaar zijn in waterig milieu zoals bloedplasma, worden de lipiden vervoerd in de vorm van lipide-eiwit complexen, lipoproteïnen genoemd. Zo'n lipoproteïne bestaat uit een kern van neutrale, apolaire lipiden (cholesterolesters, triglyceriden), omgeven door een schil van amfipatische lipiden en eiwitten. Deze eiwitten worden apolipoproteïnen (apo's) genoemd en zijn o.a. van belang voor het transport van lipoproteïnen (apoB en apoE) of ze dienen als co-faktor voor enzymen, betrokken bij het lipiden-metabolisme (bijvoorbeeld apoC2).
Er kunnen een viertal lipoproteïnen onderscheiden worden: chylomicronen, very low density lipoproteïnen (VLDL), low density lipoproteïnen (LDL) en high density lipoproteïnen (HDL). Deze lipoproteïnen verschillen onderling in dichtheid, samenstelling en grootte.
Bij het ontstaan van FD speelt het apolipoproteïne E (apoE) een belangrijke rol, evenals de chylomicronen en de VLDL lipoproteïnen. Hieronder zal derhalve alleen het metabolisme van deze twee lipoproteïnen besproken worden.

De via de voeding opgenomen triglyceriden en cholesterolesters worden samen met een aantal apolipoproteïnen in de darm verpakt tot chylomicronen. De chylomicronen worden via de lymphe naar het bloed getransporteerd. Een gedeelte van het triglyceride wordt vervolgens gehydrolyseerd door het enzym lipoproteïne lipase (LPL) resulterend in de vorming van de zogenaamde chylomicron remnants. Deze remnants bevatten

relatief veel cholesterol en apoE. Door binding aan de op de levercel aanwezige apoE receptoren, die specifiek het apoE herkennen, worden de chylomicron remnants vervolgens snel door de lever opgenomen.
Koolhydraten en vrije vetzuren worden door de lever omgezet in triglyceriden, welke samen met al in de lever aanwezig cholesterol verpakt worden in VLDL deeltjes. Nadat de VLDL deeltjes in de circulatie zijn uitgescheiden, worden ze, evenals de chylomicronen, gedeeltelijk gehydrolyseerd door het enzym lipoproteïne lipase. De ontstane VLDL remnants (ook wel intermediate density lipoproteïnen, IDL, genoemd) bevatten ook weer apoE als belangrijk apolipoproteïne. Een klein deel van de VLDL remnants wordt vervolgens omgezet in LDL, terwijl het grootste gedeelte direkt door de lever wordt opgenomen via binding aan de LDL receptor. Deze LDL receptor verschilt van de eerder genoemde apoE receptor, doch beide herkennen apoE. Voor hun onderzoek aan de LDL receptor hebben Joseph Goldstein en Michael Brown in 1985 de Nobelprijs voor Geneeskunde ontvangen.
Voor zowel de binding van de chylomicron remnants aan de apoE receptor als voor de binding van de VLDL remnants aan de LDL receptor is het apoE van groot belang. Het apoE eiwit bestaat uit 299 aminozuren. Van dit apolipoproteïne zijn een drietal vormen bekend (E2, E3 en E4), die gecodeerd worden door een aantal codominante allelen op het APOE locus op chromosoom 19. Een zestal fenotypen kan onderscheiden worden: E2E2, E3E3, E4E4, E3E2, E4E2 en E4E3. De APOE allelen worden aangeduid met *: E*2, E*3 en E*4. De apoE eiwitvarianten verschillen onderling in isoelectrisch punt, zodat ze met behulp van isoelectrisch focusseren van elkaar te onderscheiden zijn. De apoE2 en E4 varianten verschillen ieder in één aminozuur ten opzichte van de meest voorkomende variant E3 als gevolg van een puntmutatie in het APOE gen.
De apoE varianten verschillen wat betreft de binding van de chylomicron remnants en VLDL remnants aan respectievelijk de apoE- en LDL receptoren van de lever. De apoE3 en apoE4 varianten binden goed aan deze receptoren, waardoor de remnants snel uit de circulatie verwijderd worden. De meest voorkomende apoE2 variant bindt echter slecht (2% van normaal) aan de receptoren. Dit kan aanleiding geven tot ophoping (accumulatie) van de remnants. Omdat deze remnants voornamelijk bestaan uit cholesterol, leidt de ophoping van remnants tot een hoger cholesterol gehalte en tot een groter risiko op het krijgen van atherosclerose.
Bij de meeste E2E2 homozygote individuen is het mechanisme van remnant opname nog voldoende om een grote ophoping van remnants in het bloedplasma te voorkomen. Alleen vlak na een maaltijd, wanneer er een (tijdelijk versterkte) aanvoer is van chylomicronen, zullen er chylomicron remnants in het plasma aangetroffen kunnen worden.
Wanneer er echter voortdurend sprake is van een versnelde aanvoer van chylomicronen en VLDL, zal de bindingsstoring bij E2E2 personen aanleiding geven tot een permanente ophoping van de zeer atherogene remnants. Dit nu is het geval bij de ziekte familiaire dysbetalipoproteïnemie (FD).
FD patiënten hebben, als gevolg van de ophoping van remnants, een verhoogd

triglyceride en cholesterol gehalte. Hierdoor is er een groot risiko op vroegtijdige atherosclerose. Karakteristiek is verder de aanwezigheid van xanthomen (vetophopingen) op de ellebogen en in de handpalmnerven, welke geel worden.
De ziekte openbaart zich meestal na het dertigste levensjaar bij mannen en na de menopauze bij vrouwen. De ziekte is met een dieet en/of medicijnen goed te behandelen.
Aangezien de meeste FD patiënten (>90%) homozygoot zijn voor het E2E2 fenotype, maar slechts 1-4% van de E2E2 homozygoten FD onwikkelt, spreekt men van een recessieve overerving met een gereduceerde penetrantie. De oorzaak van deze gereduceerde penetrantie is niet bekend. Naast omgevingsfaktoren zoals voeding, hormonen en alcoholgebruik zijn er mogelijk (meerdere) genetische factoren noodzakelijk om FD tot expressie te laten komen. Een tweetal verklaringen voor de gereduceerde penetrantie van FD in E2E2 individuen zijn mogelijk:

1. Microheterogeniteit in het APOE gen. Op dit moment zijn er vier verschillende E2 mutaties beschreven: E2(arg158→cys), E2(lys146→gln), E2(arg145→cys) en E2(arg136→ser). Het zou kunnen dat de E2E2 patiënten andere E2 varianten bezitten dan de gezonde E2E2 invididuen.
2. De betrokkenheid van een ander gen, dat een hyperlipidemie veroorzaakt. De kombinatie van zo'n gen met het apo E2E2 fenotype zou kunnen leiden tot het ontwikkelen van FD.

Het in dit proefschrift beschreven onderzoek richtte zich op de genetische achtergrond van familiaire dysbetalipoproteïnemie (FD). In een samenwerkingsverband tussen het Instituut voor Anthropogenetica in Leiden en het Gaubius Instituut TNO, werd met name gekeken naar de rol van het apolipoproteïne E. Het belang van de rol van apoE wordt nog benadrukt door het feit dat er ook FD patiënten beschreven zijn met het E3E2 fenotype en met zeldzame apoE varianten. In deze patiënten lijkt de overerving van FD dominant.
Opheldering van de genetische achtergronden van FD is van belang omdat daarmee personen, die een hoog risiko hebben op het krijgen van FD in een vroeg stadium opgespoord kunnen worden. Met een dieet en/of medicijnen kan ophoping van remnants in het plasma, en daarmee atherosclerose op jonge leeftijd, voorkomen worden.

Resultaten:
- Met behulp van isoelectrisch focusseren van apoE werd een bevolkingsonderzoek uitgevoerd bij 2000 35-jarige Nederlandse mannen. De gevonden allelfrequenties voor de meest voorkomende varianten E2, E3 en E4 kwamen goed overeen met eerder beschreven frequenties in Westerse populaties (hoofdstuk 2). Gebleken is, dat het apoE polymorfisme sterk gecorreleerd is met de plasma spiegels van cholesterol, apoB en apoE. Aanwezigheid van het E*2 allel gaat samen met verlaagde concentraties cholesterol en apoB, terwijl het apoE gehalte verhoogd is. Het omgekeerde werd

waargenomen voor het E*4 allel.
- Tevens werd bij deze screening een nieuwe E4 variant gevonden en gekarakteriseerd (hoofdstuk 3). Deze nieuwe variant (E4* genoemd) lijkt niet geassocieerd te zijn met storingen in het lipoproteïne metabolisme.
- 43 FD patiënten werden eveneens onderzocht m.b.v. isoelectrisch focusseren. Zoals verwacht, bleek meer dan 90% van de FD patiënten E2E2 homozygoot te zijn. Drie, genetisch onafhankelijke, FD patiënten bleken het E3E2 fenotype te bezitten. Eiwitchemisch onderzoek toonde aan dat het om een E2 variant ging met slechts 1 cysteine residue (hoofdstuk 4). Deze variant erft Mendeliaans over en alle familieleden van deze 3 patiënten met deze variant hebben FD. Met andere woorden: in deze families erft FD dominant over. Met behulp van moderne DNA technieken (*in vitro* amplificatie van genomisch materiaal en hybridisatie met synthetische oligonucleotiden) kon aangetoond worden dat in de patiënten met het E3E2 fenotype de E2(lys146→gln) variant aanwezig is (hoofdstuk 5). Deze variant komt niet voor in gezonde E3E2 individuen.
Met dezelfde technieken werden de APOE genen van patienten en controle individuen met het E2E2 fenotype onderzocht om vast te stellen of microheterogeniteit in het APOE gen ten grondslag ligt aan de gereduceerde penetrantie van FD in E2E2 homozygoten. Het bleek dat alle patiënten met het E2E2 fenotype de meest voorkomende E2 variant E2(arg158→cys) bezitten. Ook vrijwel alle gezonde E2E2 individuen die onderzocht zijn, blijken homozygoot te zijn voor deze E2 variant. Toch is het niet uitgesloten, dat een nog onbekende (neutrale) mutatie in het APOE gen verantwoordelijk is voor de expressie van FD in slechts 1-4% van de E2E2 homozytoten. Om hierover meer zekerheid te krijgen zouden de volledige APOE genen van gezonde en zieke personen vergeleken moeten worden.
- Een andere mogelijke verklaring voor het feit dat slechts een klein percentage van alle E2E2 individuen FD ontwikkelt, is co-segregatie van een ander gen. Genen, coderend voor eiwitten die een funktie hebben in het lipiden-metabolisme, met name in het metabolisme van chylomicronen en VLDL, worden als mogelijke kandidaat-genen gezien. Wij hebben gekeken naar een eventuele betrokkenheid van een drietal apolipoproteïne genen: APOB, APOC1 en APOC2 m.b.v. restrictie fragment lengte polymorfisme (RFLP) onderzoek (hoofdstuk 6). ApoB, gelegen op chromosoom 2, is een belangrijk apolipoproteïne in het metabolisme van de chylomicronen en VLDL. ApoC2 is een co-faktor van het enzym lipoprotein lipase en apoC1 aktiveert mogelijk het enzym lecithine cholesterol acyl transferase (LCAT). De genen, die coderen voor apoC1 en apoC2 bevinden zich, samen met het APOE gen, in een cluster op chromosoom 19.
Als eerste werd het HpaI/APOC1 polymorfisme bekeken (met de allelen H1 en H2), omdat een eerder onderzoek een sterk verhoogde frequentie van het H2 allel bij FD patiënten aantoonde. Dit HpaI polymorfisme blijkt geassocieerd te zijn met het apoE polymorfisme: de E3 variant gaat samen met het H1 allel van het HpaI polymorfisme, terwijl over het algemeen de E4 variant en de meest voorkomende E2 variant E2(arg158→cys) samen gaan met het H2 allel. Opvallend was dat de zeldzame E2(lys146→gln) variant, welke aanwezig is in FD patienten met het E3E2 fenotype

samengaat met het H1 allel. De eerder gevonden verhoogde frequentie van het H2 allel bij FD patienten lijkt dus eerder teruggevoerd te kunnen worden op de koppeling met de apoE2 variant, dan op een koppeling met een tweede genetische faktor, betrokken bij de ontwikkeling van FD.
De andere RFLP studies toonden een significant verschil in allel frequenties aan van zowel het TaqI/APOC2 polymorfisme als het XbaI/APOB polymorfisme tussen een kontrole groep en de groep FD patiënten. In het geval van het TaqI/APOC2 polymorfisme is de associatie met FD, zoals bij het HpaI polymorfisme, vermoedelijk terug te voeren op een koppeling met apoE2. De resultaten wat betreft het XbaI/APOB polymorfisme daarentegen, duiden op een mogelijke rol van het APOB gen als tweede genetische faktor bij de ontwikkeling van FD. In de toekomst moet hierover uit familie studies meer informatie verkregen kunnen worden.
- Omdat het APOE gen op chromosoom 19 zich vlakbij twee andere APO genen bevindt (APOC1 en APOC2), was het van belang de positie van de verschillende genen ten opzichte van elkaar uit te zoeken. Met behulp van het HpaI polymorfisme (met grote fragmenten van 60 en 20 kb) en door ons geïsoleerde cosmide kloons was het mogelijk een gedetailleerde kaart van de 45 kb grote APOE-APOC1-APOC2 gencluster op chromosoom 19 te maken (hoofdstuk 7 en 8). Tevens hebben we (omdat het HpaI polymorfisme erg interessant leek voor de opheldering van de gereduceerde penetrantie van FD) de positie van de polymorfe HpaI knipplaats vastgesteld m.b.v. nucleotide sequentie analyse. De knipplaats is gelegen in het 5' flankerende gebied van het APOC1 gen. We hebben geen aanwijzingen dat deze mutatie invloed heeft op de expressie van het APOC1 gen.

Onze studies hebben aangetoond dat de genetische achtergrond van FD heterogeen is: in het geval van FD patiënten homozygoot voor het E2E2 fenotype lijkt de overerving recessief, terwijl de aanwezigheid van zeldzame varianten, zoals E2(lys146→gln), tot een dominante overerving van de ziekte leidt, zoals uit familiestudies gebleken is. Bij deze E2(lys146→gln) variant is het dus aannemelijk dat de oorzaak van FD enkel en alleen in het apoE eiwit gelegen is.
Het doel van het onderzoek was de genetische achtergronden van FD op te helderen, om vervolgens een presymptomatische diagnose van FD mogelijk te kunnen maken.
Inmiddels is het al mogelijk een vroege diagnose te stellen in families waarin een zeldzame variant, zoals E2(lys146→gln), verantwoordelijk is voor de dominante overerving van FD. Verder onderzoek zal nodig zijn alvorens het mogelijk wordt om vroegtijdig vast te kunnen stellen welke personen met het E2E2 fenotype een verhoogd risiko hebben op het ontwikkelen van FD.

Abbreviations

A	adenosine
a.a.	amino acid(s)
apo	apolipoprotein (protein)
APO	apolipoprotein (gene)
ATP	adenosine 5'-triphosphate
bp	basepair(s)
BSA	bovine serum albumin
C	cytidine
CAD	coronary artery disease
cDNA	copy DNA
CETP	cholesteryl ester transfer protein
Ci	Curie
cpm	counts per minute
dCTP	deoxy cytidine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxy nucleoside 5'-triphosphates
DTT	dithiothreitol
E2, E3, E4	apolipoprotein E isoforms
E*2, E*3, E*4	apolipoprotein E alleles
E2E2,E3E2, etc	apolipoprotein E phenotypes
EDTA	ethylene diamine tetraacetate
FCH	familial combined hyperlipidemia
FD	familial dysbetalipoproteinemia
FH	familial hypercholesterolemia
FHT	familial hypertriglyceridemia
G	guanosine
h	hours
HDL	high density lipoproteins
HL	hepatic lipase
HLP	hyperlipoproteinemia
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
IDL	intermediate density lipoproteins
IEF	isoelectric focusing
kb	kilo base (pairs)
kD	kilo Dalton
LCAT	lecitin cholesterol acyltransferase
LDL	low density lipoproteins
LPL	lipoprotein lipase
MI	myocardial infarction
min	minutes

mRNA	messenger RNA
n	number
nm	nanometer
PCR	polymerase chain reaction
PIC	polymorphism information content
RFLP	restriction fragment length polymorphism
SDS	sodium dodecylsulphate
ss	single stranded
SSC	standard saline citrate (150mM NaCl, 15 mM Na-citrate, pH7.0)
SSPE	(10mM sodiumphosphate, pH7.0, 0.18 M NaCl, 1 mM EDTA)
T	thymidine
Taq polymerase	DNA polymerase from *Thermus aquaticus*
Tm	melting temperature
Tris	tris-(hydroxymethyl)aminomethane
VLDL	very low density lipoproteins
w/v	weight per volume

Curriculum vitae

Op 24 december 1960 ben ik geboren te Amsterdam. In 1979 behaalde ik het VWO diploma aan de Scholengemeenschap Snellius te Amstelveen. In hetzelfde jaar volgde mijn inschrijving aan de Vrije Universiteit te Amsterdam voor de studierichting Scheikunde. In augustus 1982 werd het kandidaatsexamen S2 behaald. Daarna deed ik als hoofdvak Biochemie onder leiding van Dr. W.H. Mager in de groep van Prof. Dr. R.J. Planta (aan de VU). Het bijvak Immunologie heb ik gedaan onder leiding van Dr. R.J.T. Smeenk in de groep van Prof. Dr. T.E.W. Feltkamp op het Centraal Laboratorium van de Bloedtransfusiedienst. In augustus 1985 legde ik het doctoraalexamen Scheikunde af (cum laude).
Van september 1985 tot september 1988 was ik als wetenschappelijk assistente in dienst van de Rijksuniversiteit Leiden voor het verrichten van een promotieonderzoek (dit proefschrift) op het Instituut voor Anthropogenetica (hoofd: Prof. Dr. P.L. Pearson). Het onderzoek was een gezamenlijk projekt van het Instituut voor Anthropogenetica en het Gaubius Instituut TNO in Leiden. De dagelijkse supervisie berustte bij Dr. E.C. Klasen en (vanaf augustus 1988) Dr. R.R. Frants (Anthropogenetica), en Dr. L.M. Havekes (Gaubius Instituut TNO).
Sinds september 1988 volg ik de cursus Informatiekunde (GO-C) van de Stichting Gemeenschappelijke Opleiding voor archief, bibliotheek, documentatie en informatiebewerking.

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Marijke