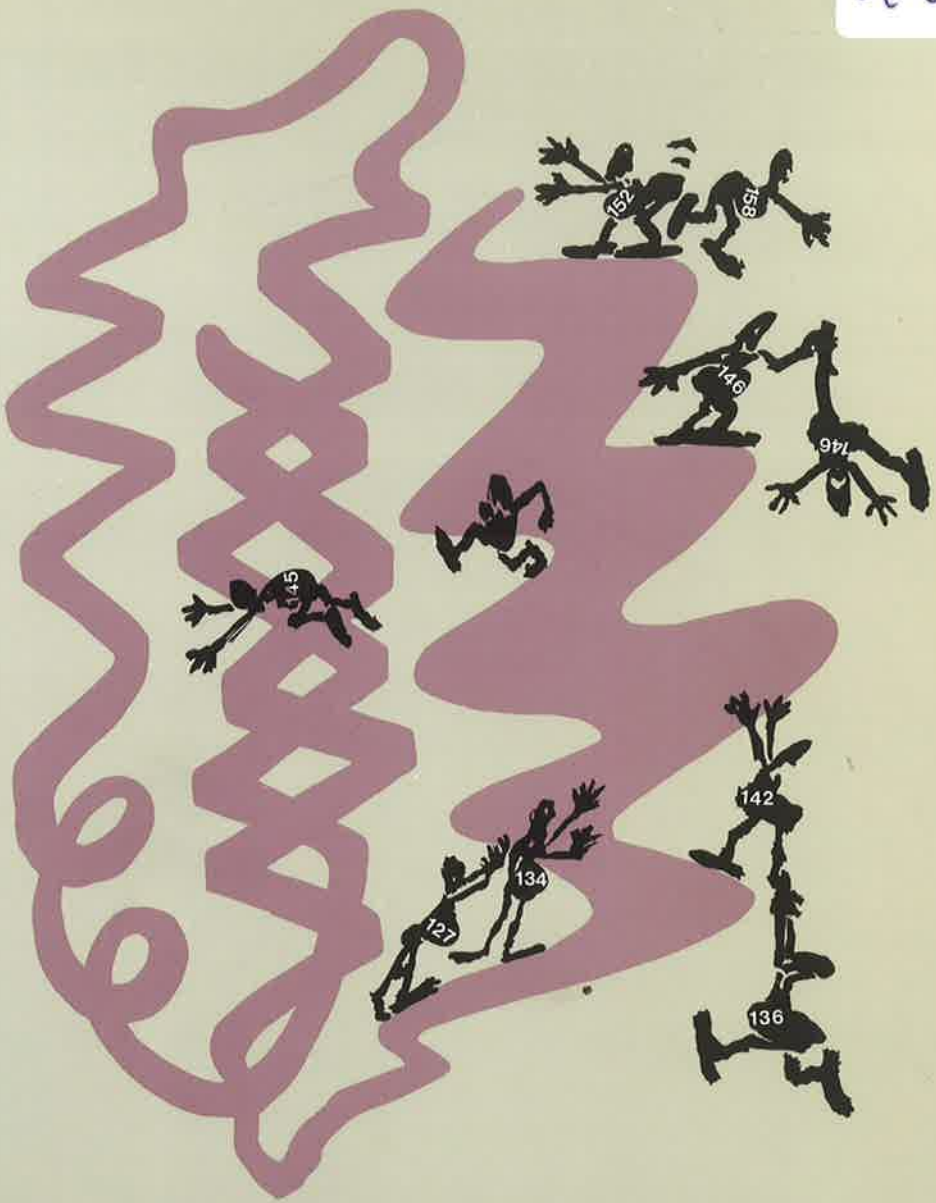
Genetic Heterogeneity of Apolipoprotein E and its Influence on Lipoprotein Metabolism



Peter de Knijff

I 1409

Genetic Heterogeneity of Apolipoprotein E and its Influence on Lipoprotein Metabolism

Stellingen

1. De genetische variatie in het APOE gen vormt een mogelijke verklaring voor de grote mate van variatie in de expressie van de ziekte van Alzheimer en de ziekte van Creutzfeldt-Jacob.
Namba et al. Brain Research 1991; 541:163-166.
Diedrich et al. J Virol 1991; 65:4759-4768.

2. De suggestie van Mahley et al. dat iedere mutatie in het receptor-bindings domein van apoE zal resulteren in een apoE variant die associeert met een dominante overerfbare vorm van FD, is onjuist.
Mahley et al. Curr Opin Lipidol 1990; 1:87-95.

3. De term "familiaire dysbetalipoproteinemie" mag niet als synoniem worden gebruikt voor de term "type III hyperlipoproteinemie".
Mahley RW, and Rall SC Jr. In: The metabolic basis of inherited disease 6th ed. 1989:1195-1213.

4. Bij veel studies naar de gunstige invloed van alcoholgebruik op de ontwikkeling van atherosclerose wordt voorbij gegaan aan de nadelige maatschappelijke gevolgen van alcoholgebruik.
Rimm et al. The Lancet 1991; 338:464-468.
Jackson et al. Br Med J 1991; 303:211-216.

5. Het zeer recent gevonden "Human Basement Membrane Sulfate Proteoglycan Core Protein" is een meer voor de hand liggende kandidaat als LPL-stimuleerbare VLDL-bindingsplaats dan de LRP-receptor.
Mulder et al. submitted. kopie op aanvraag verkrijgbaar.
Kallunki P, and Tryggvason K. J Cell Biol 1992; 116:559-571.
Beisiegel et al. Proc Natl Acad Science USA; 1991: 88:8342-8346.

6. Een ApoE genotypering welke uitsluitend is gebaseerd op een *Hha*I or *Cfo*I RFLP-analyse zal bij vrijwel alle personen met een zeldzame apoE variant resulteren in een onjuiste apoE genotypering.
Wenham et al. Ann Clin Biochem 1991; 28:599-605.

7. Bij populatie onderzoek aan Lp(a) dient men zich bewust te zijn van het feit dat het APOa gen meer dan 6 allelen heeft.
Lackner et al. J Clin Invest 1991; 87:2153-2161.
Kamboh et al. Am J Hum Genet 1991; 49:1063-1074.

8. De door Schnyder et al. beschreven "geheel nieuwe" bepalingsmethode voor plasminogeen activatoren houdt volstrekt geen rekening met de ontwikkelingen op dit gebied gedurende de afgelopen 10 jaar.
Schnyder et al. Anal Biochem 1992; 200:156-162.

9. Het ontbreken van het eiland Schiermonnikoog, het eerste Nationale Landschapspark van Nederland, in het logo van het Ministerie van Landbouw, Milieubeheer en Visserij, is illustratief voor de belangstelling voor natuurbescherming binnen dit Ministerie.

10. In Nederland broeden Kleine Mantelmeeuwen (*Larus fuscus ssp.*) met kenmerken van drie ondersoorten (*L f fuscus, L f intermedius en L f graelsii*) in gemengde paren binnen dezelfde kolonie. Dit rechtvaardigt de twijfel of dit wel "echte" ondersoorten zijn.

11. Algemeen maatschappelijke acceptatie van de man als een individu met gevoelens, emoties en intuitie, zal het proces van emancipatie beter dienen dan het ontzeggen van dezelfde eigenschappen bij de vrouw.

12. Lang leve(n) E2 !

Stellingen bij het proefschrift: Genetic Heterogeneity of Apolipoprotein E and its Influence on Lipoprotein Metabolism.

Alphen aan den Rijn, 4 juni 1992 Peter de Knijff

Genetic Heterogeneity of Apolipoprotein E and its Influence on Lipoprotein Metabolism

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 4 juni 1992
te klokke 15.15 uur

door

Pieter de Knijff

geboren te Leiden in 1956

"ALL IN" BV - KATWIJK

Promotiecommissie:

Promotor:	Prof.Dr. P. Brakman
Copromotoren:	Dr.Ir. L.M. Havekes (IVVO-TNO, Gaubius Laboratorium)
	Dr. R.R. Frants
Referent:	Prof.Dr. B.J. Vermeer
Overige leden:	Prof.Dr. G.J. van Ommen
	Prof.Dr. J.C. Birkenhäger (Erasmus Universiteit)
	Prof.Dr. A. van der Laarse

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Cover design: Monique Mulder

Ter nagedachtenis aan mijn vader

CONTENTS

CONTENTS (continued)

CHAPTER 1

General Introduction

THE LIPID METABOLISM

Introduction

In the human body, cholesterol is used for the synthesis of cell membranes, steroid hormones and bile acids, whereas triglycerides are used as a source for energy in the muscles and storage in adipose tissues. Cholesterol and triglycerides are hydrophobic lipids and thus these molecules are not soluble in an aqueous environment. In the blood they are transported in the form of lipid-protein complexes called "lipoproteins". Lipoproteins are spherical particles with a coat consisting primarily of amphiphilic phospholipids and proteins called "apolipoproteins". The core of these particles consists of non-polar lipids, mainly cholesterol esters and triglycerides.

The lipoproteins can be divided into four major classes: chylomicrons, very low density lipoproteins (VLDL[1]), low density lipoproteins (LDL) and high density lipoproteins (HDL). These lipoproteins differ in density, size, composition and electrophoretic mobility (Table 1). Besides these major lipoprotein classes, two other lipoprotein classes, particularly relevant to the present study, do exist: chylomicron remnants and VLDL-remnants or intermediate density lipoproteins (IDL).

At present, 14 distinct apolipoproteins have been characterized and are found in association with these lipoproteins (Table 1). The most important characteristics of the major apolipoproteins are summarized in Table 2 (for reviews on lipoproteins and apolipoproteins see references 1-6).

The lipoprotein metabolism can be subdivided in three different parts: i) the exogenous lipid pathway, ii) the endogenous lipid pathway and iii) the reverse cholesterol pathway (for reviews see references 7-10).

[1] A full list of abbreviations used throughout this Thesis is presented on page 194.

Table 1. Biophysical and chemical properties of human plasma lipoproteins

Lipoprotein class	Source	Biophysical Properties			
		Diameter (nm)	Electrophoretic mobility	Density (g/ml)	S_f
Chylomicrons	Intestine	75-1200	Origin	< 0.96	> 400
VLDL	Liver and intestine	30-80	pre-β	0.96-1.006	20-400
IDL	VLDL and chylomicrons	25-35	Slow pre-β	1.006-1.019	12-20
LDL	VLDL and chylomicrons	18-25	β	1.019-1.063	2-12
HDL	Liver and intestine	5-12	α	1.063-1.210	

	Chemical Properties						
	Protein	Total lipid	Triglycerides	Phospholipid	Cholesterol ester	Free cholesterol	Major Apolipoproteins
	% weight	% weight	% of total lipid				
Chylomicrons	1-2	98-99	88	8	3	1	A1, A4, B48, C1, C3, E
VLDL	6-10	90-94	56	20	15	8	B100, C1, C2, C3, E
IDL	11	89	29	26	34	9	B100, E
LDL	21	79	13	28	48	10	B100
HDL	45-55	45-55	15	45	30	10	A1, A2, E

Table 2. General properties of major human apolipoproteins

Apolipoprotein*	Plasma concentration mg/dl	Isoelectric point pI	Molecular weight kD	Metabolic function
A1	100-120	5.4-5.9	28	LCAT activator, HDL formation
A2	30-50	5.0	17.4	HL inhibitor
A4	12-20	5.5	44.5	LCAT activator
B100	70-100		550	LDLR ligand, VLDL and LDL formation
B48	3-5		264	Chylomicron formation
C1	4-6	7.5	6.6	LCAT activator
C2	3-5	4.9	8.9	LPL activator
C3	12-14	4.7-5.0	8.8	LPL and HL inhibitor
E	3-5	5.7-6.0	34.2	LDLR and LRP ligand

* In addition to these apolipoproteins, apoD, apoF, apoG, apoH and apoJ are reported, the role of which is not well understood. Sofar there are no metabolic disorders associated with malfunction of these apolipoproteins.

The exogenous lipid pathway

In the exogenous lipid transport (Figure 1), dietary triglycerides and cholesterol are absorbed and packed in the intestinal mucosal cells into large particles, called chylomicrons. These lipoproteins are very rich in triglycerides and contain apolipoproteins (apo) A1, A4 and B48 as the major protein constituents. Upon entering the circulation, chylomicrons acquire apoC1, C2, C3 and E and lose part of apoA1 and A4. Subsequently, the triglycerides are rapidly hydrolyzed by the enzyme lipoprotein lipase (LPL), lining the endothelium of the blood capillaries, with apoC2 serving as a co-factor. The resulting fatty acids are transported to muscles (for energy) and adipose tissues (for storage). During lipolysis the chylomicrons become smaller, their excess surface components (phospholipids, apoA1 and A4) are transferred to HDL, whereas more apoE is transferred from HDL to the chylomicrons. The resulting chylomicron remnants are relatively enriched in cholesterol esters and apoE. These remnants are then rapidly cleared from the circulation by the putative remnant receptor, for which the recently identified low density lipoprotein receptor-related protein (LRP) seems to be a good candidate (11,12). According to our current knowledge, apoE serves as a high affinity ligand for this receptor. The dietary cholesterol and triglycerides taken up by the liver through this route are utilized for the synthesis of bile acids and VLDL.

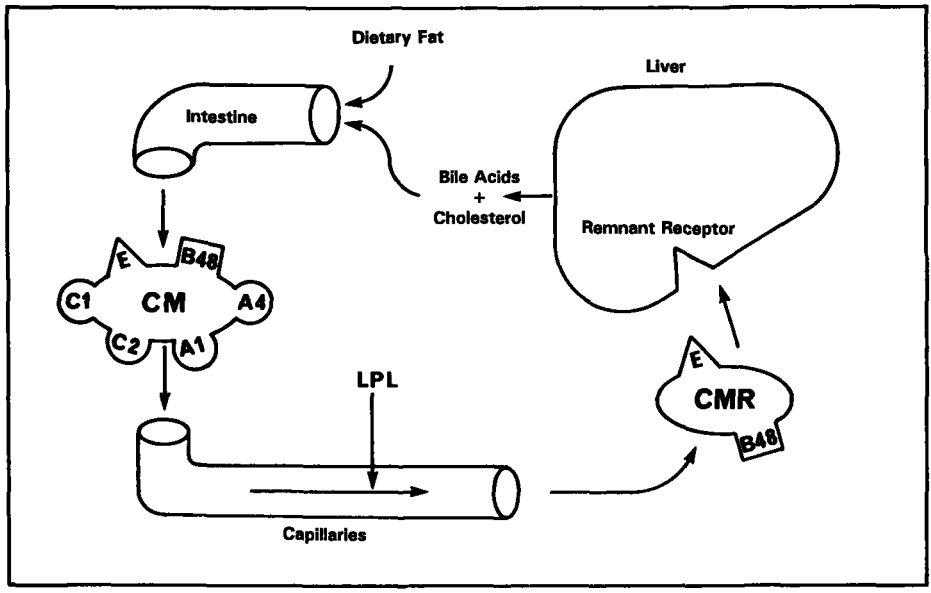

Figure 1. Exogenous lipid pathway.

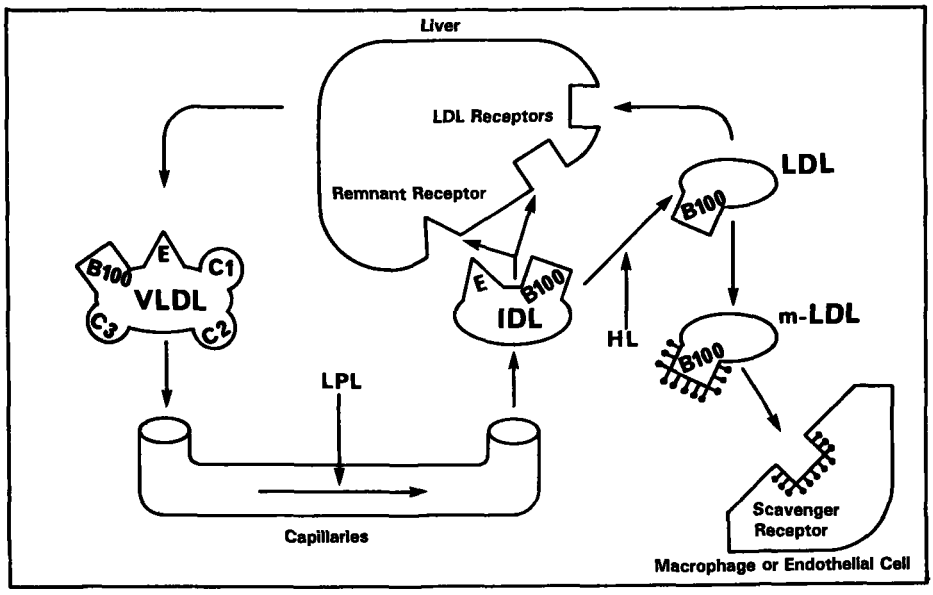

Figure 2. Endogenous lipid pathway.

The endogenous lipid pathway

The first step in the endogenous lipid pathway (Figure 2) is the production and secretion of VLDL by the liver. VLDL particles are, like chylomicrons, rich in triglycerides, but also contain a considerable amount of cholesterol, both obtained by the liver either by direct synthesis or by delivering via the exogenous lipid pathway. VLDL particles contain the apolipoproteins B100, C1, C2, C3 and E on their surface. After secretion into the circulation, VLDL particles are hydrolyzed by LPL, resulting in the formation of the smaller IDL particles (or VLDL-remnants) which are, like chylomicron remnants, enriched in cholesterol esters and apoE. The major part of these IDL particles are, like chylomicron remnants, cleared from the circulation by either the LRP or the low density lipoprotein receptor (LDLR), both using apoE as a high affinity ligand. The remaining portion of IDL particles are further hydrolyzed by a second lipolytic enzyme, hepatic lipase (HL) and converted into LDL. During this conversion, IDL loses the apoC's and apoE. The resulting LDL particles mainly contain cholesterol as lipid component and apoB100 as sole protein. This apoB100 serves as the ligand for receptor mediated endocytosis of LDL via the LDLR. This receptor is mainly present on liver cells, but also on various extra-hepatic cells (e.g. muscle, adipose tissues and adrenal gland). LDL particles normally contain 60-70 % of total plasma cholesterol. Under normal conditions about 70 % of the LDL particles are cleared from the circulation by the LDLR, mostly by the liver. The remaining LDL particles are cleared from the circulation by LDL-receptor independent processes (the scavenger pathway), mainly by cells of the reticulo-endothelial system such as macrophages in the intima and most probably after modification (mLDL). The higher the plasma LDL levels, the more LDL will be taken up by this scavenger pathway. This will, eventually, lead to an overloading of the macrophages with cholesterol esters (foam cell formation) in the intimal layers of the bloodvessels. This process of foam cell formation, is thought to be one of the major initial steps in the formation of atherosclerotic lesions (13-14).

The reverse cholesterol pathway

The reverse cholesterol pathway (Figure 3) includes the transport of cholesterol from peripheral tissues back to the liver for further processing. The major lipoprotein involved in this pathway is HDL. Nascent HDL particles, produced by the liver and by lipolysis of chylomicrons and VLDL particles, consists primarily of "flat" disc-like particles containing apoA1, apoA2 and phospholipids.

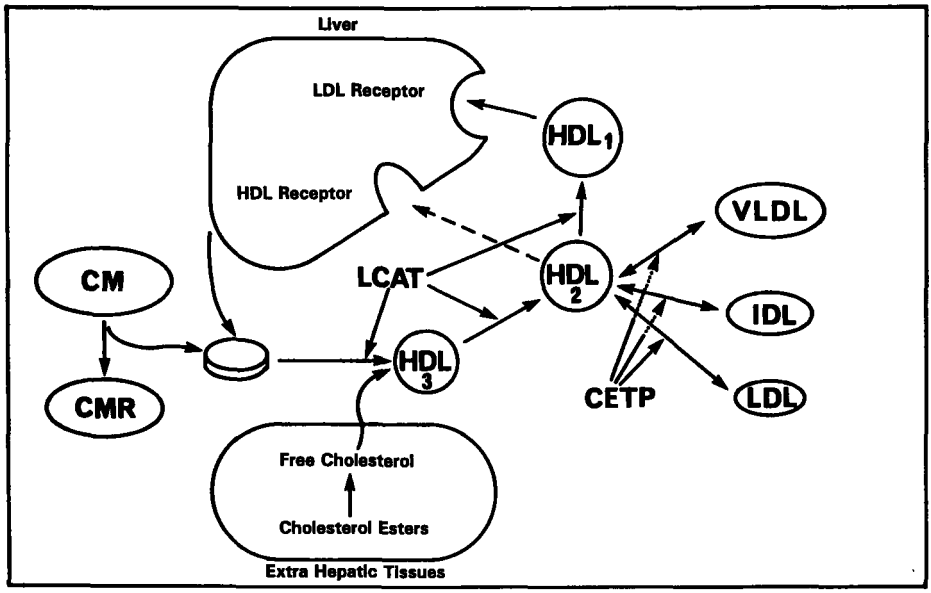

Figure 3. Reverse cholesterol pathway.

Subsequently, these nascent HDL particles take up free cholesterol from extra-hepatic tissues. Thereafter, this cholesterol is esterified by the enzyme lecithin cholesterol acyltransferase (LCAT) using the major protein constituent of HDL, apoA1, as a cofactor. The produced cholesterol esters enter the core of the HDL particle, thereby changing its shape into a spherical particle known as HDL_3 or small HDL. By further uptake of cholesterol and the action of LCAT these small HDL_3 particles will gradually increase in size which will, eventually, lead to the formation of the larger HDL_2 and apoE rich HDL_1 particles. The cholesterol esters in the HDL_2 particles are transported to the apoB containing lipoproteins (chylomicrons, chylomicron remnants, VLDL, IDL and LDL) through the action of cholesterol ester transfer protein (CETP). As a result the HDL_2 particles are converted back into the smaller HDL_3 particles. A portion of the HDL_2 and most of the apoE rich HDL_1 particles (the latter are normally found in only small quantities) are supposed to be removed from the circulation by direct interaction with HDL- and LDL-receptors using apoA1 (for HDL_2) or apoE (for HDL_1) as a ligand, respectively.

APOLIPOPROTEIN E

Introduction

Apolipoprotein E plays a central role in the metabolism of cholesterol and triglycerides. It is one of the major protein constituents of chylomicrons and VLDL and their remnants-particles and HDL. On these particles it serves as a ligand for the uptake by lipoprotein receptors. ApoE is a genetically determined polymorphic protein, with three common isoforms, E2, E3 and E4. Population studies demonstrated that the apoE polymorphism influences plasma lipid and lipoprotein levels. Mutant forms of apoE which are defective in receptor binding, are associated with familial dysbetalipoproteinemia (FD[2]), a genetic lipid disorder characterized by elevated plasma cholesterol- and triglyceride levels and an increased risk for atherosclerosis. ApoE is synthesized by various organs, and is present in relatively high concentrations in interstitial fluid, where it is thought to regulate cholesterol redistribution. ApoE is also thought to play a role in tissue regeneration, immunoregulation and cell differentiation and growth (for reviews on apoE see refs 15-17).

This thesis will pay special attention to i) the importance of the common apoE isoforms in the lipid metabolism in various populations, and ii) the influence of two rare apoE variants, associated with early developed and dominantly inherited forms of FD.

APOE Gene and Primary Sequence

Initially referred to as the "arginine-rich apoprotein", apoE was first identified by Shore and Shore (18) in 1973. It is a single polypeptide with a molecular weight of 34,200 D. Human apoE is a polymorphic apolipoprotein. Three major isoforms, E2, E3 and E4, with pI values ranging from 5.7 to 6.0, can be separated by isoelectric focusing (IEF)(19-24). The biosynthesis of these isoforms is under control of three independent alleles at a single genetic locus. As a result, three homozygous (E2E2, E3E3 and E4E4) and three heterozygous (E3E2, E4E2 and E4E3) phenotypes are found in the general population.

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[2] Throughout this thesis the term familial dysbetalipoproteinemia (FD) is used to indicate i) individuals with type III hyperlipoproteinemia (type III) diagnosed according to the criteria outlined by Mahley and Rall (166) and ii) individuals with familial dysbetalipoproteinemia who, usually, are normo- or even hypolipemic. Since both type III patients as well as FD patients display β-VLDL in the plasma, but only differ in the state of hyperlipemia, and it is generally assumed that individuals with FD are genetically predisposed to the development of type III, we prefer the use of the single term FD.

The molecular basis for the apoE polymorphism was initially revealed by amino acid sequencing (25,26). ApoE is synthesized as a prepeptide of 317 amino acids. After post-translationally cleavage of a signal peptide of 18 amino acids, it consists of a mature protein of 299 amino acids. The most common isoform, E3, contains a cysteine residue at position 112 and an arginine residue at position 158. The isoform E4 is identical to E3 but has an arginine residue at position 112. This introduces an extra single positive charge unit as compared to apoE3 and thus apoE4 focuses one charge unit more basic upon IEF. ApoE2 is also identical to E3 but contains a second cysteine residue at position 158 instead of arginine. The loss of this single positive charge unit results in the more acidic mobility of apoE2 upon IEF.

Genomic and cDNA sequence analyses revealed the molecular basis for this protein polymorphism (27-29). The APOE gene is 3597 nucleotides long and consists of 4 exons and 3 introns (Figure 4). The gene is localised on the long arm of chromosome 19 (q12-q13.2) (28,30) and forms a gene cluster together with the APOC1, APOC1' and APOC2 genes.

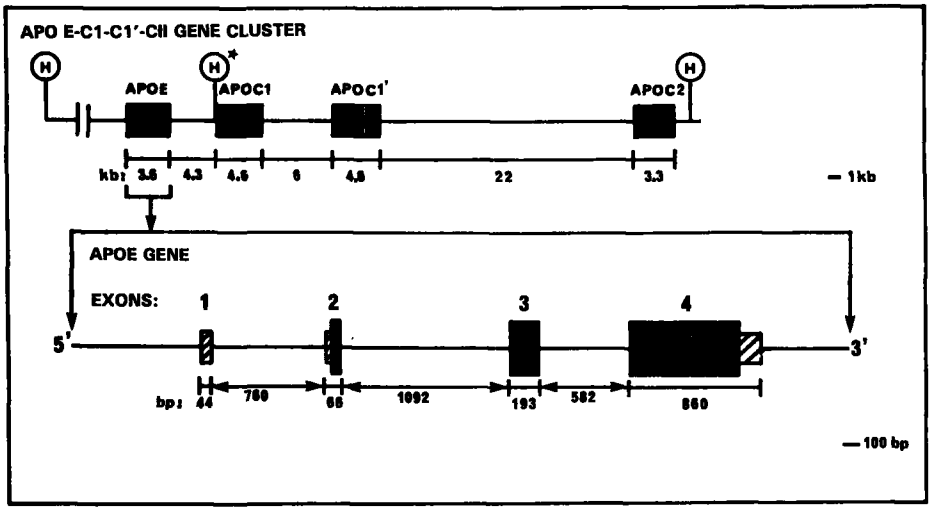

Figure 4. Top Schematic representation of the APOE-C1-C1'-C2 gene cluster. Solid boxes showing the genes. The gene sizes and intergenic distances are indicated in kilobases (kb) below the gene cluster. The constant (H) and variable (H*) *HpaI* restriction sites are also indicated.
Bottem Schematic representation of the APOE gene. Solid boxes indicate the translated parts of the exons, hatched boxes indicate the non-translated parts of the exons. Intron and exon sizes are indicated in basepairs (bp) below the gene.

This gene cluster spans about 50 kb, see Figure 4 (31-36). Using restriction fragment length polymorphism (RFLP) analysis we found almost 100% linkage disequilibrium between the APOE and APOC1 genes (37). A polymorphic *HpaI* site in the promoter region of APOC1 (Figure 4) results in two alleles: H1 (absence of the site) and H2 (presence of the site). The H1 allele is only found in association with the APOE*3 allele or in rare apoE variants most likely derived from APOE3 whereas the H2 allele is found in association with both the APOE*2- and APOE*4 alleles or in mutant alleles derived from these common alleles.

The polymorphic nature of apoE is unique for human beings. In the 10 other mammalian species studied sofar: rat (38-40), mouse (41,42), rabbit (43,44), guinea pig (45), dog (46), cow (47), chimpanzee (48), cynomologus monkey (49), baboon (50) and sea-lion (51) apoE is found as a monomorphic protein. A comparison of the amino acid sequences of these species with the human sequence reveals that apoE is E4-like (Arg-112; Arg-158) in 8 species (rat, mouse, guinea pig, dog, chimpanzee, cynomologus monkey, baboon and sea-lion) and E3-like (Cys-112; Arg-158) in only two species (rabbit and cow), whereas it is variable in humans (52). This could indicate that the APOE*4 allele is the "wild-type" or ancestral allele instead of the APOE*3 allele as is normally assumed based on its allele frequency in humans.

Structure and Function of ApoE

From physical and biochemical studies much insight in the structure and function of apoE has been gained (53-58). ApoE contains two structural domains: an amino-terminal domain (residues 1-164) and a carboxyl-terminal domain (residues 201-299) which are separated by a region of random structure (residues 165-200). The predicted secondary structure (Figure 5) clearly demonstrates these domains.

The carboxyl-terminal part of apoE has a strong α-helical character. It contains at least one heparin-binding domain, probably between residues 214-236 (54). This heparin binding domain is thought to play an important role in the interaction of apoE with proteoglycans on the arterial wall, thereby anchoring the lipoprotein particle to the endothelial layer, enabling the enzyme LPL to hydrolyse the triglycerides. The long stretch of α-helices between residues 230 and 265 is thought to be involved in lipid binding (53). However these functions are not fully elucidated and awaits further clarification.

The amino-terminal part of apoE is studied more extensively. This part contains the so-called receptor binding domain of apoE. This domain encompasses residues 130-150 and contains 9 positively charged amino acid residues, 6 of which are

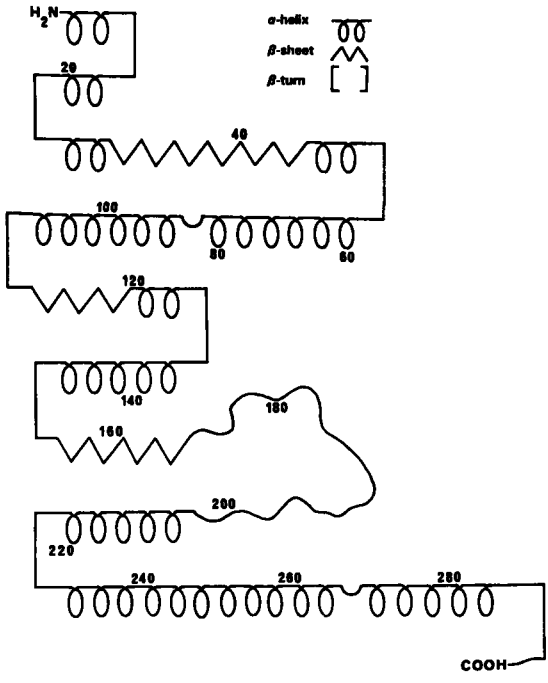

Figure 5. Schematic representation of the predicted secondary structure of apoE, showing α-helices, β-sheet structure, and β-turns. The remaining part of the molecule is predicted to have a random structure (slightly modified and adopted from ref. 56).

faced to one side, whereas the remaining positive residues are "packed" between negatively charged and neutral residues (Figure 6). It is commonly accepted that most of these positively charged residues within the apoE receptor-binding domain interact with the negative charges present in the ligand-binding domains of the LDL and LRP receptors (15).

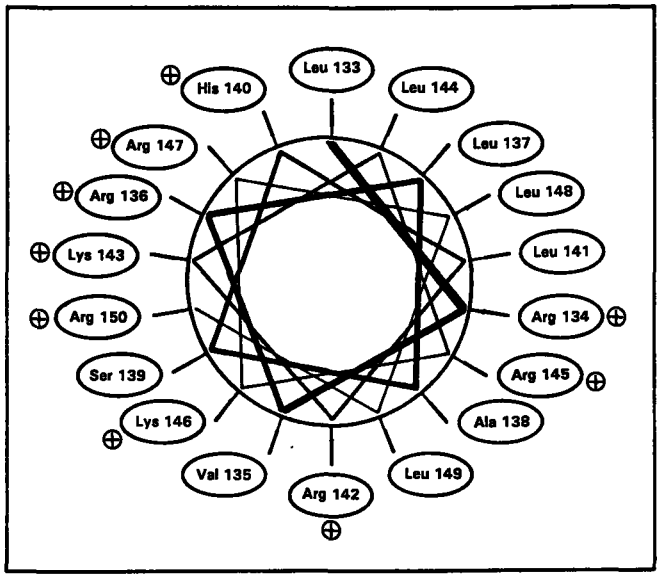

Figure 6. Helical wheel representation of apoE residues 133-150. Positively charged amino acids are indicated. The direction of the wheel in this two-dimensional projection is clockwise, and the residues are interconnected by a solid line decreasing in thickness (modified and adopted from ref. 105).

Recently, the amino-terminal part of apoE has been crystallized and the three dimensional structure in its lipid-free state has been unravelled (59). As depicted in Figure 7, the amino-terminal part of apoE has an elongated four-helix bundle structure, with the four helixes faced anti-parallel and stabilized by a tightly packed hydrophobic core which includes leucine-zipper type interactions and a large number of salt bridges. The residues 130-150 in the supposed receptor-binding domain are clustered, with most of their positive charges faced outwards on the fourth helix.

Preliminary studies suggest that this helical structure, which was determined in the apoE3-isoform, is modulated in the two most frequently occurring mutant forms: apoE4 and apoE2, thereby changing the affinity for the lipoprotein receptors (59).

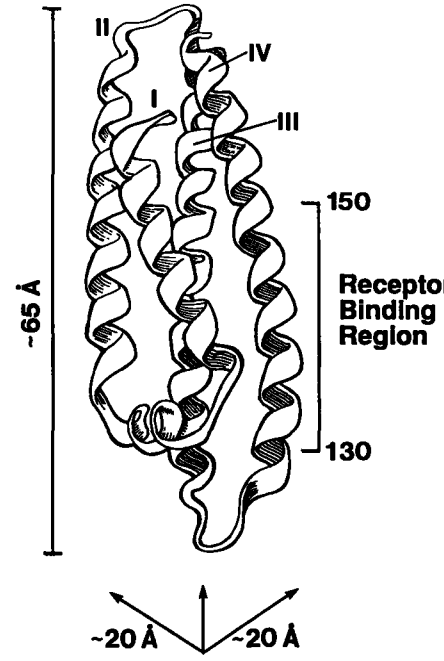

Figure 7. Three-dimensional structure of the major part of NH_2-terminal domain of apoE (residues 23-166) determined at 2.5 Å resolution by x-ray crystallography as recently publiced (ref. 57). The four helices (numbered I-IV) are faced antiparallel. The putative LDL-receptor binding domain is indicated. The estimated dimensions are indicated in angstroms (Å). This figure was kindly supplied by Dr K.H. Weisgraber and is reproduced here with his permission.

Synthesis of ApoE

ApoE is produced in various organs including the liver, brain, kidney and adrenal glands by a large number of different cell types including parenchymal cells, differentiated macrophages, astrocytic glial cells, ovarian granulosa cells, smooth muscle cells and keratinocytes both *in vivo* and *in vitro* (60-66). The major site of synthesis of circulating apoE is the liver. Significant amounts of apoE are also synthesized by the brain (67). Circulating apoE is not able to pass the "blood/brain" barrier as a result of which there are two well separated apoE pools in the human body: liver and brain derived, respectively. This was demonstrated in recent studies among patients before and after liver transplantation (68,69). It was estimated that about 90 % of the circulating plasma apoE of the recipient patient changed to the

apoE isoforms synthesized by the donor liver whereas the apoE in cerebrospinal fluid of the recipient patient remained totally unchanged.

With the help of recombinant DNA techniques, including the use of transgenic mice, it was found that the tissue specific synthesis of apoE is controlled by an array of elements scattered throughout the APOE-C1-C1'-C2 gene cluster (70-76). Only the elements in the immediate 5'-flanking region of the APOE gene have been identified in more detail (74-76) sofar. Two of these elements were found to be highly homologous to repeat 3 in the cholesterol responsive sterol regulatory region (SRE42) of the LDLR gene (77-80). In the presence of cholesterol, these elements seem to be associated with a positive regulation of the apoE synthesis, whereas in the LDLR gene SRE42 is associated with a down regulation of the LDLR synthesis. This inverse regulation was confirmed by *in vitro* experiments using mouse macrophages, human skin fibroblasts and human hepatoma cell lines (81-83).

ApoE Polymorphism

At present 24 different isoforms of apoE have been unequivocally identified by DNA and/or amino acid sequencing (Table 3 and refs 25-29,84-110). The most common isoforms, referred to as E3, E2 and E4 throughout this thesis, are the supposed wild-type isoform apoE3(Arg112,Cys-158), apoE2(Arg158→Cys) and apoE4(Cys112→Arg) and occur with allele frequencies of ~ 0.750, ~ 0.100 and ~0.150, respectively in most Caucasian populations. The other, rare, isoforms occur with allele frequencies below 0.001.

ApoE Polymorphism in Healthy Populations

Since the discovery of the polymorphic nature of apoE, a large number of studies have been published concerning APOE allele frequencies and the effect of the apoE polymorphism on plasma lipoprotein levels (111-151, chapter 6 of this Thesis). The APOE allele frequencies of 9 different population groups are presented in Table 4. These population groups were chosen according to their evolutionary relations as outlined by Cavalli-Sforza and co-workers (152-154). From Table 4 it is obvious that the APOE*4 allele frequency is high (0.254 - 0.368) in some of the oldest human population groups (Africans, Australian aboriginals and Papua New Guineans). In the more recently derived populations, the APOE*4 allele frequency is markedly lower (0.091 - 0.153). In contrast, the APOE*3 allele frequency is lower in the older human populations (0.486 - 0.682) but higher in the more recent populations (0.772 - 0.861). There is no clear tendency in the APOE*2 allele

Table 3. Characteristics of all presently sequenced apolipoprotein E variants.

IEF position	Parental allele	Amino acid substitution(s) when compared with apoE3	Trivial name	Associated hyperlipidemia*	References
E3	-	Cys112; Arg158	"Common"	N	25-29
E1	E2	Gly127→Asp;Arg158→Cys		HTG,FD	84-86
E1	E2	Arg158→Cys;Leu252→Glu		FH	87
E1	E3	Lys146→Glu	Harrisburg	FD	88,89
E2	E3	Arg158→Cys	"Common"	FD	25-29
E2	E3	Arg145→Cys		FD	90,91
E2	E3	Arg134→Gln		N	92
E2	E3	Val236→Glu		HTG	87
E2	E3	Arg228→Cys	Dunedin	HTG	93
E2	E3	Lys146→Gln		FD	94-96
E2	E3	Arg136→Ser	Christchurch	FD	91,97
E3	E3	Ala99→Thr;Ala152→Pro		N	27
E3	E4	Cys112→Arg;Arg142→Cys		FD	98,99
E3-	E3	Thr42→Ala	Freiburg	N	100
E3	E4	Cys112→Arg;7 aa insertion	Leiden	FD	101-104
E4	E3	Cys112→Arg	"Common"	N	25-29
E4	E3	Glu13→Lys;Arg145→Cys	Philadelphia	FD	105
E4+	E3	Ser296→Arg	Amsterdam	N	87
E4-	E4	Leu28→Pro	Freiburg	N	100
E4-	E4	Cys112→Arg;Arg274→His	Doetinchem	N	87,106
E5	E3	Glu3→Lys		FH	107
E5	E3	Glu13→Lys		N	108
E5	E4	Pro84→Arg;Cys112→Arg		FH	109
E7	E3	Glu244→Lys;Glu245→Lys	Suita	FH,FCH,HTG	110

* Abbreviations used in this column are N, no hyperlipidemia; HTG, hypertriglyceridemia; FD, familial dysbetalipoproteinemia; FH, familial hypercholesterolemia; FCH, familial combined hyperlipoproteinemia.

frequency. It was suggested that the APOE*2 allele is of Caucasian origin and that it is absent in pure indigenous populations (i.e. populations which are not "infected" by Caucasian genes) as is illustrated in the Australian aboriginals and Mayan indians from Yucatan, Mexico (122). A marked exception to this hypothesis are the Papua New Guineans with the highest APOE*2 allele frequency reported sofar in any population. It would be very interesting to learn if this E2 isoform has the same molecular basis as the apoE2 isoform in Caucasians.

Table 4. Apolipoprotein E allele frequencies in various ethnic populations*

Populations	sample size	APOE alleles E*4	E*3	E*2
New Guinean[1]	110	0.368	0.486	0.146
Australian[2]	64	0.260	0.740	0.000
Southeast Asian[3]	308	0.091	0.805	0.104
Northeast Asian[4]	2443	0.099	0.850	0.051
Arctic[5]	133	0.229	0.756	0.015
Amerindian[6]	1193	0.107	0.861	0.032
European Caucasoid[7]	14514	0.153	0.772	0.075
Non-European Caucasoid[8]	142	0.127	0.827	0.046
African[9]	1605	0.254	0.682	0.064

* These populations were grouped according to the regional divisions published by Cavalli-Sforza and coworkers (152-154).

[1]. Taken from reference 111.

[2]. Taken from reference 112.

[3]. Representing the weighted average allele frequencies from China and Malaysia (113).

[4]. Representing the weighted average allele frequencies from Japan (113-120).

[5]. Representing the Greenland Inuit, taken from Chapter 6 of this Thesis.

[6]. Representing the weighted average allele frequencies from South-American Indians (121), Mayans from Yucatan, Mexico (122), and Mexican Americans, USA (123).

[7]. Representing the weighted average allele frequencies from Denmark (124), Germany (125-129), Finland (113,130,131), France (132), Hungary (113), Iceland (113), The Netherlands (133-135), Norway (136, 137), Austria (113), Scotland (138,139), Spain (140), Britain (141), Sweden (142), Switzerland (143) New Zealand (144) and the USA (145-149).

[8]. Representing the allele frequencies of a population from India (113).

[9]. Representing the weighted average allele frequencies from Nigeria (150,151), Sudan (113) and American blacks from Africa (147,149,151).

Despite these large differences in APOE allele frequencies and other differences in genetic background and environmental factors such as dietary habits, a single APOE allele behaves in a rather uniform manner in different populations. Utermann (155) found that in healthy individuals the APOE*2 allele is associated with the lowest levels of plasma cholesterol and LDL-cholesterol, whereas Bouthillier et al. (156) and Davignon et al. (157) found that healthy individuals with an APOE*4 allele exhibits the highest plasma cholesterol and LDL-cholesterol levels. As outlined in Chapter 3 of this Thesis, these results were confirmed by us (133). We also showed that there was an inverse correlation between mean plasma apoB and apoE concentrations in each apoE phenotype group. Individuals with an E*4 allele showed the highest apoB- and the lowest apoE concentration whereas the opposite was true for in APOE*2 allele carriers. Since then, these results have been confirmed in a large number of other studies which are thoroughly discussed by Davignon et al. (16).

Utermann (158) was the first to propose the now well accepted model explaining (in part) these allelic influences. The effects of allelic substitution at the APOE locus on plasma levels of cholesterol, LDL-cholesterol, apoB and apoE are thought to be the result of i) a more efficient catabolism of chylomicron remnants and IDL particles in individuals with a E*4 allele and ii) a less efficient catabolism of these particles in individuals with the APOE*2 allele, due to a defect in binding of apoE2 to hepatic lipoprotein receptors (159). An enhanced uptake by the liver of either chylomicron remnants or IDL will supply the liver with extra cholesterol resulting in a reduction of hepatic LDL-receptor activity and thus leading to an elevation of plasma LDL-cholesterol and apoB levels and decreased apoE levels. Reciprocally, a diminished uptake of remnants will lead to an enhanced LDL-receptor activity which will, in its turns, lead to a reduction of plasma LDL-cholesterol and apoB levels and increased levels of apoE. In addition to this, it has been reported that the conversion of VLDL via IDL to LDL is delayed in individuals with an APOE*2 allele, resulting in lower LDL-cholesterol and apoB levels (160). These effects are rather consistent throughout the various populations studied. Recently, in a single laboratory study, Hallman et al. (113) demonstrated that the apoE polymorphism exhibited more or less comparable effects on plasma cholesterol levels in 9 different human populations: Tyrolean, Sudanese, Indian, Chinese, Japanese, Hungarian, Icelandic, Finnish and Malay.

There may be other mechanisms by means of which the apoE polymorphism could regulate the lipid metabolism, as suggested by Weisgraber et al. (161,162) and Borghini et al. (163). In their studies, it has been suggested that i) complexation of apoE either with apoA2 or with itself prevents the uptake of HDL

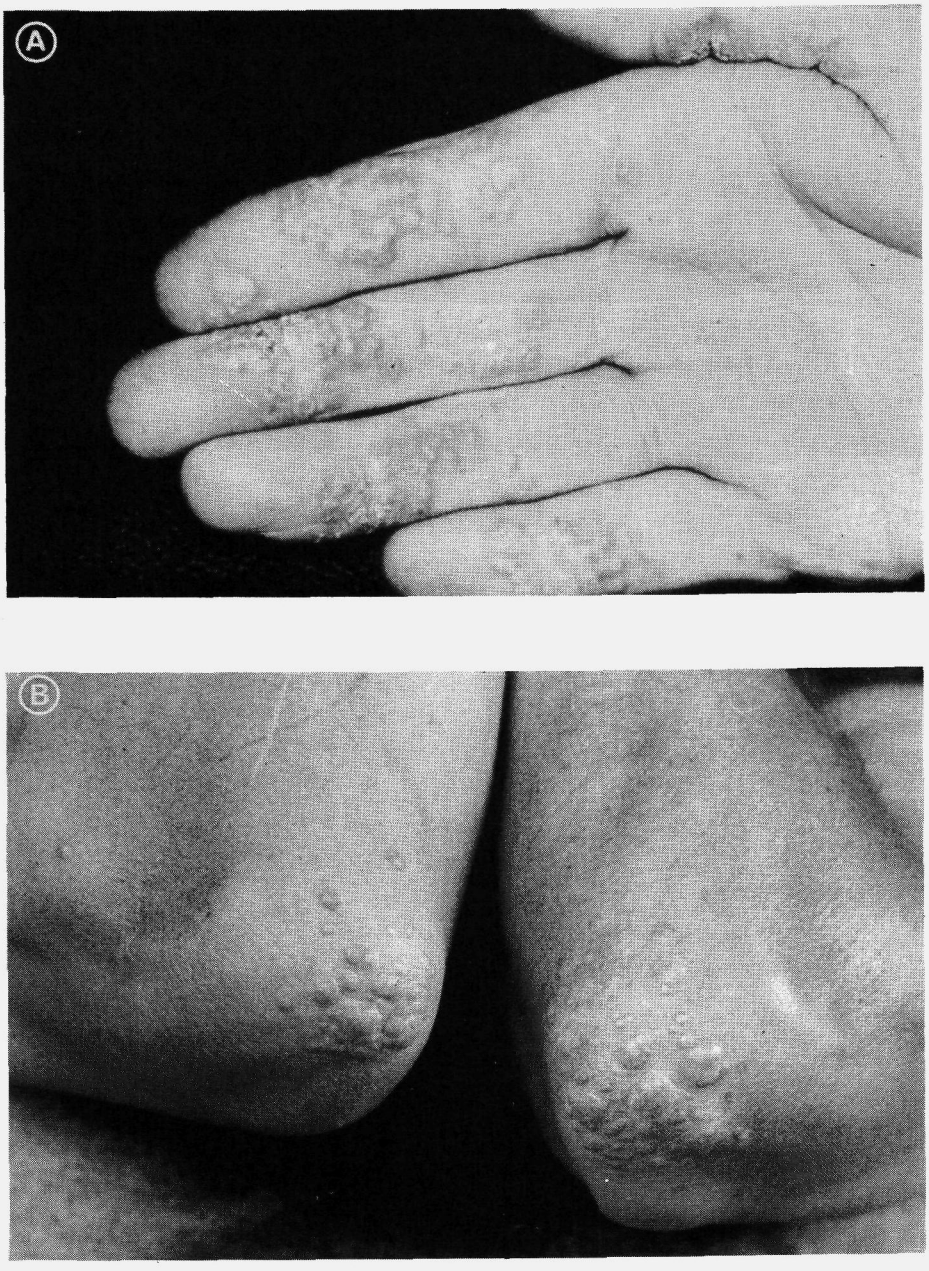

Figure 8. Xanthomas often found in association with FD; A) xanthomata palmaria, and B) tuberous xanthomas on the elbows.

by hepatic receptors, and ii) due to dimerization, a major part of plasma apoE is not readily available to be transferred from HDL to IDL and/or chylomicron remnants in case of apoE3 and E2, suggesting that in these isoforms the availability of apoE in the chylomicron remnants and IDL fractions could be a rate limiting step in the hepatic clearance of these lipoprotein particles. In addition, the apoE isoforms differ in their preference for the various lipoprotein classes. This may also lead to different clearance rates.

It also has to be taken into account that the effect of the apoE polymorphism on plasma lipid variables can be influenced by other genetic factors. Two examples of this were recently published: the *Pvu*II RFLP polymorphism of the LDLR gene in two Norwegian populations (136,137) and the *Xba*I RFLP in the APOB gene in a Finnish population sample (164,165). These polymorphisms were found to influence the plasma cholesterol, LDL-cholesterol and apoB levels and showed gene-gene interactions with the apoE polymorphism in these populations. This shows that the plasma cholesterol concentration in a single population is the result of at least three well defined genes: LDLR, APOB and APOE.

ApoE Polymorphism and Major Disorders in Lipoprotein Metabolism

In addition to the influence of the common apoE variants on the lipoprotein metabolism in healthy individuals, there are also a number of associations between apoE variants and various disorders in the lipoprotein metabolism. The best studied association is that between E2E2 homozygosity and FD.

FD is a genetic disorder of the lipoprotein metabolism predisposing to premature atherosclerosis. In the general population, FD is found with a frequency between 2-10 per 10,000. The disease rarely manifests itself before the third decade in males. In females usually it is expressed only after the menopause. The early development of FD seems also to be influenced by obesity and the presence of other clinical disorders such as diabetes mellitus or hypothyroidism (166).

It is estimated that about 50% of the patients with FD display xanthomas. One specific type, xanthoma striata palmaris, is pathognomonic for FD (Figure 8) (167). These xanthomas occur as yellowish lipid deposits in the palmar creases. In addition, other types of xanthomas are observed such as tendon xanthomas and, more pathognomonic, tuberous xanthomas on the elbows.

The primary metabolic defect in FD patients is due to mutant forms of apoE on chylomicron remnants and IDL, leading to an impaired clearance of these remnant particles by the liver. Patients with FD are, due to the accumulation of these remnants, characterized by increased levels of cholesterol (> 7.5 mmol/l) and

triglycerides (> 2.0 mmol/l), an increased ratio of VLDL-cholesterol/plasma triglycerides (>0.69 on mmol basis) and the presence of β-VLDL (166).

The vast majority of FD patients are homozygous for the apoE2(Arg158→Cys) variant (166). *In vitro* experiments showed that this variant exhibits only 1% of the receptor binding capacity of normal apoE3 (168). Since homozygosity for the apoE2(Arg158→Cys) variant is required in order to develop FD, this variant is associated with a recessive mode of inheritance of FD. In Caucasian populations homozygosity for apoE2(Arg158→Cys) occurs with a frequency of 1%, while the frequency of FD is about 2-10 per 10,000 (166). Thus, only a small percentage of these E2E2 individuals will develop FD. This indicates that FD is normally a multifactorial disease, i.e. additional factors, either genetic or environmental, are required for its manifestation (158).

Expression of FD has also been observed in individuals heterozygous for the rare apoE variants (Table 3) or with apoE deficiency (169-172). Family studies have confirmed that heterozygosity for some of these variants is associated with the development of FD despite the presence of a "normal" APOE allele, indicating that with these variants FD is dominantly inherited with a high degree of penetrance (which implies that most, but not all individuals with these rare apoE variants display FD).

OUTLINE OF THIS THESIS

The present study was initiated to further investigate the role of common and rare apoE variants on the lipid metabolism in healthy individuals (Chapters 2-6) and in patients with hyperlipoproteinemia (Chapters 7-9).

Until recently, the determination of the apoE phenotype was done by isoelectric focusing of delipidated VLDL, followed by protein staining. However this method needs fairly large amounts of serum or plasma and involves a time consuming and expensive ultracentrifugation step. Since it was expected that a large number of individuals had to be studied we developed a new, very rapid, apoE phenotyping method (Chapter 2).

Based on this new method, we were able to study the role of the common apoE variants on plasma levels of cholesterol, triglycerides, apoB and apoE in a large population of apparently healthy Dutch males (Chapter 3).

By means of twin studies with monozygotic twins, it is possible to estimate the influence of genetic and environmental factors against a genetically homogeneous background. Therefore we studied the importance of the APOE gene in determining various plasma lipid levels in 160 Dutch twin families (Chapter 4).

Lipoprotein(a) [Lp(a)] is one of the major single risk factors for atherosclerosis. It is suggested that at least part of Lp(a) is cleared from the circulation by means of the LDL-receptor. Since the apoE polymorphism influences the LDL-receptor activity *in vivo*, we were interested to study the role of the apoE polymorphism in determining the levels of Lp(a) (Chapter 5).

The Greenland Inuit do not suffer from the acute event of ischaemic heart disease to the same extent as most of the modern Western societies. They also display markedly different lipoprotein levels. However, most studies on Greenland Inuit were performed in early 70's. Since then they have changed their life style dramatically. To investigate if this change in life style showed an influence on the various lipid measures, and if these levels were influenced by the common apoE polymorphism to the same extent as is found in Caucasian populations, we studied these trends in 133 healthy Greenland Inuit (Chapter 6).

Besides the well-documented role of the apoE polymorphism in the expression of FD, the apoE polymorphism has also been reported to be associated with familial hypercholesterolemia (FH). Patients with FH have high levels of LDL-cholesterol due to the presence of defect LDL-receptors. Recently, the use of inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol synthesis, was developed for treatment of patients with FH. Upon treatment with these inhibitors, the LDL-receptor activity is increased,

leading to reduced LDL-cholesterol levels. Since the apoE polymorphism was found to influence the LDL-receptor activity in healthy individuals, we were interested to evaluate the effect of the apoE polymorphism on lipid levels in heterozygous FH patients, with special interest in the effect of the apoE variability on the response to simvastatin treatment of these FH patients (Chapter 7).

FD is a genetic disorder of the lipoprotein metabolism predisposing to premature atherosclerosis. Patients with FD show elevated plasma cholesterol and triglyceride levels due to the accumulation of β-migrating chylomicron and VLDL remnants enriched in cholesterol. ApoE, one of the major protein constituents of these remnant particles plays a central role in the receptor mediated endocytosis of these particles, functioning as a high affinity ligand for hepatic lipoprotein receptors. The accumulation of remnants in patients with FD is due to the presence of defect forms of apoE on these particles. Most patients with FD are homozygous for apoE2(Arg158→Cys). In these cases FD is inherited in a recessive mode of inheritance and the development of FD is influenced by additional factors, either genetic or environmental. In some patients however, FD is inherited in a dominant mode of inheritance. These patients are heterozygous for a number of other mutant forms of apoE. In Chapter 8 we studied the genetic heterogeneity of the APOE gene in FD patients and healthy controls in more detail using the technique of genomic DNA amplification (polymerase chain reaction, PCR) followed by hybridization with mutation-specific synthetic oligonucleotide probes. We also report on the occurance of FD in association with a rare variant of apoE, E2(Lys146→Gln), in three different families.

Finally we were able to study factors influencing the heritability patterns of FD associated with heterozygosity for the apoE3-Leiden, another rare variant of apoE associated with a dominant mode of inheritance of FD, among the relatives of five Dutch probandi sharing common ancestry in the 17th century (Chapter 9).

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CHAPTER 2

A Rapid Micromethod for Apolipoprotein E Phenotyping Directly in Serum

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Summary

A new method for the apolipoprotein E (apoE) phenotyping has been developed. The method is based on isoelectric focusing of either delipidated or guanidine-HCl-treated serum or plasma in a horizontal slab gel system followed by immunoblotting using either polyclonal or monoclonal anti-apoE antibodies as first antibody. ApoE phenotyping with this method in 200 serum samples that had been stored at -20°C for more than one year gave exactly the same results as obtained with the conventional method based on isoelectric focusing of delipidated very low density lipoproteins (VLDL) isolated from fresh serum followed by protein staining. Compared with the conventional method, the present method is less laborious because ultracentrifugation to isolate VLDL is not needed; it is suitable for large scale screening purposes; it needs only a few microliters of serum or plasma, and can easily be performed with samples with low concentrations of apoE.

Introduction

In normal subjects, chylomicron and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by means of receptor-mediated endocytosis in the liver or conversion into low density lipoprotein (LDL) (1). 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 (2).

As determined by isoelectric focusing, human apoE can be separated into three major isoforms, i.e., E2, E3, E4, and a number of minor glycosylated isoforms (3,4). The major isoforms differ in pI by a single charge unit, apoE4 being the most basic and E2 the most acidic. ApoE3 is the most commonly occurring isoform.

At present, a number of less frequently occurring apoE isoforms have been described. Some of these variants are more basic than apoE4 (5,6) or more acidic than apoE2 (7,8), and some of them have the same electric charge as E2 (9) or E3 (10-12).

Except for apoE3 and E4, apoE2 and most of the less frequently occurring apoE isoforms have reduced activity for binding to lipoprotein receptors and cause thereby the clinical picture of dysbetalipoproteinemia. This dysbetalipoproteinemia is the major defect in familial dysbetalipoproteinemia (FD) (13,14) where chylomicron and VLDL remnants accumulate. Most of the FD are homozygotes E2/E2. However, only about four percent of E2/E2 homozygotes develop FD (15).

The accumulation of chylomicron and VLDL remnant particles in the plasma often results in premature coronary artery and peripheral vascular disease and in xanthomatous lesions (16).

Until now, the determination of the apoE phenotype in order to diagnose FD is done by isoelectric focusing of delipidated VLDL (apoVLDL) followed by protein staining. This method needs a fairly high amount of serum; it is also laborious and expensive inasmuch as ultracentrifugation is required to isolate VLDL and thus it is less suitable for large scale purposes. In this report we present a new rapid method for apoE phenotyping. With this method, delipidated serum is applied directly to an isoelectric focusing slab gel whereafter the apoE polymorphism is visualized by immunoblotting using anti-apoE antibodies as first antibody. This method needs only very small amounts of serum or plasma and can be easily used for large-scale diagnosis and population studies even in less well-equipped laboratories.

Materials and Methods

Materials Serum was prepared by low-speed centrifugation (10 min; 1000 g) of clotted blood freshly obtained from hyperlipidemic donors. Ampholytes (pH 5-7) were obtained from LKB (Bromma, Sweden). Nitrocellulose paper (blotting paper) was purchased from Schleicher and Schüll (Dassel, FRG). Peroxidase conjugates of rabbit anti-goat IgG and goat anti-mouse IgG were obtained from Nordic Immunology (Tilburg, The Netherlands) and from Jackson Immunoresearch Laboratories (Avondale, PA), respectively. Dithiothreitol (DTT) was from Aldrich (Brussels, Belgium). Guanidine-HCl and 4-chloro-1-naphthol were purchased from Merck (Darmstadt, FRG). All other chemicals were reagent grade.

Preparation of polyclonal and monoclonal anti-apoE antibodies For antibody preparation, apoE was isolated from human VLDL derived from subjects with E3/3 phenotypes. For polyclonal antibody preparation, apoE was isolated from VLDL exactly as previously described (11). A goat was immunized subcutaneously with 200 μg of apoE mixed with 2 ml of complete Freund's adjuvant and then every 2 weeks with 200 μg of apoE mixed with 2 ml of incomplete Freund's adjuvant. One hundred ml of blood was collected 7 days after each injection. After seven boosters the goat was bled.

For monoclonal antibody preparation, apoE was isolated from human VLDL by preparative polyacrylamide gel electrophoresis (Desaga Apparatus) in SDS gels (13%) according to Neville (17). A female Balb/C mouse was immunized by two intraperitoneal injections (with complete and incomplete Freund's adjuvant,

respectively) and a final intravenous injection in the tail vein was given 3 days before the cell fusion. The spleen cells (including the lymphocytes) were isolated and fused with myeloma cell line P_3 x 63 Ag 8 U_1. The cells were plated in 96-well plates in HAT-DMEM (Gibco) (U. Beisiegel et al., unpublished results). A solid phase indirect binding assay with apoE coated in microtiter plates was used to detect anti-apoE in the cell supernatant. ^{125}I-labeled goat-anti-mouse IgG was used as second antibody (18). The culture supernatants that gave positive results in this assay were tested for recognition of the apoE isoforms on immunoblots of apoVLDL, separated by isoelectric focusing. The hybrid cell line EE7 (producing an IgG) was used for the immunoblot experiments shown in this paper.

Sample preparation Two methods to dissociate apoE from lipoproteins were used.

Method A. Delipidation with chloroform-methanol. A 10-μl serum sample was diluted 1:10 with distilled water and then delipidated once with chloroform-methanol 2:1 (by volume) and once with ethyl ether. The protein pellet was stored at -20°C or used immediately. The pellet was resuspended in 40 μl of buffer (0.03 M Tris-HCl, 6 M urea, pH 8.2). After 10 min of shaking, the suspension was centrifuged (10 min, 10,000 g), whereafter the supernatant was diluted fourfold, unless otherwise indicated (see Figure 4) with the same buffer.

Method B. Addition of guanidine-HCl. To avoid extensive sample manipulation, we tested the addition of guanidine-HCl in order to dissociate apoE from lipoprotein lipids. Therefore, serum samples were diluted twofold with 1 M guanidine-HCl for 15 hr at 37°C. Thereafter the samples were diluted fivefold in the buffer used in method A. To 30 μl of diluted sample, 10 μl of DTT (15 μg/ml) and 10 μl of ampholytes (pH 5-7, 10%) were added. Aliquots of 30 μl of this mixture were applied to the gel.

Cysteamine treatment When cysteamine treatment was used, 10 μl of plasma was diluted 10-fold with freshly prepared 0.4 M cysteamine solution and incubated at 37°C for 15 hr. Thereafter the samples were delipidated as in method A and treated likewise. To 30 μl of delipidated and diluted sample, 10 μl of Tris-urea buffer was added instead of 10 μl of DTT solution. After addition of 10 μl of ampholytes (pH 5-7; 10%), aliquots of 30 μl of this mixture were applied to the gel.

Experimental:

Isoelectric focusing with horizontal slab gels. The polyacrylamide gel (5% by weight) was composed exactly as described by Warnick et al. (19) containing 2% (by weight) ampholytes (pH 5-7).

The polyacrylamide gel solution was poured into a cassette of two perspex plates (10 x 24 cm) separated by a 2.3-mm spacer. On the inner side of one of the two perspex plates, 24 perspex squares (4 x 6 mm; 1 mm thick) were mounted in order

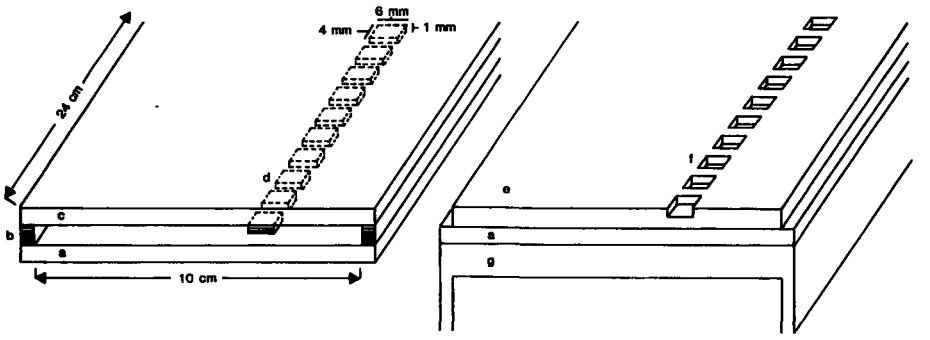

Figure 1. Schematic presentation of the preparation of the polyacrylamide slab gel. A) a, Lower perspex plate; b, rubber spacer (2.3 mm); c, upper perspex plate; d, perspex slot former, B) a, Lower perspex plate; e, polyacrylamide gel; f, slot; g, cooling plate.

to obtain individual slot formers (Figure 1A). After polymerization, the cassette was put onto a horizontal LKB 2117 Multiphor cooling plate, with the 24 slot-formers at the upper side. Thereafter the upper plate was removed carefully (Figure 1B). Both long sides of the gel were brought in contact with electrode buffers by means of strips of Whatman 3 MM filter paper hanging over in reservoirs containing the electrode buffer (0.02 M NaOH at the cathode; 0.01 M H_3PO_4 at the anode) (Figure 2). The gel between both strips of filter paper (electrodes) was covered with a perspex plate (1 mm thick) perforated at the position of the slots. The slots, located near the cathode, were completely filled with 30 μl of sample using a microsyringe and then closed with a sheet of plastic. The electrophoresis was run subsequently at 100 V for 30 min, 300 V for 2 hr, and at 500 V for another 15 hr. During the isoelectric focusing, the gel was cooled at a temperature of 6°C.

Conventional isoelectric focusing. Isoelectric focusing of apoE using the conventional method was carried out as described by Menzel, Kladetzky, and Assmann (20) using delipidated VLDL.

Immunoblotting After isoelectric focusing, the slab gel was washed in a blot buffer (192 mM glycine, 25 mM Tris-HCl, 20 volume % methanol) for 30 min, followed by electroblotting on nitrocellulose paper in the same blot buffer, but without

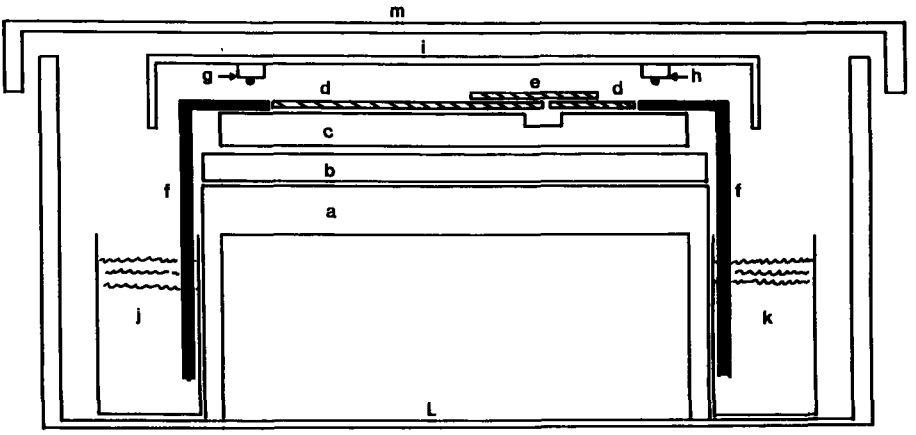

Figure 2. Diagrammatic cross-section of the horizontal slab gel isoelectric focusing system. For text see Experimental section; a, cooling plate; b, lower perspex plate; c, polyacrylamide slab gel; d, perforated covering perspex plate (1 mm thick); e, plastic sheet; f, filter paper; g, anode; h, cathode; i, electrode holder; j, anode buffer reservoir; k, cathode buffer reservoir; l, electrophoresis box; m, cover.

methanol, for 2 hr at 400 mA. After blotting, the nitrocellulose paper (blot) was incubated with a solution of bovine serum albumin (3% by weight) in buffer A (0.15 M NaCl, 10 mM Tris-HCl, 0.05 volume % Tween 20, pH 7.4) for 1 hr at room temperature in order to saturate the blot with protein. Then the blot was washed (two times, 15 min) with buffer A followed by incubation at room temperature in buffer A in the presence of 0.1% (by volume) of goat anti-apoE antiserum. After 1 hr of incubation at room temperature, the blot was washed (two times, 15 min) in buffer A and further incubated in buffer A but in the presence of 0.1% (by volume) of rabbit-anti-goat IgG conjugated to horseradish peroxidase. After 1 hr of incubation at room temperature the blot was washed (two times, 15 min) and, eventually, developed by incubation at room temperature in a substrate solution consisting of 3 mM 4-chloro-1-naphthol, 3 mM H_2O_2, 200 mM NaCl, 50 mM Tris HCl, pH 7.4, and 17% (by volume) methanol. The enzyme reaction was stopped by washing the blot with distilled water. When monoclonal anti-apoE antibodies were used, the blot was incubated with supernatant medium from the EE7 clone (diluted fivefold with buffer A) for 18 hr at 4°C. For all further steps, the blot was kept at 4°C except for the incubation with the peroxidase substrate solution. Goat anti-mouse IgG conjugated to horseradish peroxidase was used as second antibody.

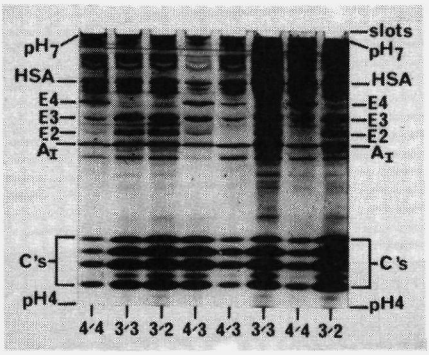

Figure 3. Isoelectric focusing of apoVLDL from eight different serum samples by the conventional method (20). VLDL was isolated by density gradient ultracentrifugation (d < 1.006 g/ml). The pH gradient was 4-7. The gel was stained with Coomassie Brilliant Blue after fixation with trichloroacetic acid and sulfonic acid. The positions of the different apolipoproteins and of residual human serum albumin (HSA) are indicated.

Results

Figure 3 shows isoelectric focusing patterns of various delipidated VLDL samples performed by the conventional method described by Menzel et al. (20). In Figure 4, isoelectric focusing slab gels loaded with various amounts of delipidated serum samples from hyperlipidemic donors are shown. Parallel isoelectric focusing slab gels were used for immunoblotting using either polyclonal (Figure 4A) or monoclonal (Figure 4B) anti-apoE antibodies as first antibody. After immunoblotting and peroxidase staining, the three major apoE isoforms were clearly visualized. With polyclonal anti-apoE antiserum as first antibody (Figure 4A), a minor band appeared between E3 and E4 when higher amounts of delipidated serum were applied to the gel. With monoclonal antibodies (Figure 4B), minor bands with apoE immunoreactive material were only found at the E1 position which are probably sialated and/or deamidated derivatives of the major apoE isoforms.

Figure 5 shows the immunoblot of the apoE isoelectric focusing patterns of 13 different hyperlipidemic serum samples using monoclonal antibodies as first antibody. These serum samples had been stored at -20° for more than 1 year.

Figure 6 shows apoE isoelectric focusing patterns of 20 different fresh hyperlipidemic serum samples using polyclonal antibodies as first antibody. It is obvious from Figure 6 that the dissociation of apoE from lipoprotein lipids by incubation of the serum samples in 0.5 M guanidine-HCl (Figure 6B) leads to apoE isoelectric focusing patterns similar to those obtained after delipidation of the serum samples with chloroform-methanol (Figure 6A). Incubation of the serum samples in 0.5 M guanidine-HCl instead of delipidation with chloroform-methanol required much less extensive sample manipulation.

Interpretation of one-dimensional isoelectric focusing pattern of apoE may be confounded by a partial sialation or deamidation of apoE isoforms. In order to validate our method relating to the possible confounding effects of sialation and/or

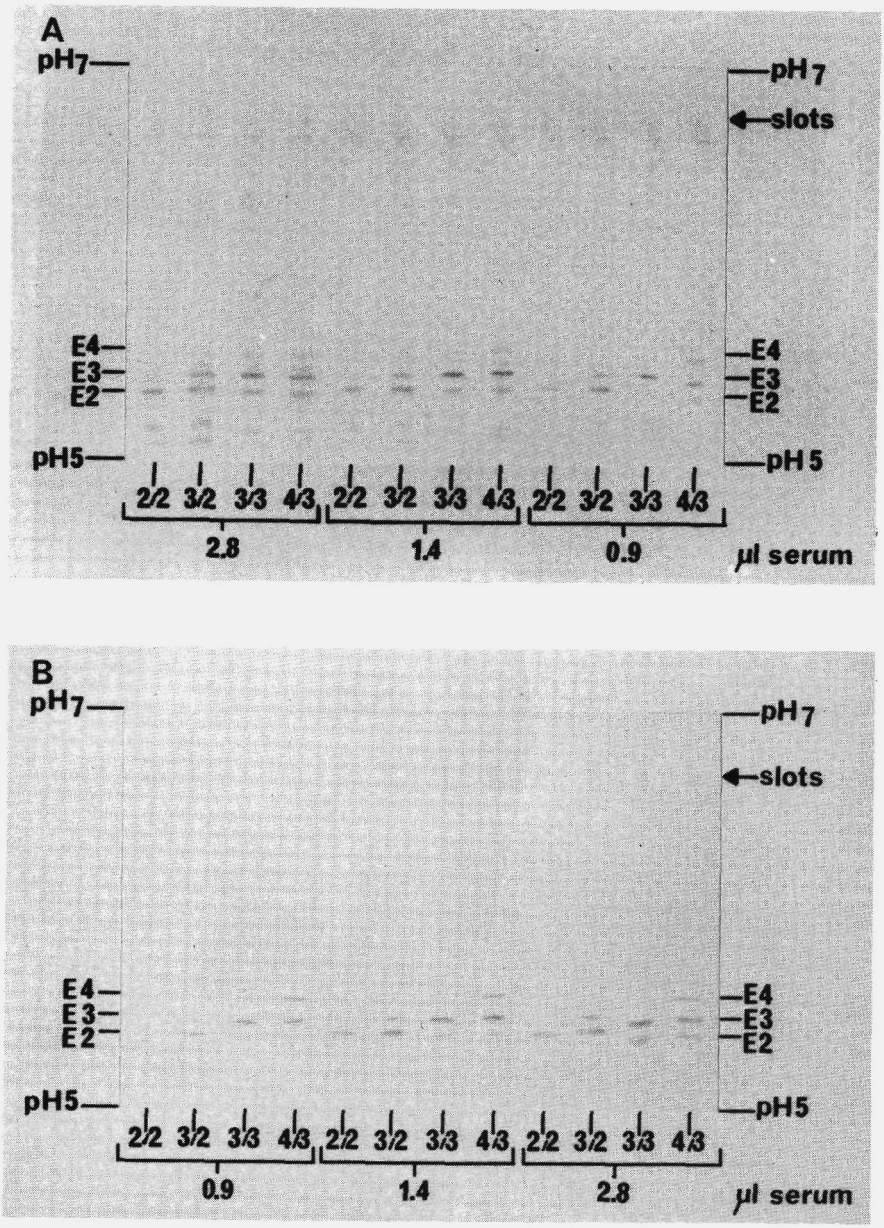

Figure 4. Isoelectric focusing slab gels applied in parallel with increasing amounts of four different chloroform-methanol-delipidated serum samples. After delipidation, the protein pellet was dissolved in the Tris-HCl buffer (Materials and Methods) and diluted appropriately in order to apply the amount of serum as indicated. Serum samples had been stored at -20°C for more than 1 year. Gels were developed by immunoblotting using either polyclonal (A) or monoclonal (B) anti-apoE antibodies. ApoE phenotypes as indicated in the figure were determined with the conventional method for apoE phenotyping using isoelectric focusing of delipidated VLDL followed by protein staining immediately after collecting the blood.

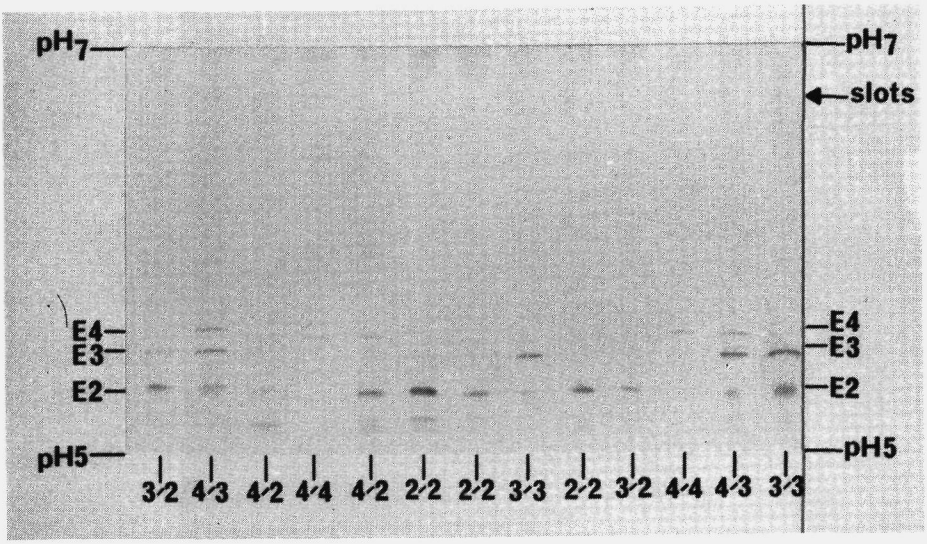

Figure 5. ApoE immunoblot of an isoelectric focusing slab gel using monoclonal antibodies. Thirteen different serum samples that had been stored at -20°C for more than 1 year were applied. ApoE phenotypes as indicated in the figure were determined with the conventional method for apoE phenotyping using isoelectric focusing of delipidated VLDL followed by protein staining immediately after collecting the blood.

deamidation, we have adapted our method to split-sample analysis with and without cysteamine treatment. After cysteamine treatment the major part of E2 and E3 focus at the position of E4 (Figure 7), suggesting that at least the major parts of E3 and E2 do not represent monosialated or deamidated derivatives of E4 and E3, respectively. Fifty plasma samples (phenotype frequency: 21, 26, 3 for E3/E3, E3/E2 and E2/E2, respectively) were analyzed with and without cysteamine treatment. For 49 plasma samples, the results obtained after cysteamine treatment were in accordance with the results obtained without cysteamine treatment. However, for one E3/E2 plasma sample the apoE isoelectric focusing pattern was classified as E4/E3 instead of E4/E4 after cysteamine treatment (Figure 7). This would suggest that in this sample a major part of E3 is monosialated or deamidated and focuses therefore at the position of E2 when analyzed without cysteamine treatment, and thus the sample would be falsely scored as E3/E2. However, DNA hybridization using synthetic oligonucleotides showed that this sample is an E3/E2 ($Lys_{146} \rightarrow Gln$) phenotype (M. Smit et al., unpublished results). E2 ($Lys_{146} \rightarrow Gln$) contains only one cys residue and will focus therefore at the position of E3 after cysteamine treatment.

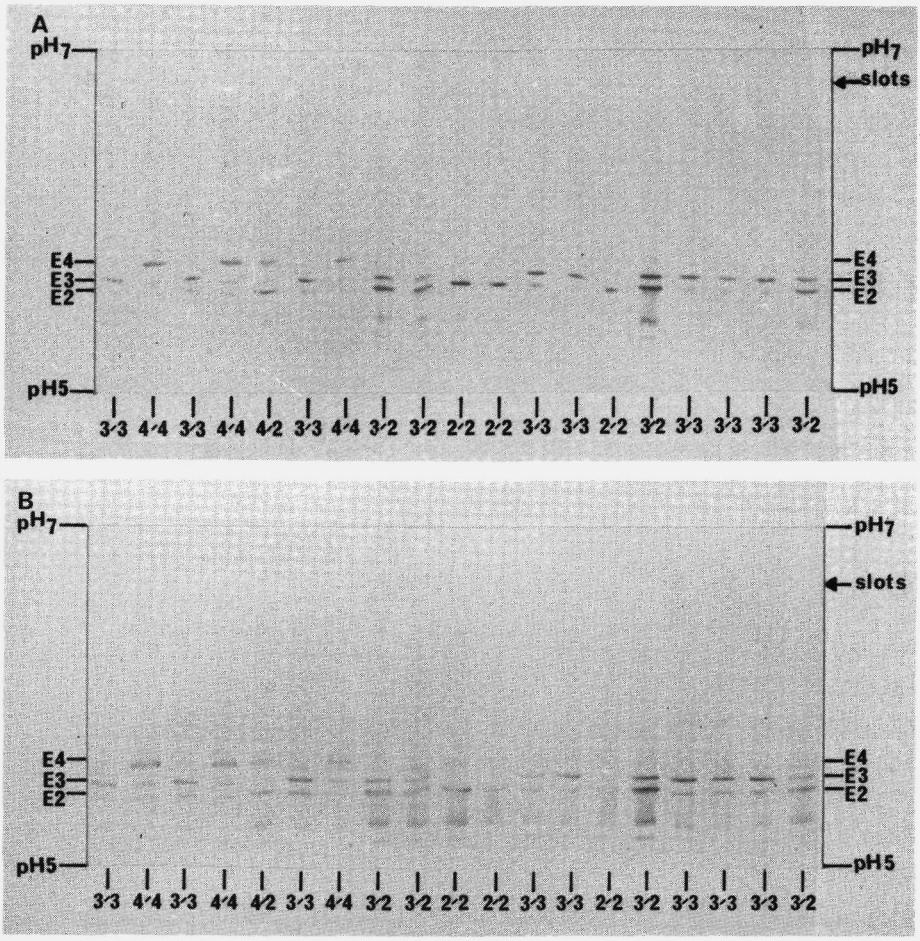

Figure 6. ApoE immunoblot of isoelectric focusing slab gels applied in parallel with 20 different serum samples that had been delipidated either with chloroform-methanol (A) or by incubation in 0.5 M guanidine-HCl (B). Polyclonal anti-apoE antibodies were used as first antibody. The serum samples had been stored at -20°C for less than 2 weeks.

Discussion

In order to prepare immunoblots, isoelectric focusing was carried out with slab gels. Several slab gel systems could be used. In our laboratory, the vertical slab gel system as used by Menzel et al. (20) (Figure 3) did not result in sharp isoelectric focusing patterns of apoE after immunoblotting. This is probably due to capillary leakage of the sample between the gel and the two glass plates. Capillary leakage of the sample is easily visualized by the highly sensitive immunoblotting technique.

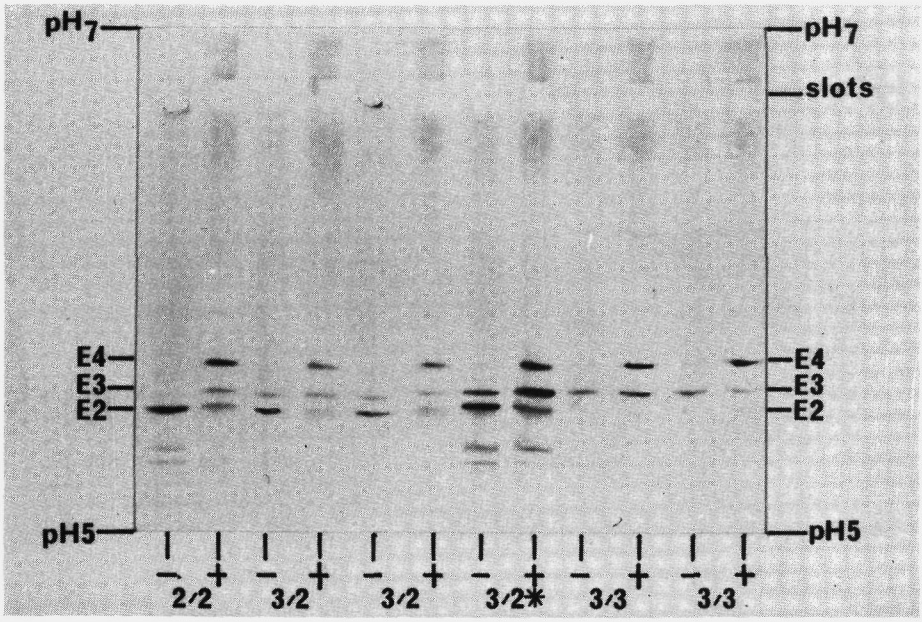

Figure 7. ApoE immunoblot of isoelectric focusing slab gels of 6 of 50 different normolipidemic plasma samples with (+) and without (-) cysteamine treatment. After treatment the samples were delipidated with chloroform-methanol. Polyclonal anti-apoE antibodies were used as first antibody. The samples had been stored for 4 to 5 months at -20°C. *, Plasma sample with E3/E2 phenotype. DNA hybridization techniques using synthetic oligonucleotides revealed that this plasma contained E2 (Lys_{146} → Gln) (M. Smit et al., unpublished results).

In the horizontal slab gel system as schematically drawn in Figs. 1 and 2, capillary leakage between the gel and the lower perspex plate cannot occur.

However, a general disadvantage of horizontal slab gel systems is either condensation of water on the gel surface or drying of the gel during electrophoresis, depending on the relative humidity in the electrophoresis box. To prevent this phenomenon, we covered the gel with a perspex plate. This plate was perforated at the position of the slots in order to be able to fill the slots afterwards. In our experiments the sharp isoelectric focusing bands were obtained when the slots remained completely filled during the electrophoresis. Therefore, after application of the samples to the slots via the perforations, the perforations of the perspex plate were covered with a plastic sheet preventing diffusion of the sample solvent into the gel.

Our results show that an additional minor band was obtained between E3 and E4 when relatively high amounts of serum were applied to the gel. It is our experience that this occurs only when old serum samples were applied, together with the use

of a polyclonal anti-apoE antiserum instead of a monoclonal antibody as first antiserum (compare Figure 4A with Figs. 4B and 5). With fresh serum samples this additional minor band did not appear (Figure 6A). This suggests that this minor band between E3 and E4 is due to proteolysis during storage. The two minor bands at the E1 position were obtained with both monoclonal and polyclonal antibodies and their appearance is independent of the duration of the sample storage. These E1 bands were also obtained when apo-VLDL was used with this immunoblot method. Since these bands partly disappeared after treatment of VLDL with neuraminidase, we strongly suggest that the presence of apoE immunoreactive material at the E1 position is due to sialation and deamidation of the major apoE isoforms. Also, with the conventional method of isoelectric focusing of apoVLDL followed by protein staining, minor bands at the E1 position are commonly found. In addition to sialated apoE isoforms that focus at position E1, mono- or di-sialated apoE4 and E3 isoforms exist and may confound the apoE phenotyping. For instance, homozygotes E4/E4 may falsely be scored as heterozygotes E4/E3 or E4/E2 and homozygotes E3/E3 may be classified as heterozygotes E3/E2. This problem of sialation arises in all one-dimensional apoE focusing methods and therefore, in this respect, the present method is as reliable as the conventional method. Usually, sialation of apoE isoforms occurs only at a minor portion, implying that a phenotype has to be classified as homozygote when the most basic band is clearly stronger than the more acidic bands. Reciprocally, when the most basic band is weaker than the more acidic one, the respective phenotype is scored as heterozygote (see Figs. 4-6). Only in those cases where two isoforms are about equally strong, special attention must be paid in order to discriminate correctly between homozygote and heterozygote. With the present method, the problems of discriminating between homozygote and heterozygote in those particular cases are mostly solved by applying smaller amounts of sample (compare in Figure 4A, phenotype 3/2 with 0.9 and 2.8 μl serum, respectively). Only with the very few samples where this is not the case, should phenotyping be repeated by a method of commonly accepted reliability such as the two-dimensional electrophoresis (4) or the split-sample cysteamine treatment technique (21). We found that the present method of immunoblotting can easily be adapted for analysis of serum samples that have been treated with cysteamine. We analyzed the apoE by isoelectric focusing in 50 plasma samples both in the presence and absence of cysteamine. The apoE phenotypes determined in the plasma samples without cysteamine treatment were in accordance with the results obtained with the respective cysteamine-treated plasma samples. This confirms the reliability of the present method.

All serum samples used in Figs. 4 and 5 had been stored at -20°C for more than

1 year. The phenotypes indicated in these figures are deduced from apoE phenotyping performed with the conventional method immediately after collecting the serum samples. Presently, we have determined the apoE phenotype with this immunoblot method in about 200 serum samples that had been stored at -20°C for 1 to 4 years and have compared the results with those obtained with the conventional method immediately after collecting the serum samples. Thus far, no false scores have been obtained. Thus, the method can safely be used with old serum samples; however, because of the possible appearance of some minor bands upon storage, we prefer the use of fresh serum samples, especially when polyclonal antiserum is used.

Although all results shown in this paper were obtained with serum samples collected from lipid clinic outpatients, the present method is also suitable for EDTA-plasma samples from either hyper- or normolipidemic subjects. With normolipidemic plasma samples we usually apply twice as high amounts of sample to the gel. With this method we have performed apoE phenotyping in plasma samples of 2000 apparently healthy and randomly selected 35-year-old men (M. Smit et al., unpublished results). We found that in this Dutch population the distribution of the apoE phenotypes was not significantly different from the expected Hardy-Weinberg distribution.

Taking these considerations of validation into account, we would like to conclude that the present method is of acceptable reliability.

In the present method, the delipidation of the serum samples with chloroform-methanol is the most laborious step. Therefore, we attempted to find a rapid and convenient method to dissociate apoE from lipoprotein lipids without the procedure of delipidation. Guided by the finding that exposure of human high density lipoproteins (HDL) to guanidine-HCl results in an irreversible dissociation of apolipoprotein A-I from HDL-lipid (22,23), we treated serum with 0.5 M guanidine-HCl instead of delipidation with chloroform-methanol. Although some background staining was obtained, our results show that pretreatment of serum with 0.5 M guanidine-HCl offers a suitable and time-saving alternative to the conventional delipidation step (compare Figure 6A with 6B).

We conclude that the present method of isoelectric focusing of delipidated or guanidine-HCl-treated serum or plasma, followed by immunoblotting using anti-apoE antibodies as first antibody, is a valid method for apoE phenotyping. When compared with the conventional method of isoelectric focusing of delipidated VLDL followed by protein staining, the present method offers the following advantages: i) the apoE isoelectric focusing patterns are easy to score since residual non-apoE proteins are not visualized (compare Figure 3 with Figs. 4-7); ii) it is less laborious

because an ultracentrifugation step to isolate VLDL is not needed; iii) it is suitable for large scale diagnosis and population studies even in less well-equipped laboratories; iv) it needs only a few microliters of serum or plasma that may have been stored for long periods of time; and v) because of the high sensitivity, minor apoE isoforms can easily be detected in serum or serum density fractions.

ACKNOWLEDGEMENTS

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CHAPTER 3

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 of America (USA). 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 USA). 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 apoB and E concentrations showed that the E*4 allele is associated with elevated plasma cholesterol and apoB levels and with decreased apoE 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 apoB levels is 6.8% and 14.2%, respectively, of the total population mean. The total average allelic effect on plasma apoE concentrations was more pronounced (50.1%), suggesting that the APOE alleles primarily affect apoE concentrations rather than plasma cholesterol and apoB levels. This hypothesis is sustained 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 is relatively low as compared with that reported for other population samples.

Introduction

The apolipoprotein E (apoE) 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 apoE can be separated by isoelectric focusing into three major isoforms, E2, E3, and E4, which differ in pI by a single charge unit, apoE4 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). ApoE3 is the most commonly occurring, or wild-type, form. ApoE4 is derived from E3 by a Cys→Arg substitution at position 112 and is designated E4(Cys112→Arg). ApoE2 is derived from E3 by an Arg→Cys substitution at position 158 and is designated E2(Arg158→Cys). Up till now a number of very rare mutants of apoE have been described. Some variants are either more basic than apoE4 or more acidic than apoE2 while others have the same electric charge as E2 or E3 (Rall et al. 1982, 1983; Havel et al. 1983; Innerarity et al. 1984; Yamamura et al. 1984a, b; Ghiselli et al. 1984; Havekes et al. 1986).

Several population studies on apoE polymorphism have been reported (Utermann et al. 1979, 1984a; Wardell et al. 1982; Menzel et al. 1983; Robertson and Cumming 1985; Ehnholm et al. 1986; Eto et al. 1986b; Utermann 1987; 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 apoE 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, triglycerides, apoB, and apoE 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 plasma was obtained by venipuncture and stored at -20°C until the assays were performed.

ApoE phenotyping ApoE 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-apoE antiserum as first antibodies.

Determination of plasma cholesterol, triglyceride, apoB, and apoE levels Plasma cholesterol and triglycerides were measured enzymatically using Boehringer test-kits (cholesterol CHOD-PAP and triglyceride GPO-PAP, respectively). ApoB concentrations were measured by immunonephelometric assay (INA) as described by Rosseneu et al. (1981). Plasma apoE 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 χ^2 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 apoB and apoE concentrations and the variance of these parameters attributable to genotypic differences were estimated exactly according to the method of Sing and Davignon (1985).

Table 1. apoE phenotype and allele frequencies in randomly selected 35-year-old males.

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	

χ^2 Hardy-Weinberg distribution is 2.82 (df=5).

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 APOE 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 χ^2 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 USA ($P < 0.005$). No significant differences were found with the German populations and the 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, measured 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 apoE 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 frequency 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,

Table 2. APOE gene frequencies in several random population samples.

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	NS	-	-	This study
Scotland	400	0.083	0.770	0.145	3.70	NS	2.31	NS	Cumming and Robertson (1984)
FRG (Munster)	1000	0.078	0.783	0.139	7.15	NS	8.71	NS	Menzel et al. (1983)
FRG (Marburg)	1031	0.077	0.773	0.150	7.24	NS	3.74	NS	Utermann et al. (1984a)
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	NS	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	NS	50.05	<0.001	Eto et al. (1986a)
Japan	319	0.081	0.849	0.067	3.51	NS	37.41	<0.001	Utermann et al. (1987)

χ^2 Values at df=2 and P values of 0.01 and 0.001 are 9.21 and 13.95, respectively.
NS, not significant (P>0.05).

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.

ApoE allele	Population (reference)				
	Japan [A]		New Zealand	Finland	USA
	(Eto et al. 1986b)	(Utermann 1987)	(Wardell et al. 1982)	(Ehnholm et al. 1986)	(Ordovas et al. 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; χ^2 =22.0; P<0.001).

Table 4. Mean plasma cholesterol, triglyceride, apoB, and apoE levels (in mg/dl) among the 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	NS	NS
	(90.9)	(71.8)	(98.2)	(110.6)	(85.0)	(98.9)	(75.1)		
ApoB	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)		
ApoE	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.
NS, not significant (P>0.05).

triglycerides, and apoB and E were assayed. Table 4 presents the mean plasma cholesterol, triglyceride, apoB, and apoE 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, apoB, and apoE 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-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 non-parametric 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, apoB, and apoE 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 apoB 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 apoE levels is the opposite to that on plasma cholesterol and apoB. The E*4 allele leads to decreased apoE levels, whereas the E*2 allele is strongly associated with an increased apoE concentration. When the mean apoB and apoE levels calculated for each phenotype group were considered separately, a strong inverse relationship was observed between apoB and apoE (Figure 1).

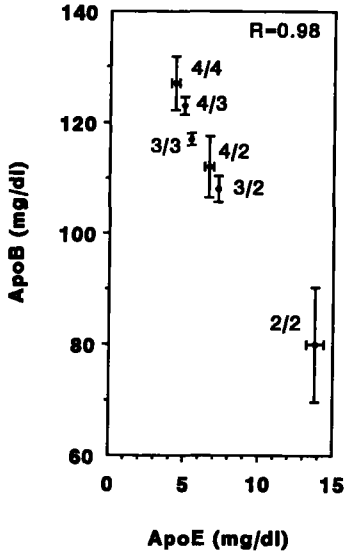

Figure 1. Inverse relationship between mean apoB and apoE levels calculated in each phenotype group. Vertical and horizontal bars represent ± SEM for apoB and apoE, respectively.

We calculated the average effects of the three APOE alleles on the lipid and apolipoprotein levels (Table 6) according to

Table 5. In pairs statistical analyses [A] of the differences in mean cholesterol, triglyceride, apoB, and apoE levels between the different apoE phenotype groups.

Different from	Cholesterol						ApoB						ApoE					
	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	204.4	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).

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 apoB (-11.4 mg/dl) and to raise apoE 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 apoB is more pronounced than the relative effect on total plasma cholesterol levels, whereas the effect on plasma apoE levels is most dramatic and the opposite to that on plasma cholesterol and apoB.

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

APOE allele	Average effect ^					
	Cholesterol		ApoB		ApoE	
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)

^ Values in parenthesis represent the average effects expressed as percentages of the population means.

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 apoE levels. The effects of the genetic variation associated with the APOE gene on the variability of total cholesterol and plasma apoB levels are much less pronounced (1.4% and 2.3%, respectively).

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
ApoB	2.3	1399
ApoE	18.1	5.7

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 representative for the whole Dutch population. This close similarity in phenotype 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 densely 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 USA. 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 USA), 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 USA 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 USA 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 t al. 1986; Eto et al. 1986b; Utermann 1987) have shown an association of the E*4 allele with elevated plasma cholesterol and apoB levels, whereas the E*2 allele appeared to be associated with decreased levels of plasma cholesterol and apoB. Reciprocally the E*4 allele is associated with a reduced plasma apoE level, whereas the E*2 allele leads to a highly significant increase in plasma apoE concentration. This effect of allelic substitution at the APOE locus on plasma cholesterol, apoB, and apoE 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 apoE2 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 apoE concentrations and thus that the metabolism of apoE-containing lipoproteins thereby regulates the LDL cholesterol and apoB 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 contribution of the genetic variance associated with the APOE locus to the total phenotypic variance of plasma cholesterol and apoB 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 apoB and apoE values outside the range of the respective mean ± 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 apoB.

Consequently, we concluded that the total genetic variance of cholesterol and apoB 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, apoB, and apoE levels, a simple relationship between apoE 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 contribution of the polymorphic APOE gene locus to the plasma cholesterol, apoB, and apoE levels.

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CHAPTER 4

The Effect of Apolipoprotein E Phenotype on Plasma Lipids is Not Influenced By Environmental Variability. Results of a Dutch Twin Study

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Summary

The present study describes the phenotype distributions and allele frequencies of the APOE gene and their influence on plasma lipid and (apo)lipoprotein levels in 160 Dutch twin families. The apoE polymorphism explained 4.5 % of the inter-individual variability of adjusted plasma cholesterol levels in the parents and 7 % in the twins. Even stronger influences were found on LDL-cholesterol (5.9, 13.0 and 10.8 %; for parents, oldest twins and youngest twins, respectively), apoB (11.1, 18.1 and 14.0 %, respectively) and apoE levels (15.8, 32.4 and 28.2 %, respectively). There was no gender difference with respect to the APOE allele frequencies and their influence on the measured parameters.

In the group of monozygotic (MZ) twins, the within-pair difference of the measured lipoprotein parameters were similar in the different apoE phenotypes classes, which indicates that the effect of the apoE polymorphism is not influenced by the environmental variability between the MZ pair-members. This identifies the APOE gene as a "level" gene and supports the concept that variability in the APOE gene is a major independent genetic factor influencing plasma lipid and (apo)lipoprotein levels.

Introduction

Apolipoprotein E (apoE) is one of the major protein constituents of chylomicron and very low density lipoprotein (VLDL) remnants. It plays a central role in the receptor mediated uptake of these particles by acting as a high affinity ligand for hepatic lipoprotein receptors (Sherril et al. 1980; Weisgraber et al. 1982). ApoE also plays a role in the conversion of VLDL via intermediate density lipoproteins (IDL) into low density lipoproteins (LDL) (Demant et al. 1991). Human apoE can be separated by isoelectric focusing into three major isoforms, E2, E3 and E4, which each differ in pI by a single charge unit, apoE4 being the most basic and E2 the most acidic form. These isoforms are encoded for by three codominant alleles, E*2, E*3 and E*4, at a single APOE gene locus on chromosome 19 (Zannis and Breslow 1981; Scott et al. 1985). ApoE3 is the most common, or wildtype isoform. ApoE4 differs from apoE3 by an arginine for cysteine substitution at amino acid residue 112 [designated as apoE4(Cys112 → Arg)], whereas apoE2 differs from apoE3 by a cysteine for arginine substitution at residue 158 [apoE2(Arg158 → Cys)]. Various population studies have demonstrated an influence of the apoE polymorphism on plasma lipid and (apo)lipoprotein levels (reviewed by Davignon et al. 1988). In these studies the APOE*2 allele is associated with lower levels of

total plasma cholesterol, LDL-cholesterol and apoB, whereas for the APOE*4 allele the opposite holds true. The apoE polymorphism was found to explain between 1.4 and 8.7 % of the interindividual variability of plasma cholesterol (Smit et al. 1988; Boerwinkle and Utermann 1988).

By means of twin studies it is possible to study the influence of genetic and environmental factors on lipoprotein parameters against a genetically homogeneous background in the case of monozygous (MZ) twins (Magnus et al. 1981; Berg 1984; Berg 1987; Berg 1990). As was first proposed by Magnus et al. (1981), the difference in a measured level between the two members of an MZ pair reflects variation in environmental factors exclusively since they share the same nuclear genes. If there are significant within-pair differences in the different genotypes or phenotypes of a single gene, then this shows that there is an interaction between this gene and environmental factors. Genes acting as such are called "variability" genes to distinguish them from "level" genes which do not exhibit a gene-environment interaction. This strongly suggests that these "level" genes have a direct effect on the measured parameters, which is not influenced by environmental variability. In the present study we used the apoE polymorphism as a genetic marker and report on the distribution of the various apoE phenotypes and their influences on plasma lipids in 160 Dutch twin families. We observed that the effect of the apoE polymorphism on lipoprotein parameters is not influenced by the variability in environmental factors. This identifies the APOE gene as a "level" gene that has an independent role in the lipoprotein metabolism.

Materials and Methods

Population description and sample collection The individuals described in this article were apparently healthy, and belong to a cohort of 160 nuclear twin families. All twins were still living with their parents. The addresses of the families were obtained from the various City Council population registries. Families were only included in this study if both parents and twins were willing to cooperate. There were 35 families with MZ boys, 35 with MZ girls, 31 with dizygous (DZ) boys, 30 with DZ girls and 29 with DZ twins of opposite sex. During the first visit, the mother was asked to tell which of the twins was born first. This person was then further denoted as "oldest" of the twins, the other twin as "youngest".

EDTA blood was obtained between 8:30 and 10:30 AM by venepuncture after overnight fasting. As soon as possible, plasma was separated from the cells by centrifugation for 10 minutes at 3,000 rpm. Part of the plasma was kept at 4°C for lipid determinations within the next 5 days. The remainder was stored in 2.5-ml

aliquots in tubes with tightly fitting screw-caps at -20°C for later use.

Plasma lipid and (apo)lipoprotein analysis Cholesterol and triglyceride levels were determined using enzymatic methods (Boehringer, Mannheim, FRG, CHOD-PAP kit no. 236691 and GPO-PAP kit no. 701904). HDL-cholesterol was measured after precipitation of VLDL, IDL and LDL according to Lopes-Virella et al. (1977). LDL-cholesterol was subsequently calculated using the formula of Friedewald et al. (1972).

Apolipoproteins A1, A2 and B were quantified by radial immunodiffusion as described by Albers et al. (1981) and Havekes et al. (1981), respectively. ApoE was quantified by enzyme-linked-immunosorbent assays (ELISA) as described by Bury et al. (1986).

Apolipoprotein E phenotyping The apoE phenotyping was performed using a rapid micromethod which is based on isoelectric focusing (pH 5-7) of delipidated plasma samples, followed by immunoblotting on nitrocellulose filter using a polyclonal anti-apoE antiserum as previously described (Havekes et al. 1987).

Statistical analysis The apoE phenotype frequencies and the APOE allele frequencies were determined in the complete set of 160 families with the exception of 2 missing fathers. For the parents and monozygotic twins the calculation of the allele frequencies was performed by gene counting procedures. For the dizygotic twins the allele frequencies were estimated according to Martin (1975) using the sib-genotype frequencies of Smith and Penrose (1955). A complete set of lipid measurements was obtained in 155 families only, 5 families of which some data were missing, were excluded for further analysis. Before analyzing the effect of the apoE polymorphism on the plasma lipid levels, three apoE phenotype groups were formed: an E2+ group including individuals with the E2E2 and E3E2 phenotypes, an E3E3 phenotype group, and an E4+ group including individuals with the E4E3 and E4E4 phenotypes. All 10 parents and 4 children with the apoE4E2 phenotype were excluded from these analysis because of opposing effects of the individual alleles. The effect of the apoE polymorphism was estimated by means of two-way analysis of covariance (ANCOVA), with apoE phenotype and gender as factors, and with age, weight, height and body mass index (BMI = weight in kg/height in m^2) as covariables, entered only if they were significantly correlated ($P < 0.05$) with the parameter studied. The proportion of variability explained by the apoE polymorphism (i.e., the R^2) was taken to be a measure of the impact of the effect. The R^2 was calculated as the sum of squares due to the polymorphism divided by the covariate-adjusted total sum of squares (sum of squares total less the covariate sum of squares). The differences in within-pair levels in the MZ twins was estimated by two-way analysis of variance (ANOVA) with sex and apoE phenotype

entered as factors without prior adjustment. ANOVA and ANCOVA procedures were performed using the programs contained in the statistical package NCSS, version 5.1 (Dr J.L. Hinze, Kaysville, Utah, U.S.A.).

Results

Apolipoprotein E phenotype distribution The apoE phenotype numbers and frequencies for the complete set of parents and twins are presented in Table 1. The observed apoE frequencies in all groups considered: parents, monozygotic- and dizygotic twins were in genetic equilibrium (results not shown). The observed allele frequencies in these three groups do not differ significantly from those found in a larger Dutch population (Smit et al. 1988). There were no significant gender differences with respect to the apoE phenotype distribution in the three groups considered (results not shown).

Table 1. Apolipoprotein E phenotype numbers, relative frequencies (in %, between brackets) and APOE allele frequencies in parents and children of 160 Dutch twin families.

		Twins	
ApoE phenotype	Parents	Monozygotic*	Dizygotic†
E2E2	3 (0.9)	0 (0.0)	1 (0.6)
E3E2	48 (15.0)	13 (18.6)	37 (20.6)
E3E3	185 (57.8)	41 (58.6)	88 (48.9)
E4E2	10 (3.1)	0 (0.0)	4 (2.2)
E4E3	67 (20.9)	15 (21.4)	46 (25.6)
E4E4	5 (1.6)	1 (1.4)	4 (2.2)
Total	318	70	180
Alleles:			
E*2	0.101	0.093	0.103
E*3	0.762	0.786	0.726
E*4	0.137	0.121	0.163

* Each monozygotic twin-pair is counted as one phenotype.
† Each dizygotic twin-pair is counted as two phenotypes, for this group the allele frequencies are calculated from sib-pair phenotype frequencies according to Martin (1975).

Table 2. Adjusted mean levels ± standard errors (sem) of plasma lipids and (apo)lipoproteins for parents and children according to apoE phenotype groups.

	Apolipoprotein E phenotype groups							
	E2+	E3E3	E4+	Combined	Covariates*	*P* sex†	*P* E-feno‡	R^2§
Parents:	*n*=50	*n*=178	*n*=72	*n*=300				
Plasma cholesterol‖	5.35 ± 0.14	5.69 ± 0.08	6.09 ± 0.12	5.71 ± 0.08	H,W	0.036	<0.001	4.5
Plasma triglycerides	1.22 ± 0.08	1.12 ± 0.04	1.22 ± 0.07	1.19 ± 0.05	H,W,B	0.003	NS	0.5
LDL-cholesterol	3.47 ± 0.14	3.92 ± 0.07	4.27 ± 0.11	3.89 ± 0.07	H	0.012	<0.001	5.9
ApoB	90.6 ± 3.02	107.6 ± 1.6	115.9 ± 2.52	104.7 ± 1.5	H,W	0.023	<0.001	11.1
ApoE	9.9 ± 0.3	7.5 ± 0.2	6.7 ± 0.3	8.1 ± 0.2		NS	<0.001	15.8
Oldest twin:	*n*=32	*n*=80	*n*=39	*n*=151				
Plasma cholesterol	3.98 ± 0.13	4.34 ± 0.08	4.63 ± 0.11	4.32 ± 0.08	A	NS	0.002	7.0
Plasma triglycerides	0.71 ± 0.05	0.70 ± 0.03	0.74 ± 0.05	0.71 ± 0.03		NS	NS	0.3
LDL-cholesterol	2.25 ± 0.11	2.74 ± 0.07	3.02 ± 0.10	2.67 ± 0.06		NS	<0.001	13.0
ApoB	68.2 ± 2.7	80.6 ± 1.7	90.2 ± 2.4	79.7 ± 1.7		NS	< 0.001	18.1
ApoE	9.5 ± 0.3	6.5 ± 0.2	5.7 ± 0.3	7.2 ± 0.2		<0.001	< 0.001	32.4
Youngest twin:	*n*=30	*n*=84	*n*=41	*n*=155				
Plasma cholesterol	3.84 ± 0.13	4.23 ± 0.08	4.49 ± 0.11	4.19 ± 0.08	A	NS	0.002	7.2
Plasma triglycerides	0.62 ± 0.05	0.64 ± 0.03	0.78 ± 0.04	0.68 ± 0.03		NS	0.014	5.2
LDL-cholesterol	2.16 ± 0.11	2.67 ± 0.07	2.85 ± 0.10	2.56 ± 0.07		NS	<0.001	10.8
ApoB	66.1 ± 2.8	78.7 ± 1.7	85.7 ± 2.4	76.8 ± 1.6		NS	<0.001	14.0
ApoE	9.2 ± 0.3	6.4 ± 0.2	5.5 ± 0.3	7.0 ± 0.2		0.043	< 0.001	28.2

* Indicating which covariates contributed significantly to the respective variables; abbreviations used are: A, age; H, body height; W, body weight; B, body mass index.
† *P* value indicating the significance of the difference in mean values for two sexes calculated by two-way ANCOVA.
‡ *P* value indicating the significance of the difference between the apoE phenotype group means calculated by two-way ANCOVA.
§ R^2 indicating the relative contribution (in %) of the apoE polymorphism to the total variance estimated from the two-way ANCOVA.
‖ All levels are expressed in mmol/l except for the apoB- and apoE levels which are expressed in mg/100 ml.
NS, not significant ($P>0.05$)

Influence of apoE polymorphism on plasma lipid and (apo)lipoprotein levels We estimated the influence of the apoE polymorphism on the levels of plasma cholesterol, plasma triglycerides, LDL-cholesterol, apoB and apoE by means of two-way ANCOVA with gender and apoE phenotype entered as factor. Age, height, weight and BMI were entered as covariate only when they displayed a significant influence on the respective variable. The adjusted mean levels and standard errors, for the parents, the oldest twins and the youngest twins are presented in Table 2. Only in the group of the parents we found a significant influence of gender on the levels of plasma cholesterol, plasma triglycerides, LDL-cholesterol and apoB. In both twin groups, gender only influenced the plasma apoE levels. We detected highly significant influences of the apoE polymorphism on plasma cholesterol, LDL-cholesterol, apoB and apoE levels in the parents as well as in the two sets of twins (oldest and youngest). In the parents the variability in the APOE gene explained 4.5 % of the total interindividual variation of plasma cholesterol, 5.9 % for LDL-cholesterol, 11.1 % for apoB and 15.8 % for apoE. In the two sets of twins even stronger influences of the apoE phenotype were found, with the highest influences on the plasma apoE level (32.4 and 28.2 %; for oldest and youngest twins respectively). In the three groups (parents, oldest- and youngest twins) there was no significant interaction between gender and apoE polymorphism (results not shown). The apoE polymorphism did not influence the plasma levels of HDL-cholesterol, apoA1 and apoA2 (results not shown)

Table 3. Mean within-pair differences (Δ) and their standard errors (SEM) for the monozygotic twins separated by apoE phenotype.

	ApoE phenotype			
	E3E2 (n=12)	E3E3 (n=39)	E4E3 (n=14)	
	Δ ± SEM	Δ ± SEM	Δ ± SEM	P^*
Plasma cholesterol†	0.45 ± 0.09	0.37 ± 0.05	0.26 ± 0.08	NS
Plasma triglycerides	0.25 ± 0.05	0.12 ± 0.03	0.18 ± 0.04	NS
LDL-cholesterol	0.38 ± 0.08	0.31 ± 0.04	0.26 ± 0.07	NS
ApoB	10.2 ± 2.2	6.6 ± 1.2	5.9 ± 2.0	NS
ApoE	1.1 ± 0.3	1.0 ± 0.1	0.8 ± 0.2	NS

* *P* value indicating the difference between the apoE phenotype groups calculated by means of two-way ANOVA with apoE phenotype and gender entered as variables

† Levels are expressed in mmol/l except for the apoB and apoE levels which are expressed in mg/100 ml. NS, not significant ($P>0.05$).

Influence of apoE polymorphism on within-pair difference in measured parameters in MZ twins The influence of the apoE polymorphism on the plasma lipid levels was further studied by comparing these levels in the two members of a MZ twin pair. For reasons of number of subjects we only considered the MZ twin-pairs with the E3E2, E3E3 or E4E3 phenotypes. We could not detect significant within-pair differences between the apoE phenotype groups in any of the measured parameters as evaluated by two-way ANOVA (Table 3). In addition, there was (i) no significant influence of gender on the within-pair differences, and (ii) there was no significant interaction between apoE phenotype and gender (results not shown). This indicates that in the MZ twins, the apoE phenotype did not influence the within-pair differences in the reported parameters.

Discussion

In the present study we describe the apoE phenotype distribution and APOE allele frequencies for the parents, the monozygotic- and dizygotic twins from 160 Dutch twin families. The apoE phenotype distribution and APOE allele frequencies in either of the three groups were comparable with the values reported previously for a large Dutch population (Smit et al. 1988).

In various studies the apoE polymorphism has been reported to influence plasma lipid and (apo)lipoprotein levels, although to a variable extent (Davignon et al. 1988). The APOE*4 allele was found to be associated with increased levels of total plasma cholesterol, LDL-cholesterol, apoB and recently also with increased levels of Lp(a) (de Knijff et al. 1991), whereas the opposite holds true for the APOE*2 allele. Conversely, in the APOE*4 allele carriers plasma apoE levels were decreased, whereas the apoE levels were increased in the APOE*2 allele carriers. The mechanism behind the effect of the apoE polymorphism on plasma (apo)lipoprotein levels is commonly assumed to be the result of the influence of the apoE polymorphism on the efficiency of chylomicron- and VLDL-remnant catabolism (Utermann 1985; Weintraub et al. 1987; Demant et al. 1991).

The effects measured in this study are markedly higher than those found previously in a much larger Dutch population (Smit et al. 1988). This is most probably due to the fact that in the present study we were able to adjust the measured variables for inter-individual variability in age, weight, height and body mass index, thereby reducing the overall variance, which was not possible in our former study (Smit et al. 1988). The values in the present study are in the same order of magnitude as found by others (reviewed in Davignon et al. 1988).

In none of the three groups considered, parents, oldest twins and youngest twins,

we could detect a gender difference with respect to the influence of the apoE polymorphism on (apo)lipoprotein levels (results not shown). Such a difference was recently reported in a population of adult males and females selected for health (Xhignesse et al. 1991). Our results confirm those of others (Sing and Davignon 1985; Ordovas et al. 1987; Eichner et al. 1990) also reporting the lack of a APOE gene-gender interaction. The reason for this discrepancy is unknown.

The within-pair differences in the group of MZ twins were not significantly different between the three apoE phenotype groups (Table 3). This indicates that the APOE gene is a "level" gene according to the definitions of Berg and coworkers. Recently, Hallman et al. (1991) published the results of a study in which the influence of the apoE polymorphism on plasma cholesterol levels was calculated in nine different populations. They concluded that, although there are marked differences in apoE phenotype frequencies and dietary habits between the various populations, the APOE gene polymorphism influences the plasma cholesterol levels in a uniform way. This indicates that the APOE gene influences the plasma cholesterol levels independently of other genetical and environmental factors. Recent studies revealed however, that there are at least two genes, showing a genetical variability, which can interact with the APOE gene. In two studies, Pedersen and Berg (1989 and 1990) reported an interaction between the *Pvu*II restriction fragment length polymorphism (RFLP) at the low density lipoprotein receptor (LDLR) locus and apoE in two Norwegian populations. They found that the increased plasma cholesterol and LDL-cholesterol levels associated with apoE4 are reduced in those individuals with the *Pvu*II-A1 allele by a sofar unknown mechanism. Conversely, in a Finish population, the raised LDL-cholesterol levels in individuals with apoE4 are further increased by the X*2 allele of the *Xba*1 RFLP in the APOB gene (Aalto-Setälä et al. 1988; Miettinen 1991). Since we did not study these DNA-RFLP's, it is unknown to what extent these factors may affect the influence of the apoE polymorphism in our study or may explain part of the variation in the intra-pair differences in lipid levels of the MZ-twins.

In summary, the present study confirms the view that the apoE polymorphism is one of the major genetic polymorphisms, explaining a substantial amount of the inter-individual variation of plasma lipid and (apo)lipoprotein levels. In addition, we have provided some evidence that the APOE gene acts as a "level" gene, which further supports the hypothesis that the apoE polymorphism plays an independent role in determining plasma cholesterol levels.

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CHAPTER 5

The Apolipoprotein E Polymorphism Affects Plasma Levels of Lipoprotein (a)

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Summary

In a group of 303 healthy Caucasian adults of both sexes we studied the influence of the apolipoprotein E (apoE) polymorphism on plasma levels of Lipoprotein (a) [Lp(a)]. The APOE*2 allele was found to decrease the mean plasma Lp(a) level by 24.8 %, whereas the APOE*4 allele increased the mean Lp(a) level by 25.7 %. These effects were parallel to the effect of apoE polymorphism on plasma cholesterol and low density lipoprotein (LDL)-cholesterol. For the Lp(a) levels, the genetic variance associated with the APOE locus contributed about 4% to the total phenotypic variance. For plasma cholesterol and LDL-cholesterol this contribution was 4.5 and 6.3%, respectively. We also found a significant positive correlation between LDL-cholesterol and Lp(a) levels. Since the apoE polymorphism effects LDL-receptor activity, we conclude that, at least in healthy normolipidemic individuals, plasma levels of Lp(a) are modulated by the LDL-receptor activity.

Introduction

Apolipoprotein E (apoE) plays a central role in the metabolism of cholesterol and triglycerides. It is one of the major protein constituents of chylomicron- and very low density lipoprotein (VLDL)-remnants, and serves as a ligand for the receptor-mediated endocytosis of these particles by the liver (1). ApoE is genetically polymorphic, showing three common isoforms, E2, E3 and E4, on isoelectric focusing and encoded by 3 different alleles, E*4, E*3 and E*2, at a single APOE gene locus on chromosome 19 (2,3). Population studies demonstrated that the apoE polymorphism affects plasma lipid and lipoprotein levels (4-7). The APOE*2 allele was found to exert a lowering effect on the levels of total cholesterol and low density lipoprotein (LDL)-cholesterol, whereas the APOE*4 allele showed the opposite effect.

Lipoprotein (a) [Lp(a)] was first described in 1963 by Berg as a distinct form of LDL (8). For detailed information about Lp(a) see reviews (9,10). It is now well established that Lp(a) represents an LDL-particle in which the apolipoprotein B (apoB)-100 molecule is covalently linked by a disulphide bridge to apolipoprotein (a) [apo(a)] (11). Plasma levels of Lp(a) in healthy Caucasian individuals vary between 0.01 and 100 mg/100 ml. Various studies have demonstrated that plasma levels of Lp(a) exceeding 20-30 mg/100 ml are positively associated with the development of premature atherosclerosis (12-14). Several distinct isoforms of apo(a) can be distinguished (15,16). These isoforms differ in size and are encoded by different alleles at a single APO(A) gene locus on chromosome 6 (17). This size

polymorphism is directly correlated with the number of kringle 4 domains in apo(a) and inversely correlated with plasma levels of Lp(a) (18,19). Although the size polymorphism of Lp(a) was found to explain 42 % of the variability in its plasma levels (20), the precise mechanisms responsible for this regulation remain unknown.

There are conflicting reports whether or not Lp(a) is cleared by the LDL-receptor. The involvement of the LDL-receptor in Lp(a) metabolism has been suggested by several groups (21-23, 26). However, other groups have disputed the importance of the LDL-receptor in the catabolism of Lp(a) (24,25,27,28).

The influence of the apoE polymorphism on plasma lipid and lipoprotein levels is thought to be due to its effect on the LDL-receptor activity in the liver. A possible influence of the apoE polymorphism on plasma Lp(a) levels would therefore support the hypothesis that the LDL-receptor is involved in Lp(a) catabolism. The data presented show that Lp(a) levels are indeed influenced by the apoE polymorphism in parallel to that of LDL-cholesterol levels.

Materials and Methods

EDTA blood samples were obtained from 303, randomly selected, healthy Dutch individuals (151 males, mean age 48.2 years and 152 females, mean age 45.7 years) after overnight fasting. Plasma was immediately separated from the cells and stored either at 4°C or -20°C until further use. Cholesterol concentrations were measured enzymatically using a Boehringer test kit (CHOD-PAP). LDL-cholesterol was calculated using the Friedewald formula (29). Lp(a) levels were measured using a bi-site "sandwich" ELISA, with rabbit anti-human Lp(a) (Behringwerke, Marburg, Germany) as a catching antibody and rabbit anti-human apoB (raised in our institute) conjugated to horse-radish peroxidase (EC 1.11.1.7) as a detecting antibody. The Immuno reference serum (Immuno AG, Vienna, Austria) was used as the standard in this procedure. LDL and plasminogen are not detected with this assay. The working range of the assay is 0.1 to 3 μg per ml. Intra- and inter-assay variation coefficients are 4.5 and 8.5%, respectively.

The apoE phenotype was determined by isoelectric focusing of delipidated plasma samples followed by immunoblotting using a polyclonal goat anti-human apoE antiserum as first antibody (30). The Hardy-Weinberg value was calculated for the male, female and combined group to test genetic equilibrium with respect to the distribution of apoE phenotypes. Differences in mean lipid concentrations between various groups and influences of the different APOE alleles were estimated using one-way analysis of variance (ANOVA). Linear correlations between the parameters considered were calculated using the Spearman rank correlation test.

Table 1. Apolipoprotein E phenotype distribution, mean levels ± SD of plasma cholesterol (mmol/l) and LDL-cholesterol (mmol/l) and the mean, median and range (minimum and maximum) values of Lp(a) (mg/100 ml) in 303 adult healthy Dutch individuals.

ApoE phenotype	*n*	Plasma-chol	LDL-chol	Lp(a)		
		Mean ± SD	Mean ± SD	Mean	Median	Range
E2E2	3	4.60 ± 0.89	2.69 ± 0.71	6.35	1.60	0.50 - 17.50
E2E3	45	5.43 ± 0.85	3.53 ± 0.75	11.34	4.40	0.50 - 57.00
E3E3	178	5.70 ± 1.07	3.92 ± 0.99	13.04	6.25	0.70 - 114.00
E4E2	9	5.74 ± 1.10	3.85 ± 1.06	4.72	3.70	1.00 - 14.20
E4E3	63	6.03 ± 1.15	4.23 ± 1.11	15.15	8.20	0.80 - 65.40
E4E4	5	6.33 ± 1.40	4.54 ± 1.54	34.04	24.00	5.50 - 70.50
all phenotypes	303	5.73 ± 1.08	3.92 ± 1.02	13.36	6.20	0.50 - 114.00
P-value*		0.020	0.002			0.017

The phenotype distribution is in Hardy-Weinberg equilibrium ($\chi^2=0.08$; $df=5$; $P<0.05$ at $\chi^2>11.07$).

* *P*-value, indicating the significance level of the difference between the phenotype means (for plasma cholesterol and LDL-cholesterol) or logarithmic transformed means (for Lp(a)) of the measured parameters, calculated by ANOVA.

The contribution of the apoE polymorphism to the total variance of the measured parameters was calculated according to Sing and Davignon (5). Since Lp(a) levels were highly skewed, we used the logarithmic transformed Lp(a) levels for these statistical comparisons. Findings were regarded to be statistically significant when the probability of these data under the null hypothesis was less than 0.05. All statistical calculations were made by use of either the SPSS program (Northwestern University, Chicago, USA) or NCSS (Dr J.L. Hintze, Kaysville, USA).

Results

Since there were no significant differences in the apoE phenotype distribution between the two sexes (data not shown), the two groups were combined into one sample of 303 individuals. The apoE phenotype distribution of this sample was in Hardy-Weinberg equilibrium ($\chi^2 = 0.08$; $df = 5$; $P < 0.05$ at $\chi^2 > 11.07$) and did not differ significantly from that found in other Caucasian populations (4-7; Table 1).

Table 2. Average effect of the different APOE alleles (in % of the population means) on plasma cholesterol, LDL-cholesterol, and Lp(a) levels

	APOE alleles		
Lipoprotein parameter	E*2	E*3	E*4
plasma-cholesterol	- 5.9	- 0.2	+ 5.4
LDL-cholesterol	- 10.9	+ 0.1	+ 8.0
Lp(a)	- 24.8	- 0.8	+ 25.7

Mean total cholesterol, LDL-cholesterol and Lp(a) levels were calculated for each apoE phenotype groups. By ANOVA, significant differences between the apoE phenotypes were found for each of these parameters. The E*2 allele exhibited a lowering effect on these parameters, while the E*4 allele showed the opposite effect. This influence of apoE polymorphism on plasma lipid levels was independent of age and sex (data not shown). We calculated the average allelic effects of the APOE alleles on total cholesterol, LDL-cholesterol and Lp(a) levels (Table 2). The APOE*2 allele decreases the mean levels of total cholesterol, LDL-cholesterol and Lp(a) by 5.9, 10.9 and 24.8 %, respectively, whereas the APOE*4 allele has an increasing influence of 5.4, 8.0 and 25.7 %, respectively. The relative contribution of apoE polymorphism to the total variance of these measured parameters was estimated to be 4.5, 6.3 and 4.1 %, respectively (Table 3).

Table 3. Relative contribution (in %) of the genetic variance (σG^2) associated with the APOE locus to the total phenotypic variance (σ^2)

Lipoprotein parameter	$\sigma G^2 / \sigma^2$ (%)
plasma-cholesterol	4.5
LDL-cholesterol	6.3
Lp(a)	4.1

Table 4. Correlation matrix indicating the Spearmans correlation coefficient and corresponding *P* levels (between brackets) for levels of plasma cholesterol, LDL-cholesterol and Lp(a).

	plasma cholesterol	LDL-cholesterol	Lp(a)
plasma cholesterol	+ 1.0		
LDL-cholesterol	+ 0.92 ($P<0.001$)	+ 1.0	
Lp(a)	+ 0.20 ($P<0.001$)	+ 0.25 ($P<0.001$)	+ 1.0

Spearman's linear correlation coefficients between these three parameters are presented in Table 4. Besides the widely-reported correlation between LDL-cholesterol and total cholesterol ($r_s=0.92$; $P<0.001$), we also found small but significant linear correlations between Lp(a) and LDL-cholesterol ($r_s=0.25$; $P<0.001$) and between Lp(a) and plasma cholesterol ($r_s=0.20$; $P<0.001$).

Discussion

It is generally assumed that the influence of the apoE polymorphism on total cholesterol and LDL-cholesterol levels is the result of its effect on the hepatic LDL-receptor activity (6,7). The present study, involving 303 healthy males and females, demonstrated also an effect of the apoE polymorphism on plasma Lp(a) levels, parallel to that on plasma total cholesterol and LDL-cholesterol levels. However, such an effect was not observed in a study involving 337 male survivors of myocardial infarction (31).

At present, there is much debate as to whether or not the LDL-receptor plays a key-role in the regulation of plasma Lp(a) levels. Using cultured human fibroblasts, several groups have reported only a minor role of the LDL-receptor in the Lp(a) catabolism (24,25). Using freshly isolated parenchymal rat-liver cells, Harkes et al (32) demonstrated that upon increasing the LDL-receptor activity in these cells by

treatment of the rats with estrogen, Lp(a) interact slightly more efficient when compared to parenchymal cells isolated from untreated rats. These results suggest that the LDL-receptor is not or only to a minor extent involved in Lp(a) metabolism. This is further supported by the observation that in heterozygous FH patients, the plasma Lp(a) levels could not be reduced upon treatment with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, which are known to effectively reduce LDL-cholesterol levels by increasing the LDL-receptor activity (27,28).

In contrast to these findings arguing against the involvement of the LDL-receptor in Lp(a) catabolism, several reports appeared in favour of such an involvement. Several groups showed that cultured human fibroblasts take up and degrade Lp(a) via the LDL-receptor (21-23). Further support for this was gained from the observation that in FH patients, being partly defective in LDL-receptor activity, plasma Lp(a) levels are 2.5 to 3 times as high as in healthy individuals, irrespective of their Lp(a) phenotype (26). In a recent study using transgenic mice with an overexpression of human LDL-receptors, Lp(a) was cleared more rapidly than in the normal mice (33).

The present data show that the apoE polymorphism does influence the plasma Lp(a) levels parallel to that of LDL. A part of the effect of the apoE polymorphism on LDL-cholesterol levels can be explained by its influence on the conversion of VLDL into LDL (34). However, this can not be the explanation for the effect of apoE polymorphism on Lp(a) levels since Lp(a) has no VLDL precursor (35,36), although it may interact with triglyceride-rich particles (37). Using primary baboon hepatocytes as an in vitro model, Rainwater and Lanford (38) found only apo(a) bound to the LDL-apoB complex in a lipoprotein with a density of 1.05 g/ml, whereas e.g. apoB was also secreted in VLDL with a density less than 1.05 g/ml (39). These authors could not detect free apo(a) in the culture medium suggesting that Lp(a) is assembled intracellularly and secreted into the circulation as an intact particle, most probably by the liver. This hypothesis is supported by the almost absence of free apo(a) in serum of patients with autosomal recessive abetalipoproteinemia (40).

Our data suggest that Lp(a) is cleared from the circulation at least partly by a mechanism involving normal functioning LDL-receptors. These data are in conflict with the results of Sandkamp et al. (31). This discrepancy might be explained by the differences in the populations studied i.e. a healthy normolipidemic population (this study) versus a population consisting of myocardial infarction survivors with increased plasma lipid levels. In previous studies, it was shown that in FH patients the apoE polymorphism fails to influence the LDL-cholesterol levels significantly

(41,42). The absence of such an effect might be due to the high LDL-cholesterol levels in these individuals, probably overruling the effect of the apoE polymorphism. Similarly, the high LDL-cholesterol levels could also explain why treatment with simvastatin failed to reduce plasma Lp(a) levels in FH patients (27,28).

In summary, we present evidence that in normolipidemic individuals the Lp(a) level is influenced by the apoE polymorphism similarly to that of the LDL-level. Hence our results sustain the hypothesis that in vivo, the LDL-receptor is involved in Lp(a) catabolism.

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CHAPTER 6

High Risk Lipoprotein Profile in a Greenland Inuit Population. Influence of Anthropometric Variables, Apolipoprotein E and A4 Polymorphism and Life Style

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Abstract

Previously it has been reported that Greenland Inuit (Eskimos) display low levels of plasma cholesterol and triglycerides and relative high levels of high density lipoprotein (HDL) when compared with healthy Danish controls (Lancet 1971;1:1143-1146). Here we present data obtained in 1989 that showed the following: In a group of 133 healthy adult Greenland Inuit, the levels of plasma cholesterol and low density lipoprotein (LDL)-cholesterol (6.39 and 4.39 mmol/l, respectively) were slightly higher than "normal" values found in western societies, whereas the HDL-cholesterol level was markedly higher (1.64 mmol/l). As compared to most Caucasian populations, the Inuit population we studied, exhibits a high apolipoprotein (APO)E*4 allele frequency (0.229), whereas the APOE*2 allele frequency was extremely low (0.015). In contrast to Caucasian populations, in the Inuit population the apoE polymorphism showed only little influence on the plasma lipid and (apo)lipoprotein levels as evaluated by multiple regression analysis with the exception for apoE levels. This absence of an effect could be explained by the low very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) cholesterol levels. The contribution of eicosapentaenoic acid and linoleic acid to the total amount of fatty acids in plasma cholesterol esters differed markedly from those reported in 1971 for another Greenland Inuit population (3.2 versus 15.8 and 49.5 versus 20.4 %, respectively) thereby resembling now values found in the average Western population. Even in Inuit who report to consume exclusively the traditional Inuit diet (13 % of the population), the fatty acids composition of the plasma cholesterol esters closely resembled the values measured in Western populations.

More than 89 % of the Inuit drink alcohol regularly. Alcohol consumption showed a strong lowering effect on the levels of (VLDL+IDL)-cholesterol, LDL-cholesterol and apoB, and an elevating effect on apoA1 and HDL-cholesterol levels. In addition, alcohol consumption showed a decreasing effect on the contribution of linoleic acid, and an increasing effect on the contribution of eicosapentaenoic acid to the total amount of fatty acids in plasma cholesterol esters. Smoking, a common habit in the present Inuit, had only a weak influence on the plasma apoB level. In conclusion, when compared with the findings of Bang and Dyerberg and coworkers in the early 70's, we found a dramatic shift in the levels of most plasma lipids and (apo)lipoproteins and in the fatty acid composition of plasma cholesterol esters towards the high-risk pattern of the common western societies.

Introduction

The Greenland Inuit population has been reported to be low-risk population with respect to ischaemic heart disease (IHD) (1). Between 1979 and 1983, mortality caused by IHD among 45-64 years old Greenland Inuit males was 6.7 %, whereas this was 32.8 % in a comparable Danish control group in 1980 (2). In a series of studies, Bang, Dyerberg and co-workers reported low plasma cholesterol, triglycerides and very low density lipoprotein (VLDL) levels, and increased high density lipoprotein (HDL) levels in both male and female Greenland Inuit (3-7). This anti-atherogenic lipid pattern was explained by the high content of long-chain polyunsaturated fatty acids (PUFA's) in the natural diet of Inuit, when compared with an age-matched Danish population (1) rather than by a difference in genetic background. At the time Bang, Dyerberg and coworkers performed their studies (between 1970 and 1978), most of the Greenland Inuit still had a traditional active lifestyle, and an Inuit diet that was mainly based on traditional sources like marine mammals such as whales and seals, and fish (6,7). Since then, lifestyle and dietary habits of the Greenland Inuit have been further subjected to western influences.

In western societies, nutrition, alcohol consumption and smoking are reported to influence plasma lipid and lipoprotein levels and risk of IHD (8-17). In addition, it has been suggested that in these populations about 50% of the variation in plasma lipid levels is due to variability in genetic factors (18), the apolipoprotein (apo) E polymorphism being one of the major genetic factors in this respect (19,20). In various studies, the apoE polymorphism was shown to influence the risk of IHD (21-23). Both in the Finnish and in the Japanese population, the APOE*4 allele predisposes for a high risk of IHD (24,25). In addition, the apoA4 polymorphism has been demonstrated to influence plasma triglycerides and HDL-cholesterol levels (26-28).

Recently, it was shown that Greenland Inuit display the same amount of atherosclerosis as seen among Caucasians on the basis of ultrasound measurements (29,30). This indicates that Greenland Inuit are not longer protected against the development of atherosclerosis, although at present the incidence of IHD is still low.

The present study was initiated to evaluate as to whether changes in plasma levels of lipids and (apo)lipoproteins, apolipoprotein polymorphisms and life style can explain the progression of the atherosclerotic process in a well-defined Greenland Inuit population, that has been adapted now to a predominant western lifestyle. Compared to the findings of Bang and Dyerberg and coworkers in the early 70's, we found a dramatic shift of most levels of plasma lipids and (apo)lipoproteins and

of the fatty acid composition of plasma cholesterol esters towards the high-risk pattern of the common western societies.

Materials and Methods

Population description and sample collection All Inuit participating in this study live in the Municipal County of Nanortalik, South-West Greenland (Figure 1). By the 1st of January 1989 the total population of Greenland Inuit in this county consisted of 2441 individuals, 1225 males and 1216 females. As a part of this population-based study, all individuals between 30 and 34 years old were invited to participate in this study, approved by the Ethics Committee for Greenland. Before entering the study, an informed consent was obtained from all participants. The age-group of 30-34 years consisted of 192 individuals. A complete set of data, encompassing a filled-in questionnaire, anthropometric measurements (both discussed below) and blood specimens were obtained from 133 individuals (62 males and 71 females). All these 133 participating subjects were born in Greenland, from mothers born in Greenland. Using this selection criterium, we could avoid inclusion of Caucasians (mainly Danes), who immigrated to this region of Greenland recently. Upon medical investigation all these 133 Inuit appeared to be healthy and did not show signs or symptoms indicating the presence of severe atherosclerosis. The Inuit population of modern Greenland still in many ways follow a traditional and active life style. This includes a major contribution from traditional food items as fish, seal and whale to their diet (although to a variable extent).

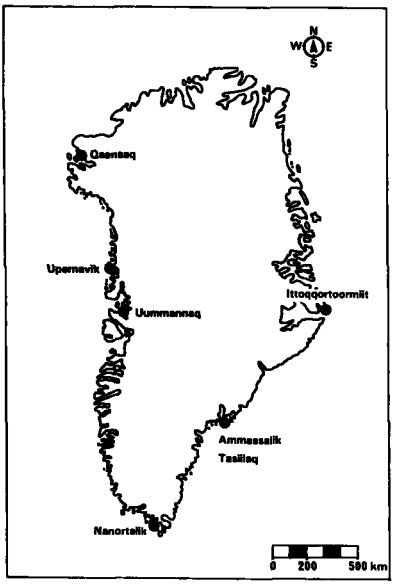

Figure 1. Map of Greenland showing localization of the places mentioned in the text.

EDTA-blood was collected after overnight fasting (including abstinence of alcohol). Plasma was separated from the cells by centrifugation at 500 g for 10 min at room temperature. After separation, plasma and cells were stored at -20 °C until use.

Questionnaire From all Inuit participating in this study we obtained information with respect to smoking habits, alcohol consumption, the type of diet, family relationships, lifestyle habits and health. The questionnaire was specially designed for use under Greenlandic conditions, but contained, essentially, the translated questions of the WHO Monica-Questionnaire (31). Based on the data collected by means of this questionnaire smoking habit was scored in three classes: less than 5 cigarettes/day, between 5 and 14 cigarretes/day and more than 15 cigarettes/day. Alcohol consumption was recorded for three types of alcohol consumptions: beer, wine and strong alcoholic beverages, separately. Since it was estimated that each of these types of alcohol consumptions contained a comparable amount of alcohol, we scored the total number of alcohol consumptions in three classes: less than 1 consumption/day, 1-2 consumptions/day and more than 2 consumptions/day. For each individual the type of diet was scored in one of three classes: Inuit (consuming the traditional Inuit diet only), Mixed (consuming a mixture of Inuit and Western diet) and Western (consuming a Western diet only).

Anthropometric measurements Body height was measured to the nearest 0.1 cm, by use of a transportable devise. Body weight was measured to the nearest 0.1 kg below the indication on a commercial scale (Seca). Body mass index (BMI) was calculated as weight (in kg) divided by height (in m^2). Waist and hip circumference were measured by a band measure to the nearest 0.5 cm below the indication. The waist/hip ratio (W/H) was calculated as waist circumference (in cm) divided by hip circumference (in cm).

Plasma lipid and lipoprotein analysis For the isolation of (VLDL+IDL), 2 ml plasma was brought to a background density of 1.019 g/ml with KBr and overlaid with 3.5 ml solution of NaCl (d=1.019 g/ml) in a 10.4 ml centrifuge tube fitting the Ti50 fixed angle rotor (Beckman Instruments, Geneva, Switzerland). After centrifugation at 106,000 g for 16 hours at 4 °C, the (VLDL+IDL) fraction was aspirated from the top. HDL was isolated from plasma after precipitation of VLDL, IDL and LDL using $MnCl_2$ and phosphotungstate (32).

Plasma cholesterol and cholesterol in the VLDL+IDL ($d < 1.019$ g/ml) and HDL fractions were measured using the CHOD-PAP kit (Boehringer Mannheim, FRG # 236691). Plasma and lipoprotein triglycerides were measured using the GPO-PAP kit (Boehringer Mannheim, FRG # 701904). LDL-cholesterol was calculated using the formula LDL-chol = plasma chol - (VLDL-chol + IDL-chol + HDL-chol).

Apolipoprotein quantification Plasma apoA1 and apoA4 concentrations were measured by enzyme-linked-immunosorbent assays (ELISA) exactly as described before (33,34). Plasma apoB levels were quantified by an immuno-nephelometric assay (INA) as described (35). ApoE levels in plasma were measured by ELISA as described by Bury et al. (36). In these measurements mean intra- and interassay variation coefficients were on average 4 and 8 %, respectively.

Apolipoprotein E and A4 phenotyping The apoE phenotyping was performed using a micromethod based on isoelectric focusing (pH 5-7) of delipidated plasma samples, followed by immunoblotting on nitrocellulose filter using a polyclonal anti-apoE antiserum as first antibody (37). ApoA4 phenotyping was performed by reusing the immunoblots after apoE phenotyping as previously described (38).

Cholesterol esters analysis The fatty acid composition of plasma cholesterol esters was analyzed by means of high pressure liquid chromatography as described before (39). Using this technique the relative amount of palmitate (C16:0), oleate (C18:1), linoleate (C18:2), arachidonate (C20:4) and eicosapentaenate (C20:5) in the cholesterol ester fraction were measured.

Statistical analyses Gender differences for the anthropometric variables and the plasma lipid, (apo)lipoprotein and fatty acid composition of the cholesterol esters were compared with the nonparametric Mann-Withney ranksum test. Differences in smoking habits, origin of diet and the use of alcohol between the sexes were evaluated by χ^2 analyses. The Hardy Weinberg χ^2 test was used to evaluate the genetic equilibrium for the apoE and apoA4 polymorphisms. The differences in APOE and APOA4 allele frequencies between the various populations were estimated by χ^2 analyses. Multiple regression analysis was used to study the influence of the anthropometric variables, the apoE polymorphism and lifestyle habits on the plasma lipid and (apo)lipoprotein levels and the fatty acid composition of the plasma cholesterol esters. All calculations were made with the commercially available statistical package NCSS (Dr. J. Hinze, Kaysville, Utah, U.S.A.).

Results

Population description The anthropometric variables and lifestyle habits of the 133 Inuit from Nanortalik are presented in Table 1. Inuit males are significantly taller and heavier than Inuit females, whereas BMI does not differ between both sexes. Inuit males had a larger waist- and hip circumference when compared with Inuit females. Regular- and excessive smoking (5-14 and > 15 cigarettes/day, respectively) was observed in 52 and 27 % of the males, and in 63 and 18 % of the females, respectively (gender difference not significant).

Table 1. Anthropometric variables, smoking habits, alcohol consumption and type of diet of Nanortalik Inuit.

	Total	Males	Females	
	Mean ± SD	Mean ± SD	Mean ± SD	*P* *
	n=133	*n*=62	*n*=71	
Height (cm)	161.8 ± 8.1	167.4 ± 6.4	156.9 ± 6.0	<0.001
Weight (kg)	64.8 ± 12.0	69.7 ± 10.9	60.6 ± 11.3	<0.001
BMI (kg/m^2)	24.7 ± 4.0	24.9 ± 3.5	24.6 ± 4.5	NS
Circumferences:				
Waist (cm)	81.5 ± 10.3	83.5 ± 8.9	79.8 ± 11.1	<0.001
Hip (cm)	90.6 ± 8.8	91.3 ± 6.8	90.0 ± 10.2	0.05
Waist/hip ratio	0.89 ± 0.05	0.91 ± 0.05	0.88 ± 0.04	<0.001
	%	%	%	
Smoking habits ‡:				
<5/day	19	21	18	
5-14/day	58	52	64	
>15/day	23	27	18	0.345 †
Alcohol use §:				
<1/day	11	5	17	
1-2/day	64	60	68	
>2/day	25	35	15	0.007 †
Type of diet ‖:				
Inuit	13	13	14	
Mixed	77	77	76	
Western	10	10	10	0.988 †

* *P* value indicating the difference between the male and female means calculated with the Mann-Whitney test.
† *P* value indicating the difference between the male and female means calculated with χ^2 analysis.
‡ Expressed in number of cigarettes / day.
§ Expressed in number of consumptions / day.
‖ Based on the Questionnaire.
SD, standard deviation; BMI, body mass index; NS, not significant ($P>0.05$).

difference ($P=0.007$) in the pattern of alcohol use between the sexes. Fewer Inuit males drank less than 1 consumption/day when compared with Inuit females (5 % vs 17 %), whereas more Inuit males consumed more than 2 consumptions/day (35% vs 15 %). A minority of the Inuit studied still consumed the original Inuit

food (13 % for males and 14 % for females). The remaining individuals either combined the Inuit food with Western food (77 and 76 % of males and females; respectively) or consumed Western food only (10 % for both males and females respectively). There was no gender difference in this respect.

Table 2. Comparison of APOE and APOA4 gene frequencies between the Nanortalik Inuit and other population samples.

		ApoE allele frequency				
Population	Sample size	E*2	E*3	E*4	P *	Reference
Greenland Inuit	133	0.015	0.756	0.229		This study
Icelandic	185	0.068	0.768	0.165	<0.001	40
Denmark	477	0.090	0.736	0.174	<0.001	41
Norway	239	0.090	0.795	0.115	<0.001	42
Sweden	279	0.119	0.675	0.206	<0.001	43
Finland	615	0.041	0.733	0.227	NS	44
The Netherlands	2018	0.082	0.773	0.167	<0.001	45
U.S.A., Whites	1204	0.075	0.786	0.135	<0.001	46
American Indians	95	0.000	0.815	0.185	0.109	47
Japan	576	0.037	0.846	0.117	<0.001	48

		ApoA4 allele frequency		
Population	Sample size	A4*1	A4*2	Reference
Greenland Inuit	133	0.970	0.030	This study
Finland	387	0.942	0.058	49
Iceland	183	0.885	0.112	28
The Netherlands	1337	0.918	0.082	38
U.S.A., Whites	159	0.908	0.088	50
U.S.A., Blacks	127	0.961	0.035	50
American Indians	105	0.967	0.000	47
Japan	614	0.998	0.002	51

* P value indicating the difference in APOE allele frequencies between the Nanortalik Inuit and the other populations as calculated by χ^2 analysis; due to low numbers of APOA*4 allele carriers in some populations the χ^2 analysis was not allowed for this polymorphism.
NS, not significant ($P>0.05$).

Apolipoprotein E and A4 polymorphism The APOE and APOA4 allele frequencies of the Nanortalik Inuit population are shown in Table 2 and are compared with the frequencies reported for other population samples (28,38,40-51). The apoE phenotype distribution is in Hardy-Weinberg equilibrium ($\chi^2=6.05$, df=5; not significant). Due to the extremely low apoA4 1/2 and 2/2 phenotype numbers it was not allowed to calculate the Hardy-Weinberg equilibrium for the apoA4 phenotype distribution. For both the apoE and apoA4 phenotype distributions no gender differences were found (results not shown). When compared with various other populations the Inuit exhibit a high APOE*4 allele frequency (0.229) concomitant with a very low APOE*2 allele frequency (0.015). The Finnish population also has a high APOE*4 allele frequency and a relative low APOE*2 allele frequency. The American Indians resemble the Greenland Inuit with respect to the extremely low APOE*2 allele frequency.

Although a statistical comparison between the various populations was not justified because of low numbers of APOA4*2 allele carriers, it is obvious that in the Inuit population the APOA4*1 allele frequency (0.970) is higher than usually found in Caucasian populations, whereas APOA4*2 allele frequency was evidently less frequent. These frequencies are comparable with the values found in American Blacks and American Indians.

Lipoprotein parameters The plasma lipid, lipoprotein and apolipoprotein levels of the Inuit population are shown in Table 3. Inuit males show a significantly higher level of plasma cholesterol and LDL-cholesterol than females. Gender differences were not found for the levels of plasma triglycerides, HDL-cholesterol, (VLDL+IDL)-cholesterol and (VLDL+IDL)-triglycerides. The levels of apoB and apoA4 were both significantly higher in males than in females, whereas the levels of apoA1 and apoE are not influenced by gender.

Factors influencing the lipid and (apo)lipoprotein parameters By means of multiple regression analysis, we evaluated the independent influence of the anthropometric variables, gender, apoE phenotype and lifestyle habits on the plasma lipid and (apo)lipoprotein levels (Table 4).

Among the anthropometric variables, body weight showed an independent significant influence on (VLDL+IDL)-cholesterol levels. The plasma apoE level was influenced by body height, body weight and the BMI. None of the lipoprotein parameters were influenced by the circumferences of waist and hip or waist/hip ratio.

Table 3. Mean values (± standard deviations) of plasma lipids and (apo)lipoproteins of Nanortalik Inuit.

	Total	Males	Females	
	Mean ± SD	Mean ± SD	Mean ± SD	P^*
	n=133	n=62	n=71	
		(mmol/l)		
Plasma cholesterol	6.39 ± 1.05	6.60 ± 0.99	6.22 ± 1.08	0.02
Plasma triglycerides	1.03 ± 0.70	1.05 ± 0.69	1.02 ± 0.71	NS
(VLDL+IDL)-chol	0.37 ± 0.28	0.40 ± 0.32	0.34 ± 0.26	NS
(VLDL+IDL)-tg	0.32 ± 0.26	0.35 ± 0.30	0.30 ± 0.21	NS
LDL-cholesterol	4.39 ± 0.97	4.54 ± 0.92	4.25 ± 0.99	0.05
HDL-cholesterol	1.64 ± 0.50	1.66 ± 0.48	1.62 ± 0.52	NS
		(mg/100 ml)		
ApoA1	149.5 ± 27.8	151.6 ± 27.5	147.6 ± 28.1	NS
ApoA4	15.3 ± 4.4	17.3 ± 4.0	13.6 ± 4.0	<0.001
ApoB	84.2 ± 17.7	87.6 ± 15.5	81.3 ± 19.1	0.01
ApoE	5.7 ± 2.0	5.6 ± 2.2	5.5 ± 1.8	NS

* *P* value indicating the difference between the male and female means calculated with the Mann-Whitney test.

SD, standard deviation; NS, not significant ($P>0.05$); chol, cholesterol; tg, triglycerides.

After multiple regression the effect of gender on the levels of apoB and apoA4 remained, whereas that for the levels of plasma cholesterol and LDL-cholesterol disappeared (compare Tables 3 and 4).

ApoE polymorphism significantly influenced the variability of the apoE levels in addition to the effects of anthropometric variables. Strikingly, we did not find an independent influence of this polymorphism on the levels of plasma cholesterol, LDL-cholesterol and apoB as is often reported for other populations (24-28).

Alcohol consumption significantly influenced HDL-cholesterol, (VLDL+IDL)-cholesterol, apoA1, and apoB levels. Higher levels of HDL-cholesterol and apoA1 were found in Inuit groups with 1-2 consumptions/day (1.63 mmol/l and 149.9 mg/100 ml) or >2 consumptions/day (1.78 mmol/l and 156.8 mg/100 ml) when compared with the Inuit group of <1 consumption/day (1.36 mmol/l and 131 mg/100 ml, respectively; see Table 5). In contrast, the levels of apoB and (VLDL+IDL)-cholesterol were significantly lower in the 1-2 consumptions/day group (82.4 mg/100ml and 0.33 mmol/l) and in the > 2 consumptions/day group (81.1 mg/100ml and 0.36 mmol/l) when compared with those levels in the <1

Table 4. Multiple regressions of the anthropometric parameters, gender, apoE polymorphism and lifestyle habits on the lipid and (apo)lipoprotein parameters of Nanortalik Inuit.

	Dependent variables									
	Plasma-chol	Plasma-tg	(VLDL+IDL)-chol	(VLDL+IDL)-tg	LDL-chol	HDL-chol	ApoA1	ApoA4	ApoB	ApoE
	(mmol/l)						(mg/100 ml)			
Independent variables:										
Height	-0.058	-0.101	-0.149	-0.084	-0.009	-0.026	-0.014	0.091	-0.112	**-0.392‡**
Weight	0.065	0.152	**0.197***	0.135	0.001	0.029	0.005	-0.111	0.118	**0.415‡**
BMI	-0.083	-0.157	-0.164	-0.098	-0.023	-0.046	-0.02	0.075	-0.106	**-0.384‡**
Waist circumference	-0.057	0.026	0.071	0.078	-0.104	0.042	0.101	-0.115	-0.115	0.030
Hip circumference	0.083	0.010	-0.062	-0.069	0.135	-0.051	-0.102	0.123	0.140	-0.020
Waist/hip ratio	0.049	-0.029	-0.051	-0.070	0.096	-0.054	-0.123	0.118	0.118	-0.044
Gender	-0.136	0.109	0.036	0.036	-0.160	-0.001	-0.072	**-0.340‡**	**-0.187***	0.003
ApoE phenotype	0.027	-0.093	-0.137	-0.134	0.120	-0.103	0.035	0.058	0.128	**-0.466‡**
Smoking habits	0.097	0.164	0.171	0.145	0.116	-0.111	-0.034	-0.014	**0.179***	-0.055
Alcohol use	-0.043	-0.080	**-0.217***	-0.162	-0.116	**0.230†**	**0.260†**	0.049	**-0.269†**	0.044
Type of diet	-0.046	-0.083	-0.019	-0.037	-0.097	0.102	0.114	0.032	-0.124	0.012
R^2	0.100	**0.212†**	**0.232†**	**0.223†**	0.145	**0.169***	0.144	**0.235‡**	**0.235‡**	**0.324‡**

A numerical code was used for gender (1=male, 2=female), apoE phenotype (1=E3E2, 2=E3E3, 3=E4E3, 4=E4E4), smoking habits (1=low, 2=moderate, 3=high), alcohol use (1=low, 2=moderate, 3=high) and dietary habits (1=Inuit, 2=Mixed, 3=Western). Data represent partial correlation coefficients, and total explained variance (R^2).

chol, cholesterol; tg, triglycerides.

Significant correlation coefficients (* $P<0.05$, † $P<0.01$, ‡ $P<0.001$) are highlighted in bold.

Table 5. Influence of alcohol consumption on adjusted levels (mean ± Standard error) of plasma lipids and (apo)lipoproteins of Nanortalik Inuit.

	Alcohol consumption (number/day)			
	<1	1-2	>2	
	Mean ± SEM	Mean ± SEM	Mean ± SEM	
	n=15	*n*=85	*n*=33	*P* *
Plasma cholesterol †	6.79 ± 0.27	6.26 ± 0.11	6.57 ± 0.18	NS
Plasma triglycerides	1.10 ± 0.18	1.04 ± 0.08	0.98 ± 0.12	NS
(VLDL+IDL)-chol	0.61 ± 0.07	0.33 ± 0.03	0.36 ± 0.04	0.001
(VLDL+IDL)-tg	0.43 ± 0.07	0.30 ± 0.03	0.33 ± 0.04	NS
LDL-chol	4.84 ± 0.25	4.29 ± 0.10	4.41 ± 0.17	NS
HDL-chol	1.36 ± 0.13	1.63 ± 0.05	1.78 ± 0.08	0.024
ApoA1	131.0 ± 7.0	149.9 ± 2.9	156.8 ± 4.7	0.011
ApoA4	15.7 ± 1.0	15.1 ± 0.4	15.7 ± 0.7	NS
ApoB	101.5 ± 4.3	82.4 ± 1.8	81.1 ± 2.9	<0.001
ApoE	5.7 ± 0.4	5.5 ± 0.2	5.7 ± 0.3	NS

Levels were adjusted for the parameters (except alcohol consumption) showing a significant correlation as evaluated by multiple regression analysis as presented in Table 4.

* *P* value indicating the difference between the three alcohol consumption groups as calculated by analysis of covariance.

† Levels are expressed in mmol/l except for the apolipoprotein levels which are expressed in mg/100 ml. SEM, standard error of the mean; chol, cholesterol; tg, triglycerides; NS, not significant ($P>0.05$).

consumption/day group (101.5 mg/100 ml and 0.61 mmol/l, respectively). We did not find a significant influence of diet on the lipoprotein values (Table 4).

Fatty acid composition of plasma cholesterol esters The contribution of different fatty acids to the total amount of fatty acids in the cholesterol ester lipid fraction is shown in Table 6. For reasons of comparison, we also present the values for the Uummannaq Inuit and Danish controls measured by Dyerberg et al. in 1970 (5,7) and recently (1989) measured values for a Belgian control sample (52). In our Inuit population, we found no significant gender differences. The relative contribution of eicosapentaenoic acid (C20:5) in the 1989 Nanortalik Inuit population sample was much lower than found in the Uummannaq Inuit by Dyerberg et al. (5,7) in 1970, but higher than the value found for the Danish population in 1970. The contribution of linoleic acid (C18:2) was much higher in the Nanortalik Inuit when compared with Uummannaq Inuit and is comparable with the values reported for the Danish population in 1970 and the Belgian population in 1989 (5,52).

Table 6. The fatty acid composition of plasma cholesterol esters of Nanortalik Inuit expressed as the relative contribution to the total amount of fatty acids in comparison with Inuit from Uummannaq and Danish controls (taken from Dyerberg et al., ref. 5) and a Belgian control population (taken from De Backer et al., ref. 52).

	Present study (collected in 1989)	De Backer et al. (52) (collected in 1988)	Dyerberg et al. (5) (collected in 1970)	
	Nanortalik Inuit	Belgian controls	Uummannaq Inuit	Danish controls
	Mean ± SD	Mean ± SD	Mean	Mean
	$n=133$	$n=134$	$n=129$	$n=29$
Fatty acid:	(% of total fatty acids)			
Palmitic acid (C16:0)	14.8 ± 2.7	13.4 ± 2.2	19.3	12.4
Oleic acid (C18:1)	25.9 ± 3.1	22.2 ± 4.7	25.3	22.8
Linoleic acid (C18:2)	49.5 ± 7.5	58.4 ± 6.2	20.4	47.8
Arachidonic acid (C20:4)	6.6 ± 2.0	6.0 ± 1.2	1.0	4.0
Eicosapentaenoic acid (C20:5)	3.2 ± 2.0	ND	15.8	0.1

SD, standard deviation; ND, not determined.

Table 7. Multiple regressions of gender, apoE polymorphism and lifestyle habits on the relative fatty acid composition in cholesterol esters of Nanortalik Inuit.

	Dependent variables				
	Eicosapentaenoic acid (C20:5)	Arachidonic acid (C20:4)	Linoleic acid (C18:2)	Oleic acid (C18:1)	Palmitic acid (C16:0)
Independent variables:					
Gender	0.063	0.122	-0.138	0.127	0.086
ApoE phenotype	-0.003	0.038	-0.020	0.023	0.000
Smoking habits	-0.040	-0.058	0.018	0.063	-0.050
Alcohol use	**0.285‡**	**0.194***	**-0.297†**	0.158	**0.282‡**
Dietary habits	**-0.210***	-0.007	0.141	-0.018	**-0.206***
R^2	**0.129‡**	0.045	**0.114‡**	0.039	**0.126‡**

A numerical code was used for gender (1=male, 2=female), apoE phenotype (1=E3E2, 2=E3E3, 3=E4E3, 4=E4E4), smoking habits (1=low, 2=moderate, 3=high), alcohol use (1=low, 2=moderate, 3=high) and dietary habits (1=Inuit, 2=Mixed, 3=Western). Data represent partial correlation coefficients, and total explained variance (R^2).
Significant correlation coefficients (* $P<0.05$, † $P<0.01$, ‡ $P<0.001$) are highlighted in bold.

Factors influencing the fatty acid composition of plasma cholesterol esters Gender, apoE phenotype and smoking habits did not influence the fatty acid composition of the plasma cholesterol esters as evaluated by multiple regression analysis (Table 7). However, both alcohol consumption as well as dietary habits did affect the relative composition of the plasma cholesterol esters significantly. As is shown in Table 8, increasing alcohol consumption resulted in a significantly higher relative contribution of palmitic acid (C16:0) and eicosapentaenoic acid (C20:5), whereas it showed a lowering influence on the relative contribution of linoleic acid (C18:2). Inuit consuming a western diet showed lower relative contributions of palmitic acid (C16:0) and eicosapentaenoic acid (C20:5) and a higher relative contribution of linoleic acid (C18:2) when compared with Inuit consuming an Inuit diet. The values from the latter group are comparable with recently measured values in a Belgian population (52).

Table 8. Influence of alcohol consumption and type of diet on the contribution of the fatty acids (mean ± standard error) in the plasma cholesterol esters of Nanortalik Inuit.

	Alcohol consumption (number/day)			
	<1	1-2	>2	
	n=15	*n*=85	*n*=33	
Fatty acid	Mean ± SEM	Mean ± SEM	Mean ± SEM	*P* *
Palmitic acid (C16:0)	13.5 ± 0.7	14.6 ± 0.3	16.0 ± 0.4	0.004
Oleic acid (C18:1)	25.6 ± 0.8	25.6 ± 0.3	26.7 ± 0.5	NS
Linoleic acid (C18:2)	52.8 ± 1.8	50.3 ± 0.8	45.9 ± 1.2	0.003
Arachidonic acid (C20:4)	5.9 ± 0.5	6.6 ± 0.2	7.1 ± 0.3	NS
Eicosapentaenoic acid (C20:5)	2.3 ± 0.5	3.1 ± 0.2	4.1 ± 0.3	0.003
	Type of diet			
	Inuit	Mixed	Western	
	n=18	*n*=102	*n*=13	
Fatty acid	Mean ± SEM	Mean ± SEM	Mean ± SEM	*P**
Palmitic acid (C16:0)	15.5 ± 0.6	14.9 ± 0.3	13.1 ± 0.7	0.041
Oleic acid (C18:1)	25.0 ± 0.7	26.3 ± 0.3	24.2 ± 0.8	0.028
Linoleic acid (C18:2)	49.1 ± 1.7	48.9 ± 0.7	53.9 ± 1.9	NS
Arachidonic acid (C20:4)	6.6 ± 0.5	6.6 ± 0.2	6.5 ± 0.6	NS
Eicosapentaenoic acid (C20:5)	3.9 ± 0.5	3.2 ± 0.2	2.1 ± 0.5	0.049

Levels are % of total fatty acids.

* *P* value indicating the difference between the three alcohol consumption or type of diet groups as calculated by analysis of variance.

SEM, standard error of the mean; NS, not significant ($P>0.05$).

Discussion

According to Persson (53), three geographically distinct populations of Greenland Inuit can be distinguished: an East-coast population living near Ittoqqortoormiit and Ammassalik Tasiilaq, a Northern population, often referred to as the Thule population, living around Qaanaaq, and the West-Coast population living between Upernavik and Nanortalik (see Figure 1). As extensively outlined by Persson (53) each of these three geographically has been subjected to different immigration waves of human populations of Inuit, Canadian Indian and Caucasian origin during the past 6000 years. The Inuit that we have included in the present study were all

living in Nanortalik, in the South-West of Greenland. This region has been inhabited by Icelanders for a short time, after Eric the Red discovered this area in 982 A.D., but was not influenced by the second immigration wave of Caucasian whalers and fisherman, mainly of Bask, Danish and Norwegian origin, starting in the early 18 th century. This second wave of immigration primarily influenced the West-coast districts around Uummannaq. Near this latter district Bang and Dyerberg and coworkers performed their study in the early 70's.

In this period Bang & Dyerberg and coworkers (1-7) reported that the Greenland Inuit population is at very low risk of IHD. Plasma lipid levels differed markedly from an age matched Danish control population. Noteworthy were the relatively high levels of HDL in both males and females and the low VLDL-levels. Based on the observation that Inuit living in Denmark showed lipid levels comparable with the normal Danish levels, the authors concluded that these remarkable differences originate from environmental, most likely nutritional, factors rather than from differences in genetical background between populations. They found that fatty acid composition of the plasma cholesterol esters differed significantly from that found in a Danish control group (5,7). Inuit showed higher levels of palmitic acid, oleic acid and eicosapentaenoic acid and lower levels of linoleic acid and arachidonic acid (5,7). At that time, the Inuit diet primarily consisted of meat from marine mammals and fish which is rich in long-chained polyunsaturated fatty acids such as eicosapentaenoic acid and relative poor in linoleic acid (6,7). They concluded that their different dietary habit was one of the major factors explaining the low-risk profile for IHD (1).

Although Bang et al. (3) stated that they were not aware of genetical difference between the Greenland Inuit and the Danish population clear evidence for the genetical difference between both populations has appeared. These data, including information on the ABO, the Rhesus and the MN bloodgroup, the HLA-system and various red cell enzymes (53-57, recently summarized by Eriksson et al. (58)), evidently show that the Greenland Inuit differ markedly from various Caucasian populations. Nevertheless, a gene flow has occurred from various Caucasian populations to different parts of the Greenland West Coast since its first discovery by the Icelanders. This will have influenced the present Inuit-gene pool to at least a minor extent (53).

Bang et al. (7) made their study in Inuit from the Uummannaq district, about 1500 km north of the Nanortalik district where we have collected data on the present Inuit population (see Figure 1). Persson (53) and Eriksson et al. (58) reported marked genetic differences between these two Greenland Inuit populations which could interfere to a certain extent with the remarkable differences existing

between the observations of Bang et al. (7) and the results presented in this study.

To our knowledge, this is the first study in an Inuit population investigating genetic markers, known to influence the lipoprotein metabolism, i.e. apolipoprotein E and apolipoprotein A4. In the Nanortalik Inuit population we observed a very low APOE*2 allele frequency (0.015) and a high APOE*4 allele frequency (0.229) when compared with other populations (Table 2). With these APOE allele frequencies the Inuit closely resembles the distantly related Finnish and American-Indian populations. Also for the APOA4 gene we observed marked differences between the Inuit and most of the Caucasian population samples (Table 2). With the virtually absence of the APOA4*2 allele the Inuit again strongly resemble the American-Indian population. These results are in agreement with the reports from Kamboh et al. (59,60) showing that the APOE*2 and APOA4*2 alleles are virtually absent in Indigenous populations thereby strongly suggesting that the APOE*2 and APOA4*2 alleles are of Caucasian origin. The differences, with respect to the APOE and APOA4 allele frequencies, between the Inuit population on the one hand and the Norwegian, Danish and Icelandic populations on the other hand support the concept that the Caucasian gene flow to the Inuit population is rather limited and allows us to conclude that from a genetical point of view the Inuit population we have studied is still of primarily Inuit origin.

The mean plasma lipid and (apo)lipoprotein levels in the Nanortalik Inuit population (Table 3) were all within the range normally found for Caucasian populations (61) and are higher than those measured by Bang et al. in 1970 (3). However, in the present Nanortalik Inuit population the HDL-cholesterol levels are still high when compared with average Caucasian levels and there are still no differences in HDL-cholesterol levels between male and female Inuit as also reported previously (3).

Strikingly, in the present study we could not find a significant influence of the apoE polymorphism on the plasma lipid and (apo)lipoprotein levels with the exception for the apoE concentration (Table 4). In the Inuit population the E*4 allele was not associated with increased plasma cholesterol, LDL-cholesterol and apoB levels like in various other populations (21-25,40,42,44-46, data not shown). In this respect, it is interesting to note that the Greenland Inuit show very low (VLDL+IDL)-cholesterol and (VLDL+IDL)-triglyceride levels when compared with a healthy Dutch control sample (preliminary results). This could explain the absence of an effect of the apoE polymorphism on plasma lipid and (apo)lipoprotein levels as only a minor amount of cholesterol is delivered to the liver via the action of apoE. Consequently, the LDL-receptor activity in the liver, and thus the plasma cholesterol and LDL-cholesterol levels are not affected by the apoE polymorphism

in this population. We are also aware of two studies among Norwegians showing that the effect of the APOE*4 allele is influenced by a *PvuII* restriction fragment length polymorphism in the LDL-receptor (LDLR) gene indicating the occurrence of an APOE-LDLR gene-gene interaction (42,62). At present, we can not exclude such a gene-gene interaction in our Inuit population.

It is obvious that the present Greenland Inuit, at least in the Nanortalik district, are regular smokers and drinkers. Smoking habits showed only a weak correlation with the plasma apoB level (Table 4). This is in marked contrast with various extensive studies performed in the USA and Israel (8,10,63) in which smoking habits correlated strongly and positively with plasma cholesterol levels and negatively with HDL-cholesterol levels, thereby potentially increasing the risk of IHD. More similar to the observations in various Caucasian populations (11-13), in the Inuit population alcohol consumption was also significantly associated with lower levels of (VLDL+IDL)-cholesterol and apoB and higher levels of HDL-cholesterol and apoA1 (Tables 4 and 5).

Also with respect to the type of diet, the Inuit have adjusted themselves rapidly to a western lifestyle, as, at present only a minority (on average 13 %) still claims to consume exclusively an Inuit diet (Table 1). However, this could not be confirmed by measuring the fatty acid composition of the plasma cholesterol ester lipid fraction (Table 6). In each of the three dietary groups (Inuit, Mixed, and Western diet) the fatty acid composition appeared to be very similar to that found in Caucasian populations (5,53) but differed markedly from the Uummannaq Inuit population studied by Dyerberg et al. in 1970 (5,7). We also found that this fatty acid composition could be influenced significantly by alcohol consumption (Table 7).

In summary, we conclude that the present Inuit, at least those from Nanortalik, now strongly resemble the Caucasians with respect to the plasma lipid and (apo)lipoprotein levels and their fatty acid composition of the plasma cholesterol esters. They also have adjusted themselves to a western way of living, including smoking, alcohol consumption. Recently, it was found that they show the same degree of atherosclerosis as healthy Danish controls (29,30). However they do not suffer from IHD to the same extend as individuals in most western societies. This suggests that the change towards a high-risk lipoprotein profile in the Greenland Inuit has started rather recently and that it is too early to observe the consequence of this change, i.e. increased mortality due to IHD.

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CHAPTER 7

The Influence of ApoE Polymorphism on the Response to Simvastatin Treatment in Patients with Heterozygous Familial Hypercholesterolemia

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Summary

In a group of 120 patients with heterozygous familial hypercholesterolemia (FH) the influence of the apolipoprotein E (apoE) polymorphism on pre-treatment plasma lipid levels and on the response to treatment with simvastatin was studied. The apoE phenotype distribution did not differ significantly between the FH group and a sample group of the Dutch population. Differences in pre-treatment lipid levels were not related to the apoE polymorphism in this FH population. After 12 weeks use of a daily dose of 40 mg simvastatin, the plasma total cholesterol, low density lipoprotein (LDL)-cholesterol and plasma triglycerides levels were reduced on average by 33 %, 38 % and 19 %, respectively. At the same time high density lipoprotein (HDL)-cholesterol concentration increased on average by 7 %. In the combined FH patient group (males and females) a considerable interindividual variation in response to simvastatin was observed, but was not related to the apoE polymorphism. However, considering males and females separately, we found that female FH patients with the apoE3E3 phenotype responded better on simvastatin treatment with respect to LDL-cholesterol than male FH patients with the apoE3E3 phenotype.

Introduction

Familial hypercholesterolemia (FH) is a well-defined autosomal dominant lipid disorder, caused by defects in the low density lipoprotein-receptor (LDLR) gene (1). FH is characterized by strongly elevated levels of low density lipoprotein (LDL)-cholesterol concomitant with a highly increased risk for premature atherosclerosis (2). In its heterozygous form, FH occurs with a frequency of 1 in 500 persons in the population and is therefore one of the most common genetic diseases in man.

Most of the LDL is cleared from the plasma through LDL-receptor mediated uptake in the liver. The LDL-receptor activity *in vivo* is regulated by the hepatic demand for cholesterol (3). This regulatory mechanism forms the basis for treatment of FH patients with bile-acid binding resins and inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol synthesis. Upon blocking the endogenous cholesterol synthesis, hepatic cells increase the production of LDL-receptors leading to an increased LDL uptake and accordingly to a reduction of plasma- and LDL-cholesterol levels (4). These inhibitors have been proven to be effective and, at least after short-term use, safe cholesterol lowering drugs (5-9).

Apolipoprotein E (apoE) is one of the major protein constituents of chylomicron- and very low density lipoprotein (VLDL)- remnants. It plays a central role in the hepatic uptake metabolism of these particles as it is recognised with high affinity by hepatic lipoprotein receptors (10). ApoE is genetically polymorphic. Three common isoforms, E2, E3 and E4, can be separated by isoelectric focusing. They each differ in isoelectric point (pI) by one charge unit, apoE4 being the most basic, and apoE2 the most acidic isoform. These isoforms are encoded for by three different alleles, E*4, E*3 and E*2, at a single APOE gene locus on chromosome 19 (11). Population studies demonstrated that the apoE polymorphism affects plasma lipid levels (12). It has been shown that individuals carrying the E*2 allele have lower total cholesterol and LDL-cholesterol levels, whereas the opposite is true for individuals with the E*4 allele (13-15). These differences were also recently found in a sample of a healthy Dutch population (16).

Besides the well-documented role of the apoE polymorphism in the expression of familial dysbetalipoproteinemia (FD) (17-18) the apoE polymorphism has also been reported to be associated with FH. A higher frequency of the E*4 allele in FH patients has been described by Eto et al (19). Furthermore, in the same study the presence of the E*4 allele resulted in higher LDL-cholesterol levels and in significantly higher triglyceride levels. In contrast to this, O'Malley and Illingworth (20) reported a significantly higher plasma triglyceride concentration in E*2 allele carrying FH patients.

With respect to the effect of the apoE polymorphism on the response of plasma lipid levels in FH patients to HMG-CoA reductase inhibitors little is known. O'Malley and Illingworth (20) reported no influence of the apoE polymorphism on the response to lovastatin.

In the present study we compared the apoE phenotype distribution in a Dutch sample of FH patients and a normal control population (16). In addition, we evaluated the effect of the apoE polymorphism on the response to simvastatin treatment in the FH patients.

Materials and methods

Subjects In this multicentre study, four Lipid Clinics in the Netherlands contributed a total of 120 Caucasian FH patients (Nijmegen 41, Utrecht 41, Leiden 29 and Amsterdam 9). Based on elevated LDL-cholesterol levels (>6.7 mmol/l), typical xanthomas (observed in two-third of the patients) and a positive family history, patients were diagnosed as heterozygous FH. The patient group consisted of 75 males (mean age 42.1 years; range 18-65) and 45 females (mean age 51

years; range 27-73). No lipid lowering drugs were administered to the subjects for four weeks or more at the onset of the study. Simvastatin was given in one daily dose of 40 mg.

Samples and analysis EDTA plasma samples were obtained after overnight fasting by venepuncture at the onset of the trial and subsequently at weekly intervals. The lipid analysis of the four patient groups was done in their respective centres. Cholesterol and triglycerides concentrations were measured enzymatically using Boehringer-test-kits (cholesterol CHOD-PAP and triglyceride GPO-PAP, respectively). High density lipoprotein (HDL)-cholesterol was measured after precipitation of VLDL and LDL (21). LDL-cholesterol was calculated using the Friedewald formula (22).

The apoE phenotype was determined by isoelectric focusing of delipidated plasma samples followed by immunoblotting (23) using a polyclonal anti-apoE antiserum (for Leiden, Amsterdam and Utrecht) or by isoelectric focusing of VLDL apolipoproteins followed by protein staining and densitometric scanning (24) (Nijmegen).

Statistical analysis The Hardy-Weinberg value was calculated for the male-, female and combined FH patient group and the control population to test genetic equilibrium with respect to the distribution of the apoE phenotype. Differences in phenotype frequencies between the male- and female FH patients and between the combined FH patient group and the control group were tested using chi-square analysis. Differences in mean lipid concentrations between the different groups were evaluated using both the non-parametric Kruskal-Wallis test and the parametric one-way analysis of variance (ANOVA). In-pair differences between the lipid values at $t=0$ and $t=12$ were tested using both the non-parametric Wilcoxon signed rank test and the parametric paired-T test. From all parameters measured, normality of distribution was tested using the Martinez and Iglewicz test (25).

Results

ApoE phenotype distribution There were no significant differences in the apoE phenotype distribution between the four groups (Leiden, Utrecht, Nijmegen and Amsterdam, data not shown). Therefore, we combined these four groups to form a FH group of 120 patients. The apoE phenotype distribution in the male-, female- and the combined FH patient groups together with that of a large control sample (16) is presented in Table 1. All groups were in Hardy-Weinberg equilibrium ($\chi^2=$ 1.99; 1.51; 3.72 and 2.82 for the male-, female- and the combined FH patient group and for the control group, respectively; $P<0.05$ at $\chi^2>11.07$; $df=5$). In the

FH patient group there was no significant difference between males and females with respect to apoE phenotype distribution ($\chi^2=1.54$; $P<0.05$ at $\chi^2>11.07$; $df=5$). In addition, the phenotype distribution of the combined FH population did not differ significantly from the previously reported control population ($\chi^2=3.46$; $P<0.05$ at $\chi^2>11.07$; $df=5$).

Table 1. ApoE phenotype distribution and allele frequencies in the FH patient group and in a control population sample[A]

Apo E	FH group						Control	
pheno-	Males		Females		Combined			
type	*n*	%	*n*	%	*n*	%	*n*	%
E4E4	3	4.0	3	6.7	6	5.0	59	2.9
E4E3	17	22.7	11	24.4	28	23.3	512	25.4
E4E2	2	2.7	1	2.2	3	2.5	45	2.2
E3E3	39	52.0	25	55.6	65	53.3	1128	55.9
E3E2	14	18.7	5	11.1	19	15.8	261	12.9
E2E2	0		0		0		13	0.7
Total	75		45		120		2018	
H-W χ^2	1.99 (NS)[B]		1.51 (NS)		3.72 (NS)		2.82 (NS)	
E*4	0.167		0.200		0.179		0.167	
E*3	0.727		0.733		0.729		0.750	
E*2	0.107		0.067		0.092		0.082	

[A] Control polpulation sample taken from ref. (16).
[B] (NS); not significant ($P<0.05$).
For Hardy-Weinberg (H-W) distribution $P<0.05$ at $df=5$ when $\chi^2>11.07$.
For the difference between the male and female FH patient groups $\chi^2=1.54$ ($df=5$; not significant; $P<0.05$ at $df=5$ when $\chi^2>11.07$.
For the difference between the combined FH patient group and the control population $\chi^2=3.46$ ($df=5$; not significant; $P<0.05$ at $df=5$ when $\chi^2>11.07$.

Effect of APOE allele substitutions on the response to simvastatin In the combined group of FH patients, we evaluated the influence of the apoE polymorphism on the lipid levels at $t=0$ and $t=12$ and on the response of plasma lipids to simvastatin treatment. The patient group was divided into three apoE groups: one group containing all patients with E4E4 and E4E3 phenotypes (E4+), a second group containing all patients with the E3E3 phenotype and a third group consisting of all

patients exhibiting the E3E2 phenotype (E2+). Because of opposing effects of the E*4 and E*2 alleles on plasma lipid parameters in normal populations (14,16,26), three patients with the E4E2 phenotype were not included in this study. In addition, because of incomplete lipoprotein data, three other patients were excluded. Table 2 represents the mean lipid values for the three apoE phenotype groups ($n=114$) at $t=0$ and $t=12$ weeks and the difference between these two values expressed as a percentage of the value at $t=0$. Results of the normality test indicated that the majority of tested parameters were not normally distributed, therefore, only the results of the non-parametric tests are given. Moreover, with the use of parametric tests no additional significant results were obtained (results not shown). Both at $t=0$ and $t=12$ weeks no significant differences were found between the three apoE phenotype groups with respect to the plasma levels of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. For all lipid parameters measured, the differences between the levels at $t=0$ and $t=12$ weeks levels were significant ($P<0.05$) in each group. With respect to plasma total cholesterol and LDL-cholesterol the response to simvastatin treatment was the lowest in the E4+ group and the highest in the E2+ group, although these differences did not reach statistical significance. With respect to plasma triglycerides the response to simvastatin treatment was the highest in the E4+ group and markedly lower in the E2+ group. However these differences were not significant. Similarly, the effect of simvastatin administration on HDL-cholesterol was not significantly different in the various apoE phenotype groups.

Effect of gender on the response to simvastatin We also evaluated possible differences in mean lipid levels and response to simvastatin treatment between male- and female FH patients. The results are presented in Table 3. We found significant differences between male- and female FH patients with respect to HDL-cholesterol levels. Both at $t=0$ as well as at $t=12$ female FH patients had higher HDL-cholesterol levels than male FH patients (0.19 mmol/l difference at both time points; $P=0.0004$ and 0.0002 respectively). There was no significant difference between the HDL response values. We also found a significant difference between male- and female FH patients in response to simvastatin treatment with respect to LDL-cholesterol. Female FH patients were able to lower their LDL-cholesterol 5 % more effectively than male FH patients ($P=0.041$). The same tendency was found for total plasma cholesterol (female FH patients responded 3.5 % better) and plasma triglycerides (female FH patients responded 5 % better) but these differences were not significant. A more detailed analysis of the difference between

Table 2. Plasma-cholesterol, LDL-cholesterol, HDL-cholesterol and plasma-triglycerides levels at $t=0$ and $t=12$ and the difference (Δ) between these two levels (expressed in % of the $t=0$ value) for the different apoE groups in the combined FH population

			Effect of simvastatin						
	ApoE groups	n	$t=0$	$t=12$	Δ (% van $t=0$)	P^A	P^B	P^C	P^D
Cholesterol (mmol/l)	E4+	32	10.91 ± 2.29	7.46 ± 1.34	-30.6 ± 10.1	0.301	0.949	0.202	<0.0001
	E3/E3	64	11.40 ± 2.13	7.40 ± 1.36	-34.3 ± 9.8				<0.0001
	E2+	18	11.39 ± 2.12	7.32 ± 1.29	-35.0 ± 9.5				0.0002
	combined	114	11.26 ± 2.17	7.41 ± 1.33	-33.4 ± 9.9				<0.0001
LDL-cholesterol (mmol/l)	E4+	32	8.96 ± 2.27	5.69 ± 1.33	-35.1 ± 12.6	0.350	0.916	0.272	<0.0001
	E3/E3	64	9.51 ± 2.12	5.70 ± 1.33	-39.0 ± 12.1				<0.0001
	E2+	18	9.54 ± 2.30	5.55 ± 1.21	-40.8 ± 9.5				0.0002
	combined	114	9.36 ± 2.18	5.67 ± 1.30	-38.2 ± 21.9				<0.0001
HDL-cholesterol (mmol/l)	E4+	32	1.09 ± 0.30	1.15 ± 0.29	+6.8 ± 15.7	0.119	0.251	0.895	0.0275
	E3/E3	64	1.03 ± 0.23	1.08 ± 0.24	+6.5 ± 17.2				0.0097
	E2+	18	0.95 ± 0.26	1.03 ± 0.30	+8.9 ± 13.8				0.0187
	combined	114	1.04 ± 0.26	1.09 ± 0.27	+6.9 ± 16.2				0.0001
Triglycerides (mmol/l)	E4+	32	1.89 ± 1.11	1.37 ± 0.84	-23.2 ± 26.0	0.507	0.455	0.758	<0.0001
	E3/E3	64	1.90 ± 1.35	1.37 ± 0.64	-19.5 ± 28.5				<0.0001
	E4+	18	0.95 ± 0.79	1.65 ± 0.97	-9.4 ± 69.6				0.0347
	combined	114	1.92 ± 1.20	1.42 ± 0.76	-18.9 ± 37.2				<0.0001

Values are mean ± SD.
+, -: Increase and decrease in lipid levels, respectively.
P: Level of significance estimated by the Kruskal-Wallis test to test the differences:
[A]: in mean lipid levels between the apoE groups for $t=0$.
[B]: in mean lipid levels between the apoE groups for $t=12$.
[C]: in response to simvastatin between the apoE groups.
P^D: level of significance estimated by the Wilcoxon test to test the differences between the $t=0$ and $t=12$ mean lipid levels.

Table 3. Plasma-cholesterol, LDL-cholesterol, HDL-cholesterol and plasma-triglycerides levels at $t=0$ and $t=12$ and the difference (Δ) between these two levels (expressed in % of the $t=0$ value) for male and female FH patients separately

			Effect of simvastatin					
	Sex	n	$t=0$	$t=12$	Δ (% van $t=0$)	P^A	P^B	P^C
Cholesterol (mmol/l)	males	71	11.03 ± 2.01	7.42 ± 1.34	-32.0 ± 9.7	0.272	0.833	0.074
	females	43	11.64 ± 2.38	7.39 ± 1.33	-35.5 ± 10.0			
LDL-cholesterol (mmol/l)	males	71	9.13 ± 2.02	5.72 ± 1.30	-36.4 ± 11.7	0.323	0.595	0.041
	females	43	9.72 ± 2.42	5.59 ± 1.32	-41.2 ± 12.0			
HDL-cholesterol (mmol/l)	males	71	0.96 ± 0.22	1.02 ± 0.23	+7.1 ± 16.7	0.0004	0.0002	0.721
	females	43	1.15 ± 0.28	1.21 ± 0.27	+6.7 ± 15.5			
Triglycerides (mmol/l)	males	71	2.05 ± 1.39	1.49 ± 0.78	-17.1 ± 43.8	0.315	0.081	0.721
	females	43	1.70 ± 0.77	1.29 ± 0.71	-22.1 ± 22.7			

Values are mean ± SD.
+, -: Increase and decrease in lipid levels, respectively.
P: Level of significance estimated by the Kruskal-Wallis test to test the differences:
[A]: in mean lipid levels between males and females at $t=0$.
[B]: in mean lipid levels between males and females at $t=12$.
[C]: in response to simvastatin between males and females.

Table 4. LDL-cholesterol levels (mmol/l) at $t=0$ and $t=12$ and the difference (Δ) between these two levels (expressed in % of the $t=0$ value) for the different apoE groups for male and female FH patients separately

			Effect of simvastatin					
	ApoE groups	*n*	$t=0$	$t=12$	Δ (% van $t=0$)	P^A	P^B	P^C
Males	E4+	19	9.03 ± 1.55	5.70 ± 1.18	-36.3 ± 11.5	0.242	0.716	0.687
	E3/E3	39	8.97 ± 2.07	5.71 ± 1.37	-35.3 ± 12.2	0.026	0.907	0.040
	E2+	13	9.81 ± 2.45	5.78 ± 1.35	-40.0 ± 10.3	0.588	0.257	0.730
P^D			0.674	0.958	0.527			
Females	E4+	13	8.85 ± 3.11	4.95 ± 1.58	-33.4 ± 14.5			
	E3/E3	25	10.34 ± 1.95	5.67 ± 1.29	-44.9 ± 9.5			
	E2+	5	8.85 ± 1.89	5.68 ± 0.46	-42.9 ± 7.3			
P^E			0.063	0.472	0.068			

Values are mean ± SD.
-: Decrease in lipid levels.
P: Level of significance estimated by the Kruskal-Wallis test to test the differences:
[A]: In mean lipid levels between males and females for the same apoE group for $t=0$.
[B]: In mean lipid levels between males and females for the same apoE group for $t=12$.
[C]: In response to simvastatin between males and females for the same apoE group.
[D]: In mean lipid levels and response to simvastatin between the different apoE groups for males.
[E]: In mean lipid levels and response to simvastatin between the different apoE groups for females.

male FH- and female FH patients with respect to LDL-cholesterol levels and apoE phenotype is presented in Table 4. Two, closely related, significant differences between male- and female FH patients could be detected. The mean LDL-cholesterol level at $t=0$ of the female FH patients with an apoE3E3 phenotype was 1.49 mmol/l higher ($P=0.026$) than the mean value of male FH patients with the same apoE phenotype. At $t=12$ this difference was reduced to 0.05 mmol/l (in favour of the female FH patients). This implies a 9.4 % better response to simvastatin treatment of LDL-cholesterol among female FH patients with an apoE3E3 phenotype ($P=0.040$). These differences could not be detected between male- and female FH patients with other apoE phenotypes.

Discussion

Eto et al. (19) reported a significantly higher frequency of E*4 allele carrying subjects in their FH patient group when compared with a control population. Neither in our study nor in that of O'Malley and Illingworth (20) a significant difference between the FH patient group and the control group, with respect to apoE phenotype distribution, was found.

In normal populations a marked difference in plasma total cholesterol- and LDL-cholesterol concentrations has been found between the different apoE phenotype groups; the highest levels are found in E*4 allele carriers and the lowest levels in subjects with the E*2 allele (14-16). This effect of the apoE polymorphism on plasma lipid levels is 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 apoE2 to hepatic lipoprotein receptors (26). 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 lower plasma LDL concentrations. In addition, it was recently shown that formation of LDL from VLDL is decreased in apoE2E2 homozygous subjects (27), leading to a reduced plasma LDL concentration. Eto et al. (19) reported a significantly higher total cholesterol level in plasma among the E*4 allele carriers in their FH patient group. However, in the same patient group they did not find significant differences in LDL-cholesterol levels between the different apoE groups. In the present study, we could not demonstrate any significant effect of the apoE polymorphism on the baseline levels of total plasma-

and LDL-cholesterol levels in the combined FH patient group, which confirms the results reported by O'Malley and Illingworth (20) and Nestruck et al. (28). Pedersen and Berg (29), reported that an interaction between the apoE gene and the LDLR gene can influence LDL concentrations. They found that individuals with an apoE*4 allele and the LDLR *Pvu*II A2A2 genotype have significant higher LDL concentrations, whereas individuals with an apoE*2 allele and the LDLR *Pvu*II A2A2 genotype have significant lower LDL concentrations. These differences could not be found among individuals with the LDLR *Pvu*II A1A1 or A1A2 genotypes. We were not able to analyse our FH patients for the LDLR *Pvu*II polymorphism, but we analysed our lipoprotein data for a bimodality within each apoE phenotype group with respect to the measured lipoprotein levels and to the responses to simvastatin treatment. Not any bimodality could be detected. Whether this difference between the results of Pedersen and Berg (29) and ours is due to the difference between the populations studied i.e healthy individuals versus FH patients, is subject to speculation.

In their FH population, O'Malley and Illingworth (20) reported a significantly higher baseline triglyceride concentration in E*2 allele carriers when compared with non-E*2 allele carriers, whereas Eto et al. (19) reported a significantly higher triglyceride concentration in E*4 allele carrying FH patients. In our study the highest basline triglyceride concentration was found in the E*2 carrying FH patients, but the differences in triglyceride levels between the different apoE groups of FH patients were not significant.

Nestruck et al. (28) reported a significantly better effect of probucol among a group of E*4 allele carrying FH patients. With respect to HMG-CoA reductase inhibitors, Davignon et al (30) found a marked difference in effect of lovastatin treatment between two siblings; the one with the apoE4E3 phenotype responded much better than the other with apoE3E3 phenotype (21.5 % vs 9.5% reduction of plasma LDL-cholesterol level). In contrast, O'Malley and Illingworth (20) did not find any significant relation between the apoE polymorphism and the lowering of total plasma and LDL-cholesterol by lovastatin in FH patients. In our combined FH population we could not identify any significant difference between the three apoE groups in the response of lipid parameters to simvastatin treatment although the individuals carrying the E*4 allele tended to respond less efficiently with respect to total plasma and LDL-cholesterol and more efficiently in reducing the plasma triglyceride levels. We also repeated all our statistical analysis regarding only two groups: E*4 allel carriers versus non E*4 allele carriers as well as subjects with the E*2 allele and without the E*2 allele. This pooling however, did not result in any additional significant difference (results not shown).

We observed significant differences between the sexes for baseline- and post-treatment HDL-cholesterol levels and LDL-cholesterol response to simvastatin treatment. Similar to the results of O'Malley and Illingworth (20), female FH patients had higher HDL-cholesterol levels at both time points than male FH patients, whereas the response of HDL-cholesterol on simvastatin treatment was the same in both sexes (Table 3). The LDL-cholesterol responded 5 % more efficiently in female- than male FH patients (Table 3). This was caused by a 9.4 % more effective lowering of LDL-cholesterol among female FH patients with the apoE3E3 phenotype. These apoE3E3 females had a significantly higher baseline LDL-cholesterol level than male FH patients with the same apoE3E3 phenotype. The data presented by O'Malley and Illingworth (20) do not allow us to compare our data with theirs with respect to sex differences. Again, no extra significant differences were found between male- and female FH patients when only two groups were considered i.e. E*4 allele carriers versus non E*4 allele carriers or E*2 allel carriers versus non E*2 allele carriers.

Our results show that in FH patients the LDL-receptor activity in the liver is not influenced by a difference in the uptake of chylomicron- and VLDL-remnants as a result of the apoE polymorphism. We suggest that the absence of such an effect of the apoE polymorphism in the FH patient population could be due to high plasma LDL-cholesterol levels in these patients. This hypothesis is sustained by our observation that in a normal population, counting only subjects with an above average plasma cholesterol level, no effect of the apoE polymorphism on plasma cholesterol level could be detected (unpublished results). We furthermore conclude that the apoE polymorphism is not responsible for the considerable interindividual variation in response of LDL-cholesterol to treatment with HMG-CoA reductase inhibitors as has been suggested by others. Recently our results have been confirmed by the results obtained by O'Malley and Illingworth (20) using lovastatin treatment.

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CHAPTER 8

Genetic Heterogeneity in Familial Dysbetalipoproteinemia. The E2(Lys146 → Gln) Variant Results in a Dominant Mode of Inheritance

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Abstract

As determined by isoelectric focusing, most patients with familial dysbetalipoproteinemia (FD) exhibit the homozygous apolipoprotein (apo) E2E2 phenotype. Only rarely does FD develop in the more common heterozygous phenotypes E3E3 or E4E2. In fact, only 1 to 4% of the E2E2 homozygotes will develop FD. We wondered whether this reduced penetrance of FD in E2E2 homozygotes could be due to additional heterogeneity in the APOE*2 allele. In the literature a number of different mutations causing an E2 isoelectric focusing variant have been described. To study the genetic heterogeneity of the APOE gene, hybridization of enzymatically amplified genomic DNA with mutation-specific oligonucleotide probes was applied. All FD patients (n= 40) with the E2E2 phenotype appeared to be homozygous for the common E2(Arg158→Cys) mutation. However, all three unrelated patients with the E3E2 phenotype exhibited the rare E2(Lys146→Gln) mutation due to an A→C substitution at nucleotide position 3.847 of the APOE gene. This mutation was not found among normolipidemic individuals with the E2E2 (n= 13) or E3E2 phenotype (n= 120) selected from a random population sample. Family studies of the three probands heterozygous for the E*2(Lys146→Gln) allele showed that this rare allele predisposes to FD with high penetrance.

We conclude that FD is a genetically heterogeneous disease entity, displaying a recessive mode of inheritance with strongly reduced penetrance in case of the common E2(Arg158→Cys) variant and with a dominant mode of inheritance with high penetrance in case of the rare E2(Lys146→Gln) mutant. It should be noted that in this dominant form presymptomatic diagnosis is possible.

Introduction

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

The apolipoprotein (apo) E present on the lipoprotein remnants plays an important role as ligand for the high affinity hepatic lipoprotein receptors (3). With isoelectric focusing apoE can be separated into three common isoforms, i.e., E2,

E3 and E4 (4,5) encoded by codominant alleles at a single APOE gene locus on chromosome 19 (6). The apoE 3 isoform is the most frequently occurring (or wild type) isoform.

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

ApoE4 differs from E3 by an amino acid substitution (Cys→Arg) at position 112 and is designated E4(Cys112→Arg). At present four different mutations, giving a band at the E2 position with isoelectric focusing, have been described. These are designated E2(Arg158→Cys), E2(Lys146→Gln), E2(Arg145→Cys), and E2-Christchurch(Arg136→Ser) (9,12-14). E2(Arg158→Cys) is the most common E2 mutation. In addition, double mutations have also been described: E3(Cys112→Arg;Arg142→Cys) (15), E3(Ala99→Thr;Ala152→Pro) (16), E1(Gly127→Asp;Arg158→Cys) (17), and E1-Harrisburg(Lys146→Glu) (18). Recently, we encountered three unrelated FD patients with the E3E2 phenotype (19). These patients exhibit a rare E2 variant containing only one cysteine residue. This E2 variant cosegregates with FD in their families, suggesting that it is a dominant trait in the expression of FD.

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

Materials and methods

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

ApoE phenotyping EDTA plasma was obtained after venepuncture and stored at -20°C until use. ApoE phenotyping was performed using a rapid micro-method based on isoelectric focusing of delipidated plasma followed by immunoblotting

using a polyclonal anti-apoE antiserum (20).

Genomic DNA isolation DNA was isolated from leucocytes of whole blood by standard methods (21).

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

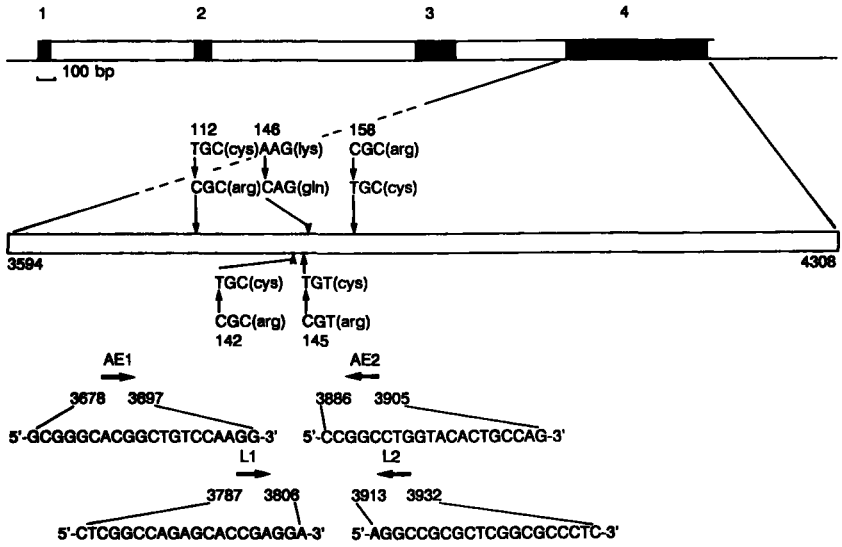

Figure 1. Schematic representation of the APOE gene; exon 4 enlarged. The most common mutations are indicated. Localization and sequence of the two sets of oligonucleotides/amplimers (AE1/AE2 and L1/L2) used for the amplification are indicated. The AE1/AE2 amplimers are identical to those described by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

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

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

temperature), and 2 min elongation (65°C) continued for 35 cycles, with 1 unit Taq polymerase extra added at rounds 10 and 20, and 0.5 units at round 30.

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

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

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

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

Hybridization was performed as described by den Dunnen et al. (25). The temperature during the hybridization and the washing conditions for the different sets of oligonucleotides were experimentally determined and are presented in Table 1. In general, the hybridization temperature was chosen 10-12 degrees below the melting 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 corresponding nucleotides in the oligomer (26).

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

DNA sequencing of the APOE gene For DNA sequencing of the relevant part of the APOE gene, genomic DNA was amplified by PCR using 20-mer oligonucleotides L3 (nucleotides 3,555-3,574 of the coding strand) and L2 (nucleotides 3,932-3,913 of the noncoding strand) as amplimers. The conditions applied for PCR were as described before. *Pst*I-digested amplified DNA, containing nucleotides 3,700-3,873 of the APOE gene, was cloned into *Pst*I-digested

M13mp19. The 174 base pairs inserts in M13 were sequenced by the Sanger method with the T7Sequencing™ Kit (Pharmacia) using [α-^{32}P]dATP.

Results

APOE genotyping For a routine screening of the various mutant alleles (genotyping), amplified DNA was subjected to electrophoresis on a 1.5% agarose gel, blotted to GeneScreen Plus filters, and hybridized with the respective mutation-specific oligonucleotide probes (for nucleotide sequences and hybridization and washing conditions of the oligonucleotides see Table 1).

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

Oligonucleotide [A]	Sequence [B]	Hybridization Temperature	Washing Procedure and Temperature [C]	
		°C		°C
112-Arg	5'-AGGCGGCCGC**G**CACGTCCTCC-3'	64	1	73.5
112-Cys	5'-AGGCGGCCGC**A**CACGTCCTCC-3'	64	1	73.5
146-Gln	5'-GGAGCCGCT**G**ACGCAGCTT-3'	53	1	63.5
146-Lys	5'-GGAGCCGCT**T**ACGCAGCTT-3'	53	1	63.5
158-Cys	5'-CTGCCAGGC**A**CTTCTGCAG-3'	53	2	63.5
158-Arg	5'-CTGCCAGGC**G**CTTCTGCAG-3'	53	2	63.5
145-Cys	5'-AGCCGCTTAC**A**CAGCTTGCGC-3'	57	1	63.5
142-Cys	5'-TCCCACCTG**T**GCAAGCTGC-3'	53	1	61

[A] Nomenclature: 112-Arg stands for the oligonucleotide specific to the allele coding for an apoE variant with an arginine residue at position 112, etc.
[B] Sequence: the sequence is deduced from Paik et al. (34). The substituted nucleotide is underlined.
[C] Washing procedures: procedure 1: 2 x 5' at room temperature in 5 x SSPE, 0.3% SDS; 10' at hybridization temperature in 1 x SSPE, 0.3% SDS; 10' at specific wash temperature in 1 x SSPE, 0.3% SDS; procedure 2: 2 x 30' at room temperature in 1.5 x SSC, at 0.1% SDS; 15' at hybridization temperature in 3 x SSC, 0.1% SDS; 10' at specific wash temperature in 3 x SSC, 0.1% SDS.

After autoradiography for 1 to 3 hours strong signals were obtained (see Fig. 2 left for examples). As the hybridization signals corresponded with the amplified fragments visible on the ethidium bromide-stained gel (not shown), the electrophoresis step could be substituted by a direct dot blot procedure (Fig. 2 right). The results presented in Fig. 2 are schematically presented in Table 2. Two rare variants are presented. Sample E represents a subject heterozygous for the wild type or E3 isoform and the rare E2(Lys146→Gln) variant, whereas sample G is heterozygous for the common E2(Arg158→Cys) variant and another as yet unknown E2 variant.

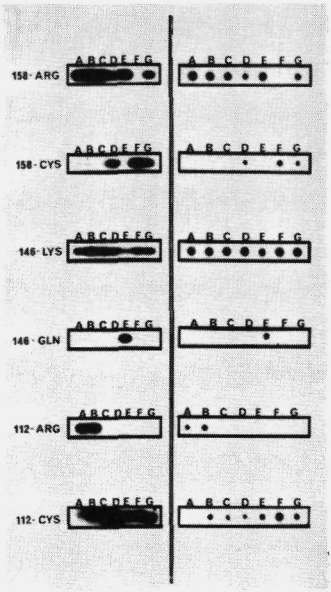

Figure 2. Detection of the APOE mutants using DNA amplification and hybridization with mutant-specific oligonucleotide probes (APOE genotyping). For detection with the 112-Arg and 112-Cys oligonucleotide probes, the samples were amplified with the AE1 and AE2 amplimers. For detection with the other oligonucleotides,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

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

	Phenotype by Isoelectric Focusing						
	A	B	C	D	E	F	G
Oligonucleotide	4/4	4/3	3/3	3/2	2/2	2/2	2/2
158-Arg	+	+	+	+	+	-	+
158-Cys	-	-	-	+	-	+	+
146-Lys	+	+	+	+	+	+	+
146-Gln	-	-	-	-	+	-	-
112-Arg	+	+	-	-	-	-	-
112-Cys	-	+	+	+	+	+	+
145-Cys [A]	ND	ND	-	-	ND	-	-
142-Cys [A]	ND	ND	-	-	ND	-	-
Genotype							
Allele 1:	Arg112	Arg112	WT	WT	WT	Cys158	Cys158
Allele 2:	Arg112	WT	WT	Cys158	Gln146	Cys158	?

[A] The hybridization results using the 145-Cys and 142-Cys oligonucleotides are not shown in Figure 2. ND; not determined; WT, wild type or E*3 allele; ?, a new unknown allele.

Screening of patients and healthy controls The method of DNA amplification followed by hybridization with mutant-specific oligonucleotides (Fig. 2) was applied for APOE genotyping of a population sample of 40 FD patients with the E2E2 phenotype and 3 FD patients with the E3E2 phenotype. The amplified DNA samples were hybridized separately with the respective mutation-specific oligonucleotides presented in Table 1. All FD patients with the E2E2 phenotype appeared to be homozygous for the common E2(Arg158→Cys) mutation, whereas all 3 patients with the E3E2 phenotype were shown to carry the E2(Lys146→Gln) mutation (Table 3).

Table 3. APOE genotyping of FD patients and healthy controls.

Subjects	Phenotype	Genotype
FD patients	E2E2 (n= 40)	E2(Arg158→Cys) / E2(Arg158→Cys)
FD patients	E3E2 (n= 3)	E3 / E2(Lys146→Gln)
Controls	E2E2 (n= 11)	E2(Arg158→Cys) / E2(Arg158→Cys)
Controls	E2E2 (n= 2)	E2(Arg158→Cys) / E2 (?)
Controls	E3E2 (n= 50)	E3 / E2(Arg158→Cys)

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

To definitely prove that these patients carry the E2(Lys146→Gln) mutation, we sequenced the relevant part of the APOE gene of one of these patients. Therefore, a 174 base pairs *Pst*I fragment of amplified APOE gene DNA was cloned into *Pst*I-digested M13mp19 (see Materials and Methods). Five independent clones were analyzed by DNA sequencing. The sequence of two clones was identical to the normal E*3 allele, whereas three clones indeed showed an A→C substitution at position 3,847 leading to an amino acid substitution Lys→Gln at position 146 (Fig. 3).

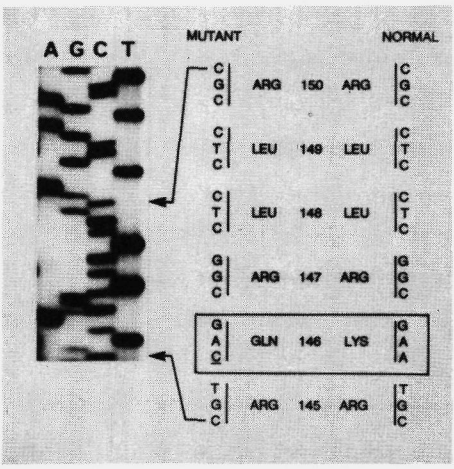

Figure 3. Sequence of the relevant part of the mutant APOE*2 allele of an FD patient (pedigree #II-3 of family N in Fig. 4). The square denotes the codon with the substitution A→C, leading to amino acid substitution Lys146→Gln. The corresponding sequence of the normal allele is derived from Paik et al. (34).

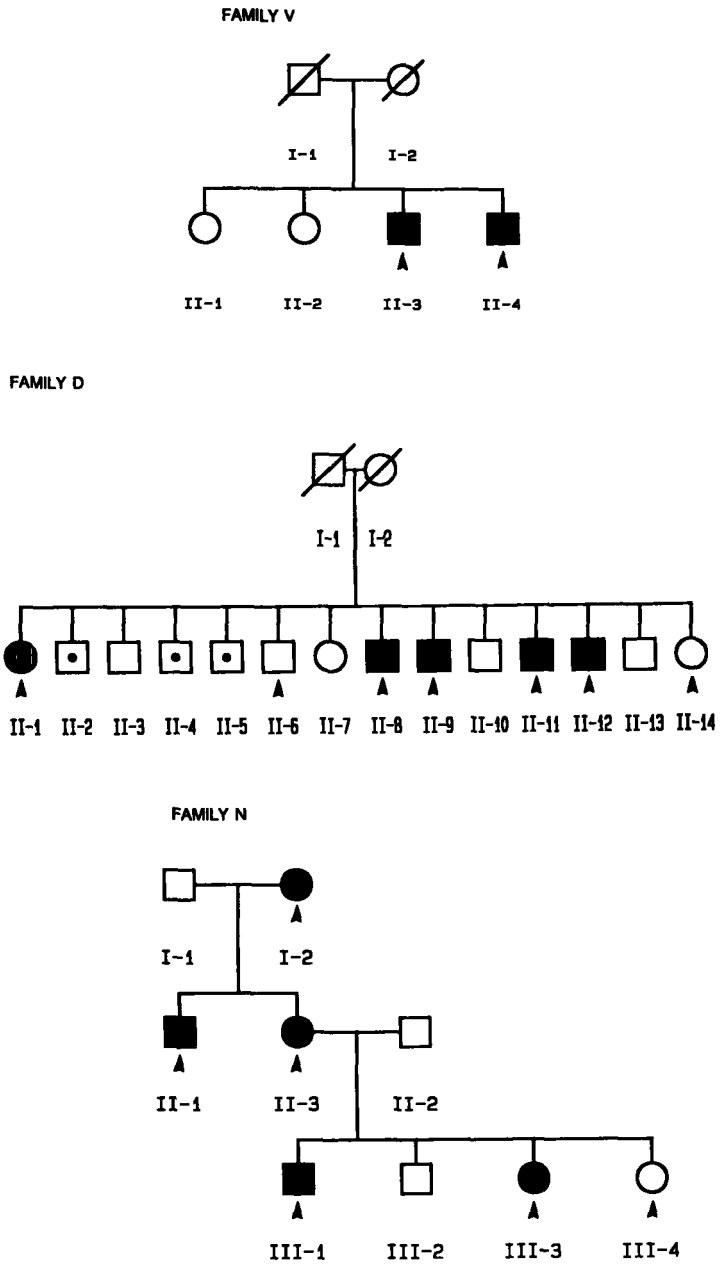

Figure 4. Pedigrees of the three families carrying the E*2(Lys146→Gln) allele (▲); (■,●) males and females with FD symptoms; (□,○) males and females not studied; (□,○) males and females deceased.

Recently we performed apoE phenotyping by IEF in a random population sample of 2,000 35-year-old males (27,28). In this population study, 13 E2E2 homozygotes were found. Eleven out of these 13 E2E2 individuals appeared to be homozygous for the E2(Arg158→Cys) mutation, whereas the remaining 2 appeared to be heterozygous for this common E2(Arg158→Cys) mutation and another, so far unknown, E2 mutation (Table 3). From this random population sample we also selected 50 individuals with the E3E2 phenotype. All these individuals appeared to be heterozygous for the common E2(Arg158→Cys) mutation. The E2(Lys146→Gln) variant was not found in these control individuals.

The results in Table 3 show that the E*2(Lys146→Gln) allele is very rare and so far found exclusively in FD patients with the E3E2 phenotype. This suggests that the E*2(Lys146→Gln) mutation is dominant in the predisposition of an individual to FD. To sustain this hypothesis we performed detailed family studies.

*Family studies of the three FD probands carrying the E*2(Lys146→Gln) allele*

Figure 4 shows the pedigrees of the three probands carrying the E*2(Lys146→Gln) allele. The clinical and genetic parameters of these families are presented in Table 4. In family V the two brothers (proband represents II-4) carrying the E*2(Lys146→Gln) allele display manifest FD with elevated plasma cholesterol and triglyceride levels. They both exhibit typical FD-associated xanthomas. Because of ethical rules, we were not allowed to study the young children of proband II-4. The two sisters (II-1 and II-2) are homozygous E3E3. Although they have slightly elevated plasma lipid levels, they were not classified as FD.

From family D we studied 10 siblings of the proband (II-9). As presented in Table 4, 5 out of 7 individuals carrying the E*2(Lys146→Gln) allele display FD. Two siblings carrying the E*2(Lys146→Gln) allele were not FD. Subject II-6 was very lean, whereas subject II-16 was a female before menopause, two conditions that may prevent or delay the expression of FD. The four siblings not carrying the rare E*2(Lys146→Gln) allele had normal plasma lipid levels. The remaining family members were not available for analysis.

In family N we were able to study three generations (proband II-3). From the results presented in Table 4 it is obvious that in this family, again, all family members carrying the E*2(Lys146→Gln) allele displayed elevated plasma cholesterol and triglyceride levels and clinical symptoms of FD, except the 19-year-old female III-4. All family members not carrying the rare E*2(Lys146→Gln) allele showed normal plasma lipid levels except subject I-1 who had an increased plasma triglyceride level.

Table 4. Clinical and genetic parameters of the families carrying the E*2(Lys146→Gln) allele.

Subject	Age/Sex	TC	TG	FD	Xanthomas	E-Phenotype	E*2(Lys→Gln)
Family V							
II-1	60/F	7.60	3.27	no	no	E3E3	-
II-2	56/F	6.45	2.18	no	no	E3E3	-
II-3	50/M	7.19	3.05	yes	yes	E3E2	+
II-4	37/M	14.45	8.30	yes	yes	E3E2	+
Family D							
II-1	54/F	9.50	3.90	yes	no	E3E2	+
II-3	50/M	3.44	1.83	no	no	E3E3	-
II-6 ^	47/M	6.20	1.77	no	no	E3E2	+
II-7	42/F	5.90	1.58	no	no	E3E3	-
II-8 ^	49/M	7.88	1.91	yes	no	E3E2	+
II-9	46/M	9.87	4.69	yes	yes	E3E2	+
II-10	42/M	4.64	1.08	no	no	E3E3	-
II-11	45/M	7.18	6.77	yes	no	E3E2	+
II-12	30/M	13.86	5.30	yes	yes	E3E2	+
II-13	37/M	6.81	1.55	no	no	E3E3	-
II-14	40/F	6.36	2.35	no	no	E3E2	+
Family N							
I-1	79/M	4.64	3.36	no	no	E3E3	-
I-2	75/F	8.87	3.75	yes	yes	E3E2	+
II-1	43/M	21.88	17.31	yes	yes	E3E2	+
II-2	49/M	5.50	2.20	no	no	E3E3	-
II-3	40/F	18.44	16.15	yes	yes	E3E2	+
III-1	25/M	6.67	2.25	yes	no	E3E2	+
III-2	27/M	4.50	1.15	no	no	E3E3	-
III-3	23/F	5.42	3.26	yes	no	E3E2	+
III-4	18/F	6.43	2.65	no	no	E3E2	+

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

^ Very lean subject.

Discussion

Most FD patients have the E2E2 phenotype. In the normal population the frequency of the E2E2 phenotype is about 1% (28), whereas the prevalence of FD in the general population has been estimated as 1-4 in 10.000. Hence, only 1 to 4% of all individuals with the E2E2 phenotype develop FD. Utermann et al. (29) suggested that another gene and/or environmental factors are required in addition to the E2E2 phenotype for the expression of FD. However, it is also possible that additional heterogeneity in the APOE*2 allele is responsible for the apparent reduced penetrance of FD in E2E2 homozygotes; i.e., a subvariant of apoE2 may be predominant in FD patients. Four apoE2 variants have been described thus far: E2(Arg158→Cys), E2(Lys146→Gln), E2(Arg145→Cys), and E2-Christchurch (Arg136→ Ser) (9,12-14). Unequivocal detection of each of these different apoE2 variants cannot be performed simply by isoelectric focusing. Therefore, in order to discriminate between the different apoE2 variants, we used DNA hybridization with mutation-specific oligonucleotides.

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

To improve the sensitivity and specificity of the method for the detection of known mutations in the APOE gene, we included an in vitro amplification step applying the heat-stable Taq polymerase. Starting with 1 μg of genomic DNA, this procedure produces strong and specific signals within 1 to 3 h of exposure and is therefore suitable for routine screening. A similar procedure, but using Klenow polymerase, was recently published by Smeets et al. (32) for the detection of the E2(Arg158→Cys) and E4(Cys112→Arg) mutations.

Using this method, we evaluated the presence of the known E4(Cys112→Arg), E2(Arg158→Cys), E2(Lys146→Gln), E2(Arg145→Cys), and E3(Cys112→Arg; Arg142→Cys) variants in FD patients and in control subjects. We demonstrated that all 40 FD patients with the E2E2 phenotype were homozygous for the mutation Arg158→Cys. The same was true for 11 out of 13 clinically normal individuals with the E2E2 phenotype. In addition, 50 normal subjects with the E3E2 phenotype also displayed heterozygosity for the Arg158→Cys mutation. Since these heterozygotes do not express FD, it is obvious that the E2(Arg158→Cys) mutation behaves like

a recessive trait in the expression of FD (both alleles need to be defective). Furthermore, since both patients and controls with the E2E2 phenotype appeared to be homozygous for the common E2(Arg158→Cys) variant, it seems that the reduced penetrance of FD in individuals with the E2E2 homozygosity is not due to heterogeneity in the E*2 allele, although the existence of additional unknown mutations within the APOE gene of E2E2 homozygous FD patients cannot be excluded by this approach. For a definite conclusion in this respect, it is necessary to determine the complete nucleotide sequences of the APOE genes of both E2E2 homozygous FD patients and E2E2 homozygous control subjects.

In a previous paper (19) we described three unrelated FD patients with the E3E2 phenotype. The E2 variant present in these patients contains only one cysteine residue and we suggested that it was the E2(Lys146→Gln) variant previously found in two E3E2 heterozygous FD patients (siblings) by Rall et al. (12). All other known E2 variants contain two cysteine residues (9,13,14). In the present study, using the method of amplification followed by hybridization with variant-specific oligonucleotide probes, we were able to prove the presence of the E2(Lys146→Gln) mutation in these E3E2 heterozygous FD patients. In one of these FD patients with the E3E2 phenotype we confirmed the E2(Lys146→Gln) mutation by DNA sequence analysis (Fig. 3). None of the 53 individuals (40 FD patients and 13 controls) with the E2E2 phenotype tested in the present study appeared to carry the E2(Lys146→Gln) mutation (see Table 3). The same was true for 50 unrelated E3E2 normolipidemic individuals randomly selected from a large population sample previously screened for apoE phenotypes (27,28). They all appeared to contain the E2(Arg158→Cys) mutation (Table 3). Additionally, another 70 E3E2 individuals selected from the same population sample were studied by the cysteamine modification followed by isoelectric focusing (19). Two cysteine residues were found in all E2 isoforms tested. Thus, in a total number of 120 E3E2 subjects selected from a random population sample, the presence of the E2(Lys146→Gln) mutation could not be detected. Based on these results, we conclude that the E*2(Lys146→Gln) allele is very rare with an allele frequency of less than 0.001 and, so far, exclusively found in FD patients with the E3E2 phenotype.

The E2(Lys146→Gln) mutation has now been found in several apparently unrelated families; one in the U.S.A. (12) and three in The Netherlands (present study). For the Dutch families, genealogical studies are presently being conducted in order to find out whether these three families share a common ancestor. The two E3E2 heterozygous subjects (siblings) exhibiting the rare E2(Lys146→Gln) variant found by Rall et al. (12) also express FD. These facts, taken together, strongly suggest that heterozygosity for the Lys146→Gln mutation is sufficient for the

expression of FD. To sustain this dominance hypothesis, we performed detailed family studies. In all three kindreds studied (Fig. 4; Table 4) we found that the E*2(Lys146→Gln) allele indeed cosegregates with FD except for three subjects (III-4 in family N and II-6 and II-14 in family D). The absence of FD in these E3E2(Lys146→Gln) heterozygotes might be due to a "healthy" life-style and/or young age. It should be noted in this respect that the FD patients carrying the E*2(Lys146→Gln) allele also respond to diet and medical treatment. We conclude that, in contrast to the common E2(Arg158→Cys) variant, the E2(Lys146→Gln) mutation behaves like a dominant trait in the expression of FD (one defective allele is sufficient for the expression of the disease). This dominance hypothesis does not necessarily imply that in the case of this rare variant the level of penetrance of FD is 100%. Nevertheless, our results show that in the case of the E2(Lys146→Gln) variant, FD is inherited with a high level of penetrance; whereas in the case of homozygosity for the E2(Arg158→Cys) mutation, the recessively inherited FD disease is commonly assumed to be inherited with a strongly reduced penetrance (1 to 4%) (29).

Recently, Mann et al. (18) described a kindred in which heterozygosity for an E1 variant (E1-Harrisburg) also cosegregates with the expression of FD. Strikingly, in E1-Harrisburg the mutation is also localized at amino acid position 146 (Lys146→Glu) suggesting that this lysine residue plays a crucial role in the removal of chylomicron- and VLDL-remnants in vivo.

Previously, we described the E3-Leiden mutation which also behaves like a dominant trait in the expression of FD (33). Recently, a second family has been discovered in which the E3-Leiden variant cosegregates with familial dysbetalipoproteinemia (Havekes et al., unpublished data). Similarly, the E3(Cys112→Arg; Arg142→Cys) also cosegregated with FD in the family in a dominant manner (15).

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

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CHAPTER 9

Familial Dysbetalipoproteinemia Associated with Apolipoprotein E3-Leiden in an Extended Multigeneration Pedigree

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Abstract

By carefully screening of Familial dysbetalipoproteinemic (FD) patients, five probands showing heterozygosity for the APOE*3-Leiden allele were found. Genealogical studies revealed that these probands share common ancestry in the seventeenth century. In a group of 128 family members, spanning three generations, 37 additional heterozygous APOE*3-Leiden gene carriers were detected. Although with a variable degree of severity, all carriers exhibited characteristics of FD such as (i) elevated levels of cholesterol in the VLDL and IDL fractions, (ii) elevated ratios of cholesterol levels in these density fractions over total plasma levels of triglycerides and (iii) strongly increased plasma levels of apolipoprotein E. Multiple linear regression analysis revealed that most of the variability in expression of FD in APOE*3-Leiden allele carriers can be explained by age. Body mass index showed a less significant influence on the expression of FD. Gender had no effect on the expression in E*3-Leiden allele carriers, nor did it influence the age of onset of FD. In the group of APOE*3-Leiden allele carriers, we found that the E*2 allele enhances the expression of FD, whereas the E*4 allele had the opposite effect. Isoelectric focusing of plasma and of isolated VLDL, IDL and HDL density fractions showed that in E*3-Leiden allele carriers the apoE3-Leiden variant largely predominates over its normal apoE counterpart, especially in the VLDL and IDL density fractions. We conclude that in APOE*3-Leiden allele carriers FD is dominantly inherited with a high rate of penetrance, i.e. the presence of normally functioning apoE molecules in the plasma does not prevent the age-related expression of this disease.

Introduction

Familial dysbetalipoproteinemia (FD) is a genetic disorder of the lipoprotein metabolism predisposing to premature coronary and/or peripheral vascular disease (1). Patients with FD are characterized by elevated plasma cholesterol and triglycerides levels due to the presence of β-migrating chylomicron- and VLDL-remnants enriched in cholesterol and apoE (2-4). As a consequence, the ratio of VLDL-cholesterol to plasma triglyceride levels is elevated. Approximately 50 % of the patients show lipid deposits like palmar streaks and tuberous xanthomas (1).

ApoE, one of the major protein constituents of chylomicron- and VLDL-remnants, plays a central role in the receptor-mediated endocytosis of these particles functioning as a high affinity ligand for hepatic lipoprotein receptors (5,6). The primary metabolic defect in FD patients is due to mutant forms of apoE on

chylomicron- and VLDL-remnants leading to an impaired clearance of these remnant particles by the liver (1,7,8).

By isoelectric focusing, three common genetic variants of apoE, designated E2, E3 and E4 according to their mobility upon isoelectric focusing, can be recognised. They each differ in isoelectric point by one charge unit, apoE4 being the most basic and apoE2 the most acidic isoform. These isoforms are encoded for by 3 codominant alleles, E*2, E*3 and E*4, at a single APOE gene locus on chromosome 19 (9). The common apoE2 is derived from the wild type apoE3, by a cysteine for arginine substitution at amino acid residue 158 [designated as apoE2(Arg158→Cys)], while apoE4 is derived from apoE3 by an arginine for cysteine substitution at residue 112 [apoE4(Cys112→Arg)].

The vast majority of FD patients are homozygous for the apoE2(Arg158→Cys) variant (1). In vitro experiments showed that this variant exhibits only 1% of the receptor binding capacity of normal apoE3 (10). Since homozygosity for the apoE2(Arg158→Cys) variant is required in order to develop FD, this variant is associated with a recessive mode of inheritance of FD. In Caucasian populations homozygosity for apoE2(Arg158→Cys) occurs with a frequency of 1%, while the frequency of FD is about 1: 2500 (1). Thus, only a small percentage of these E2E2 individuals will develop FD (1). This indicates that, in general FD is a multifactorial disease, i.e. additional factors, either genetic or environmental, are required for its manifestation (11,12).

Expression of FD has also been observed in individuals heterozygous for the rare apoE variants like apoE3(Cys112→Arg; Arg142→Cys) (13,14), apoE2(Lys146→Gln) (15-17) and apoE1-Harrisburg(Lys146→Glu) that focuses one charge unit more acidic than E2 (18,19). Family studies have confirmed that heterozygosity for these rare variants is associated with the development of FD despite the presence of a "normal" APOE allele, indicating that with these variants FD is dominantly inherited with a high degree of penetrance.

Previously, we have reported on a patient with FD exhibiting heterozygosity for a rare apoE variant focusing at the E3 position, lacking cysteine residues and designated as apoE3-Leiden (20,21). By analyzing first degree relatives, we were able to show that in this family the apoE3-Leiden variant was strongly associated with the occurrence of FD (21). Recently, DNA and protein sequencing analyses revealed that the APOE*3-Leiden allele was identical to the APOE*4(Cys112→Arg) allele, but included an in-frame repeat of 21 nucleotides (coding for seven amino acids) in exon 4. Since it was impossible to exactly define the duplication unit because of identical sequences at bp positions 3768-3772 and 3789-3793 of the normal apoE sequence (numbering according to Paik et al. (24)) the insertion leads

to a tandem repeat of codons 120-126 or 121-127 (22,23). The seven amino acids insert introduces one extra negatively charged glutamyl residue when compared with the common apoE4(Cys112→Arg) variant and thus leads to a focusing on the apoE3 position.

In the present paper we describe four additional probands showing FD in association with heterozygosity for apoE3-Leiden. Genealogical studies revealed that the five probands share common ancestry. A detailed characterization of the effect of heterozygosity for the APOE*3-Leiden allele on lipoprotein levels has been performed by studying family members of the five E3-Leiden pedigrees. All APOE*3-Leiden allele carriers showed clinical signs of FD, although with a variable degree of severity. Because of the relatively large number of APOE*3-Leiden allele carriers available (n=42), we were able to estimate the effects of age, body mass index (BMI) and gender on the expression of FD in apoE3-Leiden carriers by statistical analysis. We also determined the influence of the accompanying "normal" APOE allele on the expression of FD in these E3-Leiden subjects.

Materials and methods

Subjects. The initial proband (CV) is a Caucasian male who has been previously described as a FD patient with heterozygosity for the apoE3-Leiden variant (20). Subsequently, his first degree relatives members have been studied and five more gene carriers were found, all exhibiting FD (21). Later, four other, apparently unrelated probands with FD and heterozygosity for apoE3-Leiden have been found in lipid clinics in Nijmegen and Leiden.

Genealogical studies. Information presented to us by the five probands enabled us to perform genealogical studies. This was done by checking the parish records from the 18th century and the civil registration-, population- and census records from the 19th and 20th centuries in a region south-east of The Hague. We learned that in this region there is no high degree of consanguinity.

Collection of blood samples. From the five probands and 123 relatives, EDTA blood samples were collected. Individuals were allowed to consume a low calorie (fat free) breakfast. Plasma was separated from the cells by centrifugation at 500 g for 10 min at room temperature and used for lipid and lipoprotein analysis; the cells were used to isolate genomic DNA.

Lipid and lipoprotein analysis. For the isolation of VLDL (d < 1.006 g/ml), 2 ml plasma was overlaid with 2.5 ml solution of NaCl (d=1.006 g/ml) in a 5 ml tube fitting the 40 Ti swing out rotor (Beckman, Geneva, Switzerland). After

centrifugation at 90,000 g for 16 hours at 4 °C, VLDL was aspirated from the top. HDL was determined in the infranatant after precipitation of IDL and LDL (25). For the isolation of VLDL + IDL ($d < 1.019$ g/ml), 2 ml plasma was brought to a background density of 1.019 g/ml with KBr and overlaid with 3.5 ml solution of NaCl ($d = 1.019$ g/ml) in a 10.4 ml centrifuge tube fitting the 50 Ti fixed angle rotor (Beckman). After centrifugation at 106,000 g for 16 hours at 4 °C, VLDL + IDL were aspirated from the top.

Plasma cholesterol and cholesterol in the VLDL ($d < 1.006$ g/ml), VLDL+IDL ($d < 1.019$ g/ml) and HDL cholesterol were measured using the Boehringer (Mannheim, FRG) CHOD-PAP kit (# 236691). Plasma and lipoprotein triglycerides were measured using the Boehringer (Mannheim, FRG) GPO-PAP kit (# 701904). IDL-cholesterol was calculated as the difference between VLDL-cholesterol ($d < 1.006$ g/ml) and VLDL+IDL-cholesterol ($d < 1.019$ g/ml). LDL-cholesterol ($1.019 < d < 1.063$ g/ml) was calculated using the formula LDL-chol = plasma chol - (VLDL-chol + IDL-chol + HDL-chol).

Agarose electrophoresis was performed as described by Demacker (26).

Apolipoprotein E quantification, phenotyping and genotyping. Apolipoprotein E concentrations in plasma were measured by ELISA as described by Bury et al. (27). In short, affinity-purified polyclonal Goat anti-human apoE antibodies, raised in our Institute as described before (28), were used for both coating and for preparing the antibody-peroxidase conjugate. The pool-plasma's of healthy individuals, used as internal standards, were calibrated with a standard curve constructed with purified human apoE as primary standard. Mean intra- and interassay CV's were 4.3 and 8.2 %, respectively.

The apoE phenotype was determined by isoelectric focusing of delipidated plasma samples before and after cysteamine treatment followed by immunoblotting as described (28). For apoE phenotyping in the different lipoprotein fractions, VLDL, IDL and HDL fractions were isolated by density-gradient ultracentrifugation of freshly prepared plasma by the method of Redgrave et al. (29) followed by extensive dialysis of the fractions against PBS.

For genotyping, genomic DNA was isolated from leucocytes by standard methods (30). The 5' part of exon 4 of the APOE gene was amplified by the polymerase chain reaction (PCR) using the primers 402 (nucleotides 3,555-3,574, coding strand) and 401 (nucleotides 3,932-3,913, non-coding strand) (24) (Figure 1) giving a fragment of 378 bp (or 399 bp in the case of the APOE*3-Leiden allele). This fragment encodes the amino acid residues 61-174 of the mature protein (see Figure 1 and ref. 22).

After PCR, 5 μl aliquots were separated on a 2% agarose gel by electrophoresis,

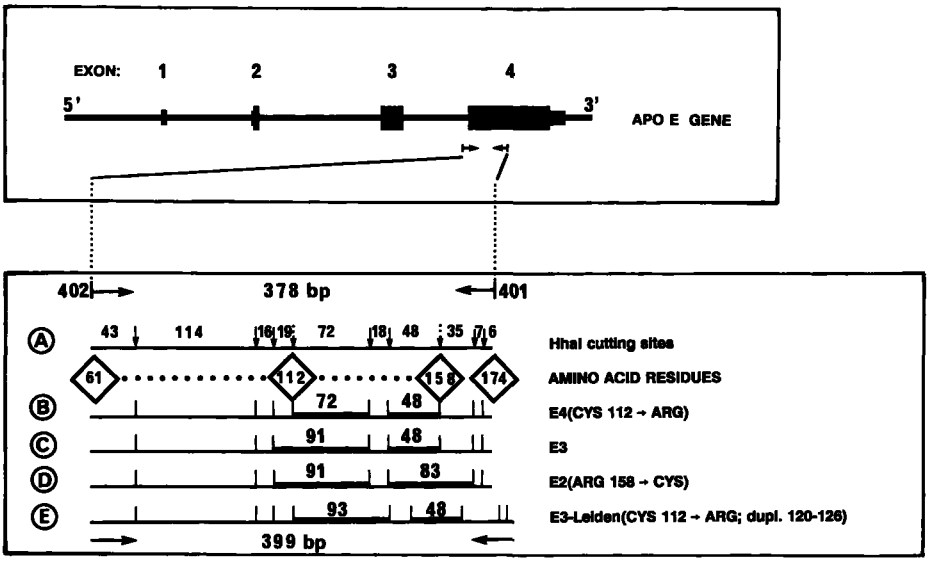

Figure 1. Top: Schematic representation of the APOE gene. Narrow boxes indicate the non-translated part and broad boxes indicate the translated parts of the exons. **Bottom:** Enlarged 5' part of exon 4 of the APOE gene showing the position of the two amplification primers 402/401 (→ and ←). Lane A shows the 378 bp PCR product of normal apoE using these two amplification primers. The constant (↓) and variable (▼) *Hha*I restriction endonuclease cutting sites are indicated. The positions of the amino acid residues 61, 112, 158 and 174 are also indicated (◇). Lane B represents the 378 bp PCR product of apoE4(Cys112→Arg) with the 72 bp *Hha*I restriction fragment length unique for this allele. Lane C represents the 378 bp PCR product of apoE3 with the combination of the 91 bp and 48 bp *Hha*I restriction fragment lengths unique for this allele. Lane D represents the 378 bp PCR product of apoE2(Arg158→Cys) with its unique combination of a 91 and 83 bp *Hha*I restriction fragment lengths. Lane E shows the 399 bp PCR product of apoE3-Leiden. Since the apoE3-Leiden mutation adds 21 bp to the 72 bp *Hha*I restriction fragment length normally found for the APOE*4(Cys112→Arg) allele, the resulting 93 bp fragment is a unique feature of this apoE variant.

the gel was stained with ethidium bromide and photographed. Thereafter the DNA was transferred to a Biotrace membrane ™ RP (Gelman Sciences, Annarbor, USA) and hybridized with a [γ-^{32}P]ATP labelled synthetic allele specific oligonucleotide probe directed against the junction region of the APOE*3-Leiden gene duplication (22).

For allele specific restriction endonuclease genotyping as described first by Hixson and Vernier (31), 15 μl PCR product was digested with 7.5 U *Hha*I at 37 °C for 16 hours according to recommendations of the supplier (Pharmacia, Uppsala, Sweden). Thereafter, the digested material was separated on a 10 %

neutral polyacrylamide gel for 3 h at 10 V/cm, stained with ethidium bromide and photographed.

Statistics All statistical calculations were performed with the use of the commercially available statistical package Number Cruncher Statistical Systems, developed by Dr. J. L. Hinze (Utah, USA). In order to avoid the possibility of ascertainment bias, the 5 probands were omitted from all calculations. Since the studied population involves family members (not randomly selected individuals), and most of the measured parameters showed non-gaussian distributions, differences in these parameters between groups were calculated using either the non-parametric analysis of variance (ANOVA) test of Kruskal-Wallis (in the case of more than 2 groups) or the Mann-Whitney test (in the case of 2 groups). Differences in gender distribution between 2 groups were evaluated with χ^2 analysis. Spearman's rank correlation was calculated to test for a linear correlation between age and body mass index (BMI). Multiple regression analysis to determine the independent effects of age, BMI and gender was performed on logarithmic transformed data in order to avoid non-gaussian distribution of the dependent variables. *P* values lower than 0.05 were considered as indicative of significant differences.

Results

Probands. Besides the original proband (CV), four additional, apparently unrelated, probands (GW, AB, JB and JE) showing heterozygosity for the APOE*3-Leiden allele have currently been identified among Dutch FD patients. One of these (AB) was previously reported as apoE3-Nijmegen (32). Table 1 represents the results of the lipid analysis and clinical characteristics of the five probands at their first visit to a lipid clinic. Three apoE3-Leiden probands show the signs and symptoms typical for FD. The remaining two probands had the clinical signs only. ApoE isoelectric focusing patterns of the five probands are shown in Figure 2. All probands showed only partial modification with cysteamine, indicating heterozygosity for apoE3-Leiden (21). As shown in Figure 2, four probands (GW, AB, JB and JE) have the apoE3E3-Leiden phenotype while the original proband CV exhibits the apoE2E3-Leiden phenotype. The presence of the APOE*3-Leiden allele in these subjects was further proven by genotyping using agarose electrophoresis of amplified DNA corresponding to the 5' part of exon 4 of the APOE gene. All five probands exhibited a 399 bp band in addition to the common 378 bp band indicating the presence of the 21 bp insert reported for apoE3-Leiden (Figure 3a, ref. 22). By subsequent blotting and hybridization of this amplified DNA fragment with a synthetic oligonucleotide probe directed against the junction region of the

Table 1. Clinical characteristics of the five apoE3-Leiden probands at their first visit to the lipid clinic

	FD probandi with apoE3-Leiden*				
	CV	GW	AB	JB‡	JE
Age	42	46	41	55	64
CVD / PVD§	- / -	+ / -	- / -	- / -	+ / -
Xanthomas‖	+	+	+	-	-
Plasma cholesterol¶	11.1	14.6	14.6	7.0	8.6
Plasma triglycerides	3.9	7.3	4.5	3.1	1.9
VLDL-chol/TG**	0.8	0.67	1.38	1.03	1.74
β-VLDL#	+	+	+	+	+
ApoE phenotype§§	E2E3-L	E3E3-L	E3E3-L	E3E3-L	E3E3-L

All five probands are excluded form these statistical calculations.
* All probandi are males.
‡ Proband JB was on lipid lowering drugs when first examined by us.
§ CVD = coronary vascular disease; PVD = peripheral vascular disease.
‖ Xanthomas include palmar streaks and tuberous xanthomas.
¶ Levels of plasma cholesterol and plasma triglycerides are given as mmol/l.
** VLDL-cholesterol measured in VLDL d < 1.006 g/ml, TG = total plasma triglycerides (mmol/mmol).
# As evaluated by agarose electrophoresis in VLDL d < 1.006 g/ml fraction.
§§ E3-L: E3-Leiden.

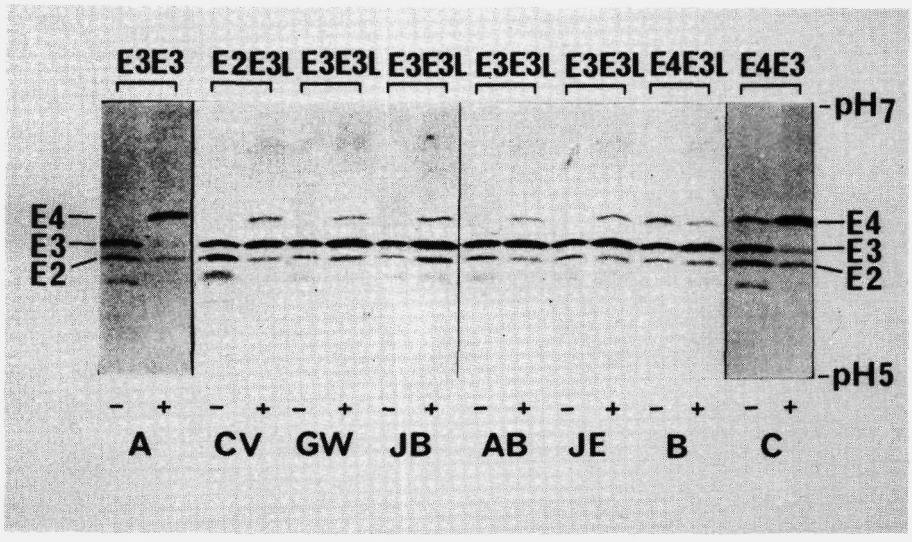

Figure 2. ApoE isoelectric focusing (pH 5-7) patterns of delipidated serum before (-) and after (+) cysteamine treatment of the five probands (indicated below the lane with their initials), of a family member of one of the probands (indicated with B below the figure) and of two healthy control subjects (indicated with A and C below the gel). The corresponding apoE phenotypes are indicated above the lane. The abbreviation E3-L was used to indicate the APOE*3-Leiden allele.

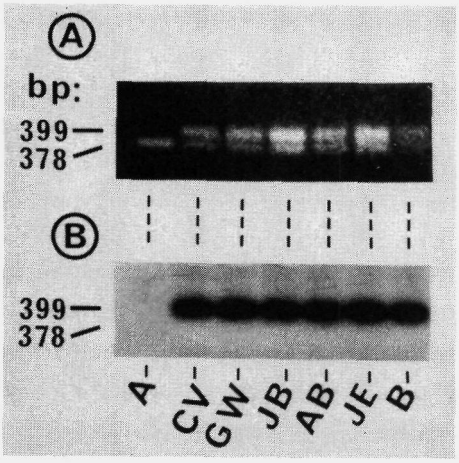

Figure 3. Agarose gel electrophoresis of PCR products using the 402/401 set of amplification primers followed by ethidium bromide staining (panel A) or by hybridization with a synthetic oligonucleotide probe directed against the junction region of the E3-Leiden duplication (panel B). The lanes represent the five apoE3-Leiden probands (lanes CV, GW, JB, AB and JE), a healthy control subject with apoE3E3 phenotype (lane A) and a subject with the apoE4E3-Leiden phenotype (lane B).

APOE*3-Leiden gene duplication it was shown that DNA from the probands does hybridize with this allele specific oligonucleotide probe (Figure 3b). Finally, digestion of this amplified DNA with *Hha*I restriction endonuclease followed by polyacrylamide gel electrophoresis enabled genotyping of apoE. Since the apoE3-Leiden mutation adds 21 bp to the 72 bp *Hha*I- fragment which is normally found in individuals with the APOE*4 allele the resulting 93 bp *Hha*I-fragment is typical for the APOE*3-Leiden allele (Figure 1 and 4).

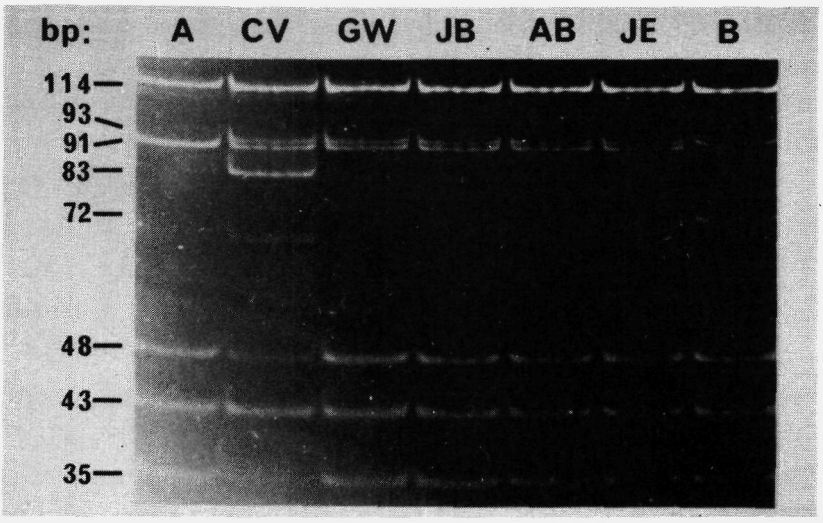

Figure 4. Polyacrylamide gel electrophoresis of PCR products using the 402/401 set of amplification primers followed by *Hha*I restriction endonuclease digestion. The lanes represent the five apoE3-Leiden probands (lanes CV, GW, JB, AB and JE), a healthy control subject with apoE3E3 phenotype (lane A) and a subject with the apoE4E3-Leiden phenotype (lane B).

Genealogical studies. Although the five probands were ascertained independently in two different lipid clinics (Leiden and Nijmegen), we learned that they had relatives living in the same area of the Netherlands. Based on information presented to us by the probands, genealogy was done by checking the parish records from the 18th century and the civil registration-, population- and census records from the 19th and 20th centuries in a region south-east of The Hague. We learned that in this region there is no high degree of consanguinity. This genealogical study proved that the five probands share common ancestry in the seventeenth century (Figure 5). It was found that the grandfather and grandmother of proband CV were related, giving the possibility of homozygosity for the E3-Leiden variant in the parental generation of this proband; the mother of proband CV, however, was heterozygous for this variant.

Family studies. As well as the five probands, 123 family members were studied (Appendix and Figure 5). After apoE phenotyping and genotyping, performed as described above for the probands, 37 additional individuals were found to be heterozygous for the APOE*3-Leiden allele (filled symbols in Figure 5). In all statistical calculations the five probands were excluded in order to avoid the possibility of ascertainment bias. As indicated in Table 2, there were no significant differences between the group of APOE*3-Leiden allele carriers and the group of non-carriers with respect to BMI (P=0.656; NS), age (P=0.778; NS) and gender distribution (χ^2 = 1.020; P=0.315; NS). This allowed us to compare both groups with respect to lipoprotein levels without prior correction.

Agarose electrophoresis of VLDL (d < 1.006 g/ml) revealed that all carriers showed β migrating VLDL particles, whereas β-VLDL could not be detected in any of the non-carriers (results not shown). In the group of non-carriers 14 individuals were found with more or less severe hyperlipoproteinemia other than FD based on lipoprotein analysis and agarose electrophoresis (see Appendix).

All mean plasma lipid and lipoprotein levels were significantly different between both groups (Table 3). In the carriers, the ratio VLDL-cholesterol to plasma triglycerides and the plasma level of IDL-cholesterol was much higher than in the non-carrier group (mean values 0.67 versus 0.30 and 1.41 versus 0.24, respectively). However, a range of overlap still exist (see Appendix). Regarding the ratio of (VLDL + IDL)-cholesterol to plasma triglycerides, the range of overlap between both groups was much smaller (Appendix). All carriers display high plasma apoE concentrations (> 11 mg/100 ml) (Appendix). Only one individual in the group of non-carriers showed an apoE level above 11 mg/100 ml (see Appendix).

Figure 5. The extended apoE3-Leiden pedigree. I, II, III and IV represent the successive generations. Each of the five families is indicated with the initials of the proband. The probands are indicated with an arrow. The numbering of the individuals corresponds with the numbering given in the Appendix. ● / ■ , subject (female/male) carrying the APOE*3-Leiden allele; ○ / □ , individuals not carrying the APOE*3-Leiden allele; ◎ / ⊡ , living subject, not studied; ◈ , variable number of living subjects, not studied; ⌀ / ⊠ , deceased subjects, not studied. The individuals in the inner 6 generations (not numbered) were all deceased and could not be studied.

Table 2. Comparison of the group of APOE*3-Leiden allele carriers (n= 37) and non-carriers (n= 86) with respect to body mass index, age and sex distribution

	Non-carriers		Carriers		
	Mean	SD	Mean	SD	P *
Body Mass Index‡	22.8	3.4	22.8	3.3	0.656
Age	39.9	18.6	39.8	19.8	0.778
No. of males/females	48/38		17/20		0.315§

All five probands are excluded from these statistical calculations.
* P value indicating the difference between the two groups as calculated with the Mann-Whitney test.
‡ Body mass index represents weight (in kilograms) / height^2 (in meters).
§ P value calculated by χ^2 analysis comparing the difference in number of males and females between the two groups.

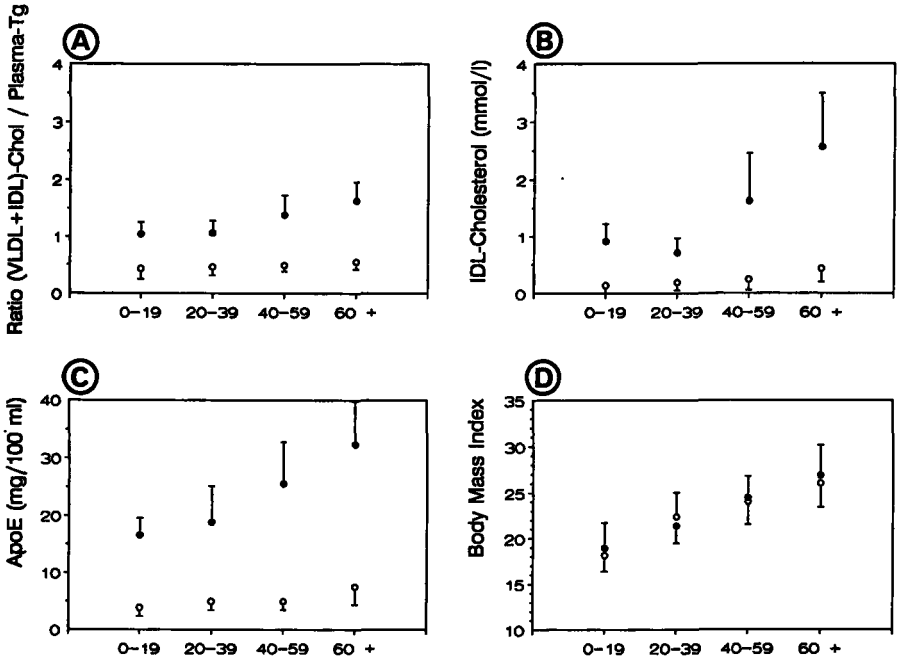

Figure 6. The ratio (VLDL + IDL)-cholesterol / plasma triglycerides (A), IDL-cholesterol level (B), plasma apoE level (C) and body mass index (D) of the APOE*3-Leiden allele carriers (●) and non-carriers (○). Each individual was assigned to one of the four age classes: 0-19 years (n = 6 and 10, respectively); 20-39 years (n = 10 and 34, respectively); 40-59 years (n = 17 and 27, respectively) and 60 years or older (n = 4 and 15, respectively). The figures between brackets in the legend represent the number of carriers and non-carriers, respectively. Mean values and standard deviation (indicated in one direction) are shown. The five probands are excluded from these calculations.

Effect of age, BMI and gender. From our data it is clear that there is a strong association between the presence of the APOE*3-Leiden allele and the development of FD, although there is a considerable variability in the expression of FD among the five probands and the other 37 APOE*3-Leiden allele carriers. Additional factors that may influence the expression of FD in E2(Arg 158→Cys) homozygotes include age, BMI and gender (1). Since a relatively large number of APOE*3-Leiden allele carriers was available, we were able to estimate the effects of these additional factors on the expression of FD in these subjects by statistical analysis. For estimating the effect of age on plasma lipid and lipoprotein levels in the APOE*3-Leiden allele carriers and non-carriers, we divided both groups into four age sub-classes: 0-19; 20-39; 40-60 and >60 years old. In the group of carriers we found a strong influence of age on the ratio (VLDL + IDL)-cholesterol / plasma triglycerides and an even more pronounced effect on plasma IDL-cholesterol and plasma apoE concentration (Figure 6a, b and c, respectively). A similar, but much less evident, effect was found in the group of non-carriers.

Table 3. Comparison of the group of APOE*3-Leiden allele carriers (n= 37) and non-carriers (n= 86) with respect to plasma lipid and lipoprotein levels

	Non-carriers		Carriers		
	Mean	SD	Mean	SD	P *
Plasma triglycerides‡	1.48	1.0	2.24	0.9	<0.001
Plasma cholesterol	5.44	1.3	7.14	2.1	<0.001
VLDL-cholesterol	0.48	0.4	1.65	1.1	<0.001
(VLDL + IDL)-cholesterol	0.73	0.5	2.96	1.7	<0.001
IDL-cholesterol	0.24	0.2	1.41	0.9	<0.001
LDL-cholesterol	3.34	1.1	2.85	0.8	0.010
HDL-cholesterol	1.43	0.3	1.33	0.3	0.034
VLDL-chol/Plasma Tg§	0.30	0.1	0.67	0.2	<0.001
(VLDL + IDL)-chol/Plasma Tg	0.49	0.1	1.26	0.3	<0.001
ApoE	5.31	2.1	22.76	7.7	<0.001

All five probands are excluded from these statistical calculations.
* P value indicating the difference between the two groups as calculated with the Mann-Whitney test.
‡ All levels are given in mmol/l except apoE which is given in mg/100 ml.
§ TG = triglycerides.

Table 4. Multiple linear regression analysis of age, body mass index and sex for log-transformed plasma lipid and lipoprotein levels in the group of APOE*3-Leiden allele carriers.

Dependent variable	Independent variable	P *	R ‡	F ‖	P ‖
Ln([VLDL+IDL]-chol/Plasma Tg¶)	Age	0.71			<0.0001
	BMI	-0.38			0.0273
	Sex**	0.32			0.0654
	All variables		0.69	11.52	<0.0001
Ln(IDL-cholesterol)	Age	0.69			0.0001
	BMI	-0.28			0.1293
	Sex	0.24			0.1970
	All variables		0.73	11.57	<0.0001
Ln(apoE)	Age	0.50			0.0029
	BMI	0.01			0.9793
	Sex	-0.17			0.3558
	All variables		0.66	9.67	<0.0001

All five probands are excluded from these statistical calculations.
* P value represents the partial correlation coefficient. ‡ R value represents the multiple correlation coefficient. ‖ F value obtained from F-statistic. ‖ P value indicating the probability for t-statistics (partial correlation) or F-statistic. ¶ TG = triglycerides. ** For sex a numerical code (1 = females, 2 = males) was entered in this analysis.

Both in the group of carriers, as well as in the group of non-carriers, we found a strong linear correlation between age and BMI (Spearman's correlation coefficients of 0.61 and 0.66 for carriers and non-carriers, respectively; $P < 0.001$, results not shown), indicating that BMI is strongly influenced by age. The strong influence of age on BMI is also illustrated in Figure 6d for the four age sub-classes for both groups. Multiple linear regression analysis was used to estimate the influence of age, BMI and gender on the ratio (VLDL+IDL)-cholesterol / plasma triglycerides, IDL-cholesterol and plasma apoE concentration (Table 4). It is clear that in the group of carriers, age has a strong influence on these parameters. BMI showed an additional influence on the expression of FD in this group, whereas gender had no effect.

In case of individuals with homozygosity for the E2(Arg158→Cys) variant, a marked difference in the onset of expression of FD exists between males and females due to the difference in hormonal status (1). In males the FD is normally expressed between 30 and 40 years of age, whereas in females FD is expressed after the menopause. To investigate whether this holds true also for the APOE*3-Leiden allele carriers, we compared the plasma lipid and lipoprotein levels between

Table 5. Comparison of males and females carrying the APOE*3-Leiden allele with respect to plasma lipid and lipoprotein levels after subdivision in two age classes.

	< 45 years					≥ 45 years				
	Males $n=13$		Females $n=7$			Males $n=4$		Females $n=13$		
	Mean	SD	Mean	SD	P *	Mean	SD	Mean	SD	P*
Plasma triglycerides‡	2.14	0.77	1.80	0.66	0.405	2.30	0.96	2.57	0.96	0.610
Plasma cholesterol	6.25	1.93	6.02	1.50	0.905	7.70	1.54	8.45	2.15	0.571
VLDL-cholesterol	1.62	0.97	1.19	0.92	0.322	1.79	1.61	1.88	1.31	0.651
IDL-cholesterol	1.05	0.72	0.73	0.29	0.342	1.76	0.36	1.96	1.05	0.734
VLDL-chol/Plasma Tg§	0.71	0.21	0.59	0.27	0.285	0.68	0.32	0.68	0.26	1.000
(VLDL + IDL)-chol/Plasma Tg	1.16	0.33	1.01	0.22	0.362	1.52	0.12	1.43	0.31	0.308
ApoE	18.45	4.99	19.88	6.20	0.501	21.53	2.23	29.00	8.03	0.113

All five probands are excluded from these statistical calculations.

* P value indicating the difference between the males and females in each group as calculated with the Mann-Whitney test.

‡ All levels are given in mmol/l except apoE which is given in mg/100 ml.

§ TG = triglycerides.

Table 6. Comparison of the different apoE phenotypes with respect to mean plasma lipid and lipoprotein levels in the group of APOE*3-Leiden allele carriers.

	ApoE phenotype			
	E2E3-L	E3E3-L	E4E3-L	*P* *
Number of subjects	4	30	3	
Plasma triglycerides‡	2.98	2.24	1.27	0.019
Plasma cholesterol	9.25	7.07	5.05	0.025
VLDL-cholesterol	2.98	1.58	0.58	0.024
(VLDL + IDL)-cholesterol	5.01	2.85	1.25	0.017
IDL-cholesterol	2.04	1.39	0.66	0.100
VLDL-chol/Plasma Tg§	0.96	0.66	0.46	0.014
(VLDL + IDL)-chol/Plasma Tg	1.60	1.25	0.98	0.035
ApoE	30.75	22.62	13.55	0.008

All five probands are excluded from these statistical calculations.
* *P* value indicating the difference between the three phenotypes as calculated with the Kruskal-Wallis test.
‡ All levels are given as mean values in mmol/l except apoE which is given in mg/100ml.
§ TG = triglycerides.

male and female carriers subdivided in two age classes: < 45 years and ≥ 45 years of age, respectively. In both age classes no significant difference in levels of any plasma lipid and lipoprotein parameter measured was found between males and females (Table 5), indicating that in the group of APOE*3-Leiden allele carriers there is no influence of gender on the age of onset of FD.

Effect of the second APOE allele. All individuals with FD found in this study are heterozygous for the APOE*3-Leiden allele and thus, they are heterozygous for a common APOE allele as well. We wondered whether this common APOE allele influences the plasma lipid and lipoprotein levels in the APOE*3-Leiden allele carriers.

The results presented in Table 6 show that in the group of APOE*3-Leiden allele carriers the E*2 allele enhances the expression of FD (which is reflected by the higher lipid and lipoprotein levels), whereas the E*4 allele showed the opposite effect. This effect is consistent with previous data obtained by Weintraub et al (33). These results could be less evident as the numbers of individuals with either the E2E3-Leiden- or the E4E3-Leiden phenotype were rather small (4 and 3, respectively; Table 4). In addition, there could be some bias of the results since all E4E3-Leiden individuals come from one generation in a single kindred, and three of the four individuals with the E2E3-Leiden phenotype come from one generation of another kindred (see Figure 5 and the Appendix).

Irrespective of the common accompanying second allele in the E*3-Leiden allele carriers, FD is dominantly inherited, i.e. the presence of normally functioning apoE molecules does not prevent the expression of FD in these subjects. To evaluate whether this might be due to a difference in distribution among the different lipoprotein fractions between apoE3-Leiden and the common apoE variant, we performed apoE phenotyping in cysteamine treated and untreated VLDL, IDL and HDL fractions. The cysteamine treatment enabled us to distinguish "normal" apoE (either E2, E3 or E4) from apoE3-Leiden (Figure 2) since apoE3-Leiden contains no cysteine residues (21).

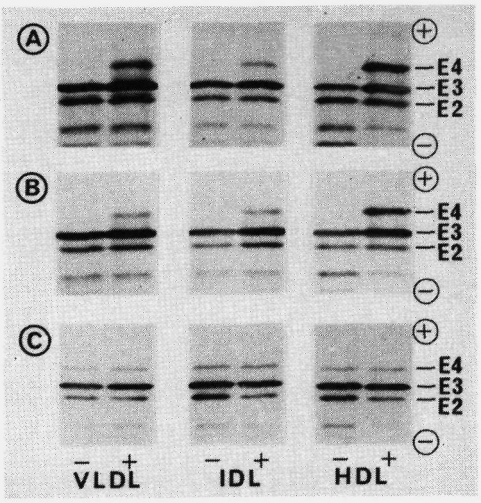

Figure 7. Isoelectric focusing pattern (pH 5-7) of VLDL, IDL and HDL of subjects with the apoE2E3-Leiden phenotype (**A**), the apoE3E3-Leiden phenotype (**B**) and the apoE4E3-Leiden phenotype (**C**) before (-) and after (+) cysteamine treatment.

As can be seen in Figure 7 the majority of APOE protein in the VLDL and IDL density fraction consists of the apoE3-Leiden variant. In the case of both the E*2 and E*3 allele as second allele, the HDL density fraction is relatively enriched with the common apoE isoform as compared with its relative amount in the VLDL and IDL density fraction (Figure 7a and b). In the case of the E*4 allele, the relative contribution of the E4 variant to the total amount of APOE protein does not differ between the VLDL, IDL and HDL density fraction (Figure 7c).

Discussion

FD has been reported earlier in association with heterozygosity for E3(Cys112→Arg; Arg142→Cys), E2(Lys146→Gln) and E1-Harrisburg(Lys146→Glu) (13-19). Both apoE3(Cys112→Arg;Arg142→Cys) as well as E1-Harrisburg (Lys146→Glu) have been reported in one family each (13,14,18) whereas apoE2(Lys146→Gln) has been studied in three apparently unrelated kindreds (17). With the exception of three very lean APOE*2(Lys146→Gln) allele carriers and one individual with the APOE*1-Harrisburg(Lys146→Glu) allele who was on lipid lowering diet, all family members showing heterozygosity for one of these rare

mutant forms of apoE had manifest FD. Thus, for these variants FD is inherited in a dominant fashion with a high penetrance. In addition to the rare variants described above, the apoE variants E2(Arg145→Cys) (8,34,35) and E2-Christchurch (Arg136→Ser) (35,36) are also reported to be associated with the development of FD. However it is not known whether these mutants are associated with either a dominantly or recessively inherited form of FD, as family studies have yet to be performed.

In previous studies (20,21) we have shown that dominant expression of FD also occurs in the family of an FD patient who has the apoE3-Leiden mutation. Recently, DNA and protein sequence analysis revealed that the molecular basis of apoE3-Leiden is due to a partial gene duplication leading to an in-frame insertion of 21 nucleotides forming a tandem repeat of the codons 120-126 or 121-127 (22,23). In this paper, four additional, apparently unrelated patients with FD carrying the APOE*3-Leiden allele in a heterozygous form were ascertained. Genealogical studies revealed that the probands share common ancestry in the seventeenth century (Figure 5). Using the families of all five probands, we were able to perform the most extensive family study described so far with respect to a dominantly inherited form of FD. In this family study we obtained a total of 42 individuals carrying the APOE*3-Leiden allele among a total number of 128 family members. We could clearly prove that the apoE3-Leiden variant is invariably associated with the expression of FD in all five families, although with a variable degree of severity.

In subjects with homozygosity for apoE2(Arg158→Cys) FD only rarely develops (1 to 4 %) and is commonly assumed to be highly influenced by additional factors such as age, body mass index, nutritional status and gender (1). As all subjects exhibiting heterozygosity for the APOE*3-Leiden allele express clinical symptoms of FD, we wondered whether in these subjects the expression of FD is also influenced by these additional factors. The relatively high number of FD subjects ascertained in this study (37 individuals in addition to the five probands), together with the absence of significant differences between the apoE3-Leiden carriers and non-carriers with respect to age, BMI and gender distribution (Table 2), gave us the unique opportunity to estimate the possible association of the expression of FD with these factors by means of statistical analysis.

We found that in APOE*3-Leiden allele carriers the development of FD is strongly correlated with age (Figure 6a-c) as is BMI (Figure 6d). Multiple regression analysis revealed that there was a weak effect of BMI on the expression of FD in addition to the highly significant effect of age (Table 4). Because of the strong correlation between BMI and age, part of the effect of age on the expression

of FD could be derived from the effect of BMI. Although the E3-Leiden heterozygotes resembles the E2(Arg158→Cys) homozygotes with respect to the effect of age on the expression of FD, we found that in E*3-Leiden allele carriers there is, strikingly, no influence of gender on the expression and on age of onset of FD (Table 5).

A number of studies conducted by Mahley and co-workers (10,37-40) strongly suggest that the conformation of the 130-150 α-helical region of apoE, the putative receptor-binding domain, can be easily modulated, leading to a change in receptor-binding activity. For instance, an increase in receptor-binding activity of apoE2(Arg158→Cys) can be obtained in vitro by cleavage with thrombin (38) or by cysteamine treatment (a reagent that converts cysteine to a positively charged lysine analogue) (10,38). The binding activity of apoE2(Arg158→Cys) may also be modulated in vivo to some extent, by changing the lipid composition of the lipoprotein particle (40). Their results suggest that the conformation of apoE2(Arg158→Cys) is sensitive to its environment and that the cysteine at residue 158 has a secondary, rather than a direct, effect on receptor-binding activity. Mahley et al. (41) hypothesize that this property could explain the requirement for additional environmental and/or genetic factors for the expression of FD. Only when exacerbating factors are present, does the receptor-binding activity of β-VLDL decrease and, eventually, will result in the development of FD.

In contrast to the apoE2(Arg158→Cys) variant, the apoE variants E3(Cys112→Arg;Arg142→Cys), E2(Lys146→Gln) and E1-Harrisburg(Lys146→Glu) are almost absolutely associated with FD, even at young age (13-19). These variants have a common molecular defect i.e. a substitution of a basic amino acid residue for a neutral or acidic residue in the predicted α-helical receptor binding domain of apoE (residues 130-150). It is suggested that the basic amino acid residues in this particular domain provide direct ionic interaction with the LDL-receptor (42). Mahley et al. (41) suggest that loss of any positively charged amino acid residue within the putative α-helical segment affects the binding of apoE by reducing the ionic interaction. This reduction probably is not easily influenced by environmental factors like lipid composition of the lipoprotein particle. As a consequence, they hypothesize that mutations in the 130-150 segment of apoE result in "permanent" receptor-binding defects. This hypothesis is sustained by experiments using apoE variants made by site-directed mutagenesis (39).

The present results convincingly show that the apoE3-Leiden variant is also invariably associated with the development of FD, although age exerts a significant effect on the severity of hyperlipidaemia (Figure 6a-c), like in E2(Arg158→Cys) homozygotes. Previously we have shown that apoE3-Leiden possesses a reduced

receptor-binding activity (20,21). Recently it was found that upon cleavage with thrombin, the 22-kDa fragment of apoE3-Leiden possesses a nearly normal binding activity (23). This suggests that, due to the insertion of seven amino acids, close to the receptor-binding domain, and constituting nearly two turns of alpha-helix, the structure of the entire protein is changed in such a way that the carboxyl-terminal domain irreversibly prevents the receptor binding region of apoE to interact with the LDL-receptor, and as a consequence, interferes with the binding activity, the latter being sensitive to environmental factors such as age.

ApoE3-Leiden, described in this paper, as well as the E3(Cys112→Arg; Arg142→Cys), E2(Lys146→Gln) and E1-Harrisburg(Lys146→Glu) represent binding defective apoE mutants that are associated with dominantly-inherited FD, i.e. the subjects who are heterozygous for these variant alleles display FD, irrespective of the presence of normal apoE molecules. As chylomicron- and VLDL-remnants possess several apoE molecules per particle, it may be expected that both normal and mutant apoE molecules are present on each particle. This raises the question as to why the normal apoE molecules do not prevent the expression of FD in these subjects. Mahley et al. (41) postulates two mechanisms for the phenomenon of dominantly-inherited FD: (i) the mutant apoE disrupts the organization of the apoE molecules on the surface of the lipoprotein, including that of the normal apoE molecules; (ii) the presence of defective apoE molecules reduce the effective concentration of active apoE molecules on the surface of the particles, thereby reducing its affinity for the receptor. There is some evidence that plasma levels of apoE may become rate-limiting in the clearance of remnant lipoproteins (43). Thus, a low efficient concentration of normally active apoE molecules on the remnant particles might be the underlying molecular defect of dominantly inherited forms of FD.

In subjects with the E3(Cys112→Arg; Arg142→Cys) variant, equal amounts of normal and variant apoE3 were found in the VLDL density fraction (14). In contrast, our results clearly show that in plasma of all apoE3-Leiden subjects, the apoE3-Leiden variant largely predominates over its normal apoE counterpart (Figure 2). Preliminary results, obtained by quantitative isoelectric focusing and immunoblotting using ^{125}I-labelled Rabbit anti Goat anti-apoE antibodies followed by counting the radio-activity in the respective bands after cysteamine modification, showed that approximately 75 % of the total plasma apoE consists of apoE3-Leiden (results not shown). Separate analysis of the different lipoprotein fractions, revealed that this predominance, is most pronounced in the VLDL and IDL density fractions (Figure 7) except in the case of apoE4 as normal variant. ApoE4 distributes preferentially to VLDL (44), which should be ascribed to the arginine at residue

112 (45). Similarly, the preferential association of apoE3-Leiden with VLDL and IDL density fractions may be due to the fact that apoE3-Leiden is apoE4-like in that it contains arginine at residue 112. In fact, this predomination of apoE3-Leiden renders the chylomicron- and VLDL-remnants into "apparent" homozygosity for apoE3-Leiden. Whether the "extent of homozygosity". i.e. the relative amount of active apoE on these particles is associated with the degree of severity of FD expression is currently under investigation. We found that the APOE*2(Arg158→Cys) allele as second allele in apoE3-Leiden subjects enhances the expression of FD, whereas the opposite was true for the APOE*4(Cys112→Arg) allele (Table 6). This observation indicates that the second common APOE allele in apoE3-Leiden subjects does influence the development of FD.

The present paper reinforces the concept that FD is a genetically-heterogeneous disease entity with a recessive mode of inheritance in case of the common E2(Arg158→Cys) variant and with a dominantly-inherited form in case of rare apoE mutants. It is shown that these dominantly inherited forms of FD displays high rates of penetrance, making early diagnosis in these families feasible. From a clinical point of view, we recommend that all patients with elevated plasma cholesterol and triglyceride levels concomitant with increased cholesterol/triglyceride ratios in the VLDL fraction should be analyzed for apoE phenotype and/or genotype. In case of E2E2 homozygosity this analysis will only sustain the FD diagnosis. However, when suspected FD patients do not exhibit the common E2E2 homozygosity, the patients might carry a rare APOE allele, and thus family studies are indicated.

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APPENDIX
Individual data

No	BMI kg/m²	Age yr	Sex	Plasma Tg	Plasma Chol	VLDL Chol	Ratio	IDL Chol	LDL Chol	HDL Chol	Plasma ApoE	β-VLDL	ApoE pheno	Clinical symptoms	Remarks
				mmol/l				mmol/l			mg/dl				
Family proband CV															
I-1	22.4	82	F	2.50	10.28	1.35	1.97	3.58	3.98	1.37	34.37	+	3/3L*	X	III
II-1	24.9	60	M	4.07	7.15	1.32	0.51	0.74	3.92	1.17	9.75	-	3/3		II
II-2	24.0	57	M	2.27	7.85	1.26	1.49	2.13	3.47	0.99	23.14	+	3/3L	X, C	III,Med
II-3	19.4	55	F	4.25	12.82	5.11	2.05	3.62	2.77	1.32	39.69	+	2/3L		III
II-4	23.2	52	F	1.48	6.59	1.24	1.40	0.83	2.83	1.69	19.26	+	2/3L	X	III,Med
II-5	22.1	50	F	3.63	10.43	3.16	1.60	2.66	3.43	1.18	35.93	+	2/3L		III
II-6	21.5	48	M	2.82	6.74	2.30	1.01	0.55	2.77	1.12	23.35	+	2/3L	X	III,Med
II-7	22.0	46	F	1.13	5.75	0.37	0.62	0.33	3.62	1.43	4.81	-	3/3		
II-8	23.0	44	M	2.18	7.99	1.78	1.56	1.63	3.51	1.07	23.24	+	3/3L	X	III
III-1	20.4	34	F	1.48	5.48	0.38	0.51	0.37	3.38	1.35	5.97	-	3/3		
III-2	24.7	25	M	3.09	4.90	1.09	0.44	0.26	2.88	0.67	3.99	-	3/3		
III-3	20.3	23	F	1.09	5.47	0.21	0.39	0.21	2.80	2.25	7.43	-	4/2		
III-4		28	M	1.03	4.89	0.33	0.72	0.41	2.45	1.70	6.56	-	3/2		
III-5	22.0	23	M	0.90	3.88	0.35	0.42	0.03	2.38	1.12	3.96	-	3/2		
III-6	20.4	19	M	0.64	3.48	0.20	0.34	0.02	2.11	1.15	5.06	-	4/2		
III-7	22.6	21	F	0.75	3.97	0.24	0.39	0.05	2.27	1.41	7.41	-	4/2		
Family proband GW															
I-1		82	M	1.21	4.78	0.37	0.41	0.13	2.83	1.45	4.07	-	3/3		
I-2		78	F	3.32	9.95	2.72	1.50	2.27	3.67	1.29	27.90	+	3/3L	C	III
II-1	27.0	51	F	1.44	8.67	0.66	1.27	1.17	4.91	1.93	30.02	+	3/3L		III
II-2	20.7	47	M	1.69	5.37	0.40	0.47	0.39	3.26	1.32	3.85	-	3/3		

No	BMI	Age	Sex	Plasma	Plasma	VLDL	Ratio	IDL	LDL	HDL	Plasma	β-VLDL	ApoE	Clinical	Remarks
	kg/m^2	yr		Tg	Chol	Chol		Chol	Chol	Chol	ApoE		pheno	symptoms	
				mmol/l				mmol/l			mg/dl				
II-3	20.3	45	F	1.02	5.37	0.21	0.58	0.38	2.77	2.01	3.16	-	3/3		
II-4	26.8	46	M	3.55	7.77	2.07	1.03	1.60	2.91	1.19	22.39	+	3/3L	X, C	III,Med
II-5	26.3	41	M	2.93	5.41	0.76	0.34	0.24	3.47	0.94	2.53	-	4/3		
II-6	25.6	43	F	1.83	4.81	0.37	0.67	0.85	2.06	1.53	5.54	-	3/3		
II-7		40	F	1.43	4.96	0.53	0.78	0.58	2.82	1.03	18.32	+	3/3L		III
III-1	26.4	28	F	0.96	4.80	0.15	0.31	0.15	2.70	1.80	4.36	-	3/3		
III-2	20.1	24	F	1.52	5.33	0.39	0.91	0.99	2.35	1.60	15.99	+	3/3L		III
III-3	16.8	19	F	0.63	3.96	0.15	0.25	0.01	2.06	1.74	7.12	-	3/3		
III-4	20.3	21	M	2.42	5.08	0.69	0.40	0.28	2.99	1.12	5.03	-	3/3		
III-5	20.5	18	F	1.32	3.28	0.33	0.26	0.01	1.73	1.21	2.95	-	3/3		
III-6	17.4	17	M	0.74	3.20	0.21	0.35	0.05	1.57	1.37	2.64	-	3/3		
III-7	21.5	22	M	2.14	5.36	0.69	0.42	0.20	3.32	1.15	3.26	-	4/3		
III-8	24.9	21	F	1.70	6.18	0.55	0.47	0.25	3.65	1.73	3.61	-	4/3		
III-9	17.6	19	F	2.25	6.16	0.69	0.32	0.03	4.13	1.31	3.58	-	3/3		
Family proband JE															
II-1	30.1	80	F	3.92	9.04	2.10	1.43	3.51	2.31	1.12	44.11	+	3/3L		III
II-2	27.7	74	F	2.28	7.04	1.04	1.26	1.83	3.10	1.07	30.45	+	3/3L		III
II-3	26.8	73	F	1.79	6.02	0.71	0.62	0.40	3.75	1.16	9.47	-	3/3		
II-4	24.7	71	M	1.44	5.92	0.48	0.57	0.34	3.94	1.16	7.55	-	3/3		
II-5	26.9	70	F	5.80	8.29	2.91	0.63	0.75	3.59	1.04	15.70	-	4/3		II
II-6	27.5	70	F	1.04	5.30	0.18	0.52	0.36	3.22	1.54	9.84	-	3/3		
II-7	23.5	68	M	0.98	6.11	0.21	0.52	0.30	3.92	1.68	8.99	-	3/3		
II-8	24.9	65	F	3.43	9.40	0.96	0.55	0.91	6.18	1.35	9.09	-	4/3		II

No	BMI	Age	Sex	Plasma	Plasma	VLDL	Ratio	IDL	LDL	HDL	Plasma	β-VLDL	ApoE	Clinical	Remarks
	kg/m²	yr		Tg	Chol	Chol		Chol	Chol	Chol	ApoE		pheno	symptoms	
				mmol/l				mmol/l			mg/dl				
II-9	27.4	64	M	1.99	8.62	2.37	2.01	1.62	3.42	1.21	25.09	+	3/3L	C	III
II-10		62	F	2.44	6.06	0.82	0.45	0.27	3.67	1.30	5.24	-	3/3		
III-1	28.4	52	M	3.52	6.48	1.33	0.37		3.90	1.29	6.24	-	3/3		II
III-2	26.5	47	F	1.68	5.46	0.42	0.92	1.12	1.94	1.98	18.24	+	3/3L		III
III-3	24.2	42	F	1.87	6.01	1.35	1.01	0.54	2.50	1.62	22.21	+	3/3L		III
III-4	25.2	46	M	1.82	6.46	1.20	1.38	1.31	2.60	1.35	20.71	+	3/3L		III
III-5	30.4	40	F	0.82	5.74	0.27	0.35	0.02	3.89	1.56	4.13	-	4/2		
III-6	26.1	46	F	2.22	5.85	1.35	1.09	1.06	2.32	1.12	23.03	+	3/3L		III,Med
III-7	23.8	49	M	5.18	5.20	1.56	0.36	0.32	2.56	0.76	7.78	-	4/4		IV
III-8	22.6	45	F	1.25	6.00	0.39	0.54	0.29	3.84	1.48	5.15	-	4/3		
III-9	22.3	41	M	1.51	5.35	0.56	0.51	0.21	2.88	1.70	6.48	-	3/3		
III-10	25.9	43	M	1.01	5.26	0.38	0.64	0.27	3.23	1.38	4.87	-	4/3		
III-11	21.4	36	M	0.54	2.68	0.07	0.54	0.22	1.57	0.82	4.89	-	3/3		
III-12		33	F	1.14	4.08	0.43	0.41	0.04	2.32	1.29	4.19	-	4/3		
III-13	23.5	39	M	2.24	5.96	0.89	0.58	0.40	3.59	1.08	4.22	-	4/3		
III-14	21.2	37	F	0.67	4.20	0.22	0.49	0.11	2.21	1.66	3.47	-	4/3		
III-15	22.0	40	M	0.52	4.56	0.18	0.65	0.16	2.63	1.59	5.60	-	4/3		
III-16	21.5	38	F	0.58	5.74	0.24	0.66	0.14	3.66	1.70	6.54	-	3/3		
III-17	23.4	41	M	1.96	8.87	0.57	0.51	0.42	6.45	1.43	7.48	-	4/3		II
III-18	29.4	38	F	1.09	6.79	0.36	0.40	0.08	4.86	1.49	6.24	-	4/3		
III-19	22.3	38	M	0.70	4.58	0.13	0.54	0.25	2.41	1.79	7.14	-	3/3		
III-20	21.7	33	M	1.87	4.94	1.52	1.06	0.47	1.88	1.07	18.14	+	3/3L		III
III-21	23.3	30	M	1.86	6.25	1.83	1.41	0.80	2.48	1.14	17.89	+	3/3L		III

No	BMI	Age	Sex	Plasma	Plasma	VLDL	Ratio	IDL	LDL	HDL	Plasma	β-VLDL	ApoE	Clinical	Remarks
	kg/m²	yr		Tg	Chol	Chol		Chol	Chol	Chol	ApoE		pheno	symptoms	
				mmol/l				mmol/l			mg/dl				
IV-1	20.5	19	M	2.69	5.84	2.13	1.04	0.66	1.92	1.13	17.73	+	3/3L		III
IV-2		24	M	1.14	5.70	0.60	0.55	0.03	3.78	1.29	6.57	-	4/3		
IV-3	21.5	23	F	1.79	6.56	0.53	0.41	0.21	3.74	2.08	6.91	-	4/3		
IV-4		20	M	3.67	4.51	0.84	0.28	0.18	2.45	1.04	6.26	-	4/3		
Family probandi AB and JB															
I-1	25.7	83	F	1.30	5.27	0.28	0.45	0.31	3.28	1.40	4.29	-	3/3		
I-2	25.4	85	F	1.60	7.68	0.57	0.46	0.16	5.75	1.20	6.48	-	4/3		II,Med
I-3	33.1	74	F	2.61	6.38	0.99	0.50	0.32	3.41	1.66	7.09	-	3/3		Med
I-4	24.8	72	M	2.04	8.52	0.85	0.63	0.44	6.11	1.12	4.47	-	4/3		II
I-5	27.8	71	M	3.03	6.79	1.20	0.50	0.33	4.44	0.82	7.09	-	4/3		
II-1	24.1	56	M	3.65	9.82	4.15	1.67	1.93	2.95	0.79	23.53	+	3/3L		III
II-2	25.4	55	M	3.14	7.06	1.80	1.28	2.23	2.00	1.03	30.07	+	3/3L		III,Med
II-3	25.3	49	F	0.53	4.90	0.09	0.28	0.06	3.07	1.68	2.59	-	4/3		
II-4	24.5	53	M	0.60	4.74	0.12	0.53	0.20	3.15	1.27	4.25	-	4/3		
II-5	22.5	52	F	1.17	6.11	0.20	0.38	0.25	3.61	2.05	6.98	-	3/3		
II-6	22.5	69	M	0.82	4.52	0.11	0.96	0.68	2.71	1.02	3.91	-	4/2		
II-7	28.7	52	F	1.59	6.24	0.69	1.27	1.33	2.74	1.48	19.28	+	3/3L		III,Med
II-8	24.2	51	M	1.93	6.23	0.62	0.57	0.48	3.56	1.57	4.17	-	4/3		
II-9	20.8	47	M	1.44	6.68	0.55	1.53	1.65	3.15	1.33	18.76	+	3/3L		III
II-10	22.7	49	F	1.02	5.66	0.23	0.42	0.20	3.80	1.43	6.10	-	4/3		
II-11	23.5	44	M	1.20	5.28	0.37	0.41	0.12	3.33	1.46	5.12	-	4/3		
II-12	21.3	46	M	0.65	5.53	0.21	0.58	0.17	3.87	1.28	4.52	-	4/3		
II-13		41	F	0.69	5.21	0.16	0.30	0.05	3.02	1.98	3.50	-	4/3		

No	BMI	Age	Sex	Plasma	Plasma	VLDL	Ratio	IDL	LDL	HDL	Plasma	β-VLDL	ApoE	Clinical	Remarks
	kg/m²	yr		Tg	Chol	Chol		Chol	Chol	Chol	ApoE		pheno	symptoms	
				mmol/l				mmol/l			mg/dl				
II-14	24.6	42	M	2.16	4.34	0.67	0.40	0.20	2.60	0.87	3.54	-	4/3		
II-15	26.3	40	M	2.52	10.05	1.97	1.94	2.93	3.93	1.22	27.25	+	3/3L		III
II-16	24.7	37	F	1.17	5.29	0.21	0.34	0.19	2.83	2.06	4.39	-	3/3		
II-17	24.8	53	M	0.83	5.68	0.39	0.48	0.01	4.06	1.22	7.59	-	3/2		
II-18	24.3	48	F	2.96	8.69	2.86	1.40	1.28	3.15	1.40	28.68	+	3/3L		III
II-19	28.1	49	M	2.28	6.08	1.03	0.48	0.06	4.34	0.65	4.73	-	3/3		
II-20	24.7	46	F	2.18	8.81	1.75	1.39	1.27	3.73	2.06	26.06	+	3/3L		III
II-21	24.0	45	M	1.26	6.72	0.54	0.66	0.29	4.17	1.72	4.62	-	4/3		II
II-22	20.0	44	F	0.98	6.60	0.44	0.50	0.05	4.07	2.04	3.42	-	4/3		
II-23	25.7	42	M	2.05	9.00	0.95	0.60	0.27	6.11	1.67	6.31	-	4/3		II
II-24	25.9	41	M	4.57	14.68	6.29	1.97	2.70	4.19	1.50	44.83	+	3/3L	X	III,Med
II-25	20.3	38	F	0.69	4.28	0.14				1.38	2.94	-	4/3		
II-26	26.9	39	M	1.87	7.74	0.87	0.58	0.22	5.27	1.38	5.62	-	4/3		II
II-27	27.5	36	M	1.41	7.69	0.79	1.04	0.67	4.90	1.33	5.60	-	4/3		II
II-28	22.4	34	M	0.47	5.05	0.11	0.32	0.04	3.23	1.67	2.58	-	4/3		
II-29	23.6	33	F	0.67	6.62	0.18	0.51	0.16	4.44	1.84	6.05	-	4/3		
II-30	19.8	29	F	2.83	8.80	2.52	1.27	1.06	3.17	2.05	27.51	+	3/3L		III
III-1	19.6	27	F	0.70	4.65	0.10	0.26	0.08	2.39	2.08	5.95	-	3/3		
III-2	17.8	24	F	1.45	4.81	0.37	0.42	0.24	2.53	1.67	5.23	-	3/3		
III-3	20.0	23	F	1.25	4.43	0.72	0.87	0.37	1.97	1.37	14.30	+	3/3L		III
III-4	19.6	26	F	1.14	5.46	0.44	0.87	0.55	2.59	1.88	12.74	+	4/3L		III
III-5	25.0	25	M	1.37	5.17	0.61	0.83	0.53	2.85	1.18	15.82	+	4/3L		III
III-6	19.6	23	M	0.95	3.94	0.45	1.09	0.59	1.72	1.18	11.11	+	3/3L		III

No	BMI	Age	Sex	Plasma	Plasma	VLDL	Ratio	IDL	LDL	HDL	Plasma	β-VLDL	ApoE	Clinical	Remarks
	kg/m²	yr		Tg	Chol	Chol		Chol	Chol	Chol	ApoE		pheno	symptoms	
				mmol/l				mmol/l			mg/dl				
III-7	19.6	20	F	0.40	3.67	0.06	0.45	0.12	1.77	1.72	4.76	-	3/3		
III-8	19.6	18	M	0.66	3.64	0.18	0.53	0.17	2.00	1.29	2.41	-	4/3		
III-9	*19.0*	*17*	*M*	*1.31*	*4.51*	*0.70*	*1.23*	*0.91*	*1.87*	*1.03*	*12.10*	+	*4/3L*		*III*
III-10	22.3	21	F	1.09	4.02	0.25	0.45	0.24	1.76	1.77	2.20	-	4/3		
III-11	19.4	20	M	0.71	3.86	0.18	0.51	0.18	2.17	1.33	2.18	-	3/3		
III-12	17.3	15	M	0.66	4.70	0.11	0.74	0.38	2.44	1.77	2.50	-	3/3		
III-13	18.7	11	M	2.30	6.29	1.17	1.08	1.31	2.70	1.11	20.90	+	3/3L		III
III-14	16.6	9	M	1.66	5.34	1.04	1.30	1.12	2.21	0.97	14.72	+	3/3L		III
III-15	15.6	7	M	2.62	5.27	1.41	0.75	0.56	2.03	1.27	15.90	+	3/3L		III
III-16	15.7	5	M	0.70	3.98		0.61	0.43	3.55		4.30	-	3/3		
III-17	21.7	25	M	0.90	4.89	0.32	0.46	0.09	2.98	1.50	5.76	-	3/2		
III-18	22.0	23	F	2.56	7.16	2.39	1.34	1.04	2.09	1.64	28.11	+	2/3L		III
III-19	23.3	19	M	2.56	5.75	2.29	0.85		2.66	0.92	18.17	+	3/3L		III
III-20	21.5	23	M	1.22	4.71	0.46	0.45	0.09	3.13	1.03	3.60	-	3/3		
III-21	22.6	20	M	3.89	9.96	4.17	0.90		5.24	1.21	26.88	+	3/3L		III
III-22	*20.4*	*20*	*M*	*0.86*	*4.32*	*0.21*	*0.26*	*0.01*	*2.71*	*1.39*	*4.18*	-	*3/3*		
III-23		14	M	0.95	4.03	0.19				1.34	3.50	-	4/3		
III-24		12	M	0.98	4.89	0.29				1.69	5.12	-	3/3		

Clinical variables of the 128 family members of the extended Dutch ApoE3-Leiden pedigree. The subject numbering corresponds with the one presented in the pedigree (Fig. 5). Subject number (No), body mass index (BMI), age, sex, total plasma triglycerides (Plasma Tg), total plasma cholesterol (Plasma Chol), VLDL-cholesterol (VLDL-Chol), ratio (VLDL+IDL)-cholesterol/total plasma triglycerides in mmol/mmol (Ratio), IDL-cholesterol (IDL-Chol), LDL-cholesterol (LDL-Chol), HDL-cholesterol (HDL-Chol), plasma apoE concentration, presence (+) or absence (-) of β-VLDL evaluated by agarose electrophoresis (β-VLDL), apoE phenotype (ApoE pheno), clinical symptoms of the individuals observed at their first visit to lipid clinic and remarks are shown. Abbreviations used under clinical symptoms are: X, xanthomas including palmar streaks, tendinous xanthomas and tuberous xanthomas; C, coronary vascular disease. Abbreviations used under remarks are: II, III, IV, subject was classified as having either Type IIa or IIb, Type III or Type IV hyperlipoproteinemia, based on the observed lipid and lipoprotein levels, agarose electrophoresis and other clinical symptoms; Med, subject using lipid lowering drugs.

* 3L, apoE3-Leiden.

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CHAPTER 10

General Discussion

General Discussion

The aim of this thesis was first to study the influence of the common apoE variants on various aspects of the lipoprotein metabolism in a number of different population samples. In addition to this, we also investigated the influence of two rare apoE variants on the lipoprotein metabolism, with special emphasis to the development of familial dysbetalipoproteinemia (FD).

Initially, apoE phenotyping was performed using isolated and delipidated very low density lipoproteins (VLDL) samples which were subjected to isoelectric focusing (IEF) followed by protein staining. There were three disadvantages to this former phenotyping technique, (i) it involved laborious and time consuming VLDL isolation procedures (ii) the use of VLDL sometimes results in incorrect phenotyping due to different preferential distribution of the apoE isoforms among the lipoprotein subclasses, and (iii) protein staining is not limited to the apoE isoforms bands but will stain other proteins that are present in the isolated VLDL fraction such as apoA1, serum albumin and human serum amyloid A, an acute phase protein sometimes present in human plasma after myocardial infarction (1,2).

In order to avoid these disadvantages, we developed a rapid and very sensitive apoE phenotyping method which is based on IEF of plasma followed by immunoblotting (**Chapter 2**). This method offers the following advantages: (i) the apoE isoelectric focusing patterns are easy to score since residual non-apoE proteins are not visualized; (ii) it is less laborious because an ultracentrifugation step to isolate VLDL is not needed; (iii) it is suitable for large scale diagnosis and population studies even in less well-equipped laboratories; (iv) it needs only a few microliters of serum or plasma that may have been stored for long periods of time; and (v) because of the high sensitivity, minor apoE isoforms can easily be detected in serum or serum density fractions (3).

In addition to this phenotyping method we sometimes perform apoE phenotyping after a chemical modification of the plasma samples with cysteamine. By means of this modification it is possible to introduce one positive charge specifically to each cysteine residue of the protein in study (thus in this case apoE). This enables to distinguish some rare apoE variants from common isoforms with the same relative charge, but with a different number of cysteine residues.

Although the inclusion of a cysteamine treatment in the phenotyping method may indicate the presence of rare apoE variants, as shown by us in **Chapters 8** and **9** of this thesis, it does not provide definitive prove, nor does it present the exact amino acid substitution.

However, rare apoE variants do differ on the level of DNA. Therefore apoE genotyping methodologies on the level of DNA have been developed (4-10) that are based on the polymerase chain reaction (PCR) (11). These methods include the use of amplified DNA for allele-specific oligonucleotide (ASO) hybridization (6) or allele-specific restriction fragment length polymorphism analysis (AS-RFLP) (10). A variant technique described for APOE genotyping involves allele-specific amplification (12-14).

In our hands, the combination of apoE phenotyping with and without cysteamine modification and PCR of genomic DNA followed by AS-RFLP and/or ASO resolves most of the apoE variants described so far. Therefore, we strongly recommend the combination of apoE phenotyping and genotyping to screen for the common and rare apoE variants. In addition to this, we recently developed a very sensitive denaturating gradient gel electrophoresis technique (DGGE), originally described by Myers et al. (15). Using this technique we identified 4 new variants of apoE (16).

We were able to confirm the concept that the common APOE alleles influence plasma lipid and lipoprotein levels in a large Dutch population study (**Chapter 3**) and in a study among the parents, the monozygotic (MZ)- and dizygotic (DZ) twins from 160 Dutch twin families (**Chapter 4**). In both studies, we found an association between the E*4 allele and elevated plasma cholesterol and apoB levels, whereas the E*2 allele appeared to be associated with decreased levels of plasma cholesterol and apoB. Reciprocally the E*4 allele is associated with a reduced plasma apoE level, whereas the E*2 allele leads to a highly significant increase in plasma apoE concentration. 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 apoE2 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 low density lipoproteins (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 (17,18), who showed that the APOE gene primarily affects apoE concentrations and thus the metabolism of apoE-containing lipoproteins thereby regulating the LDL-cholesterol and apoB concentrations in plasma.

In addition, studying a group of monozygotic (MZ) twins (**Chapter 4**) we found no effect of the apoE polymorphism on the within-pair differences of (apo)lipoprotein levels. This indicates that the APOE gene is a "level" gene according to the definitions of Magnus and Berg (19) rather than a "variability" gene, implying that the APOE gene exhibits a direct effect on the lipid measures which is independent of environmental factors. This agrees with the results of Hallmann et al. (20) showing the influence of the apoE polymorphism in nine different populations. They concluded that, although there are marked differences in apoE phenotype frequencies and dietary habits, in each population the influence of the apoE polymorphism displayed similar effects on the plasma cholesterol levels.

We learned that the Greenland Inuit differ significantly from most of the Caucasian populations with respect to their APOE and APOA4 allele frequencies (**Chapter 6**). Strikingly, in the Inuit the apoE polymorphism did not influence the plasma lipid and (apo)lipoprotein levels at all. In this respect, it is interesting to note that the Greenland Inuit show very low VLDL-cholesterol and triglyceride levels. This could explain the absence of an effect of the apoE polymorphism on plasma lipid and (apo)lipoprotein levels as only a minor amount of cholesterol is delivered to the liver via the action of apoE. Consequently, the LDL-receptor activity in the liver, and thus the plasma cholesterol and LDL-cholesterol levels are not affected by the apoE polymorphism in this population.

The importance of the apoE polymorphism is further illustrated by our recent finding in a subset of 303 apparently healthy subjects that plasma levels of Lp(a) are influenced by the APOE gene, parallel to that on plasma total cholesterol and LDL-cholesterol levels (**Chapter 5**). At present, there is much debate as to whether or not the LDL-receptor plays a key role in the regulation of plasma lipoprotein (a) [Lp(a)] levels. Several groups have reported only a minor role of the LDL-receptor in the Lp(a) catabolism (21-23). This seems to be supported by the observation that in heterozygous familial hypercholesterolemic (FH) patients, the plasma Lp(a) levels could not be reduced upon treatment with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, which are known to effectively reduce LDL-cholesterol levels by increasing the LDL-receptor activity (24,25). However, other groups showed that cultured human fibroblasts take up and degrade Lp(a) via the LDL-receptor (26-27). Additional support for the involvement of the LDL-receptor in the Lp(a) catabolism was gained from the observation that in FH patients, being partly defective in LDL-receptor activity, plasma Lp(a) levels are 2.5 to 3 times as high as in healthy individuals, irrespective of their Lp(a) phenotype (28). Also, in a recent study using transgenic mice with an

overexpression of human LDL-receptors, Lp(a) was cleared more rapidly than in the normal mice (29). Our data, showing that the apoE polymorphism influences the Lp(a) levels parallel to that of LDL also indicates that Lp(a), at least in normolipidemic individuals, is cleared from the circulation by a mechanism involving normal functioning LDL-receptors. Our results sustain the hypothesis that in vivo, the LDL-receptor is involved in Lp(a) catabolism.

The Greenland Inuit population was of special interest for several reasons. The Inuit display one of the lowest mortality rates due to atherosclerotic processes (30) and they were reported to consume a diet entirely different from Caucasian populations (31). Besides a difference in APOE allele frequencies, we learned that nowadays they display more or less the same lipid profile as is normally found in the various western societies with only one significant difference: both male, as well as female Inuits still have increased HDL-cholesterol levels (**Chapter 6**). We also found that they display significant lower mean levels of Lp(a) (32). During the last 20 years Greenland Inuits have adjusted themselves to the modern western way of living, including the regular consumption of alcohol and excessive smoking. They also do not consume their traditional diet, which was rich in long-chained poly-unsaturated fatty acids and supposed to be anti-atherogenic (33), to the same extent as 20 years ago. Others have reported that modern Inuit can not be distinguished from Danish controls with respect to the presence of atherosclerotic lesions in their arteries (34,35). Despite all this, they still do not suffer from the acute event of ischemic heart disease to the same extent as e.g. American, Danish or Dutch adults. Whether this indicates that they still have other protective factors (perhaps in the fibrinolytic cascade), or that it will be a matter of time before they will start suffering from atherosclerosis as severe as in our Western societies, is subject of further investigation.

The above mentioned studies were performed in normolipidemic populations. This raises the question as to whether the apoE polymorphism is also able to influence the plasma lipid levels in individuals with raised cholesterol levels. Therefore we studied the effect of the apoE polymorphism on the plasma lipid levels in FH patients (**Chapter 7**). We learned that in contrast to what is found in healthy populations except the Greenland Inuit, the apoE polymorphism does not influence the plasma lipid levels in FH patients, as was also reported by others (36,37). In addition, the apoE polymorphism did not explain the variability in response to lipid lowering drugs, as was suggested (38). We postulate that the absence of such an effect of the apoE polymorphism in the FH patient population could be due to high plasma LDL-cholesterol levels in these patients. A high amount of LDL-cholesterol delivered to the hepatocyte will probably overrule the

effect of the apoE polymorphism on the hepatic demand for cholesterol (and thus the hepatic LDL-receptor activity) through the uptake of chylomicron and VLDL remnants. This hypothesis is sustained by our observation that in a normal population, considering only subjects with an above average plasma cholesterol level, no effect of the apoE polymorphism on plasma LDL-cholesterol level could be detected (unpublished results). Recently our results have been confirmed by the results obtained by O'Malley and Illingworth (39) using lovastatin treatment.

In normal individuals chylomicron- and VLDL remnants are cleared from the circulation by receptor-mediated endocytosis via the liver. In patients with FD, the normal uptake of chylomicron- and VLDL remnants is impaired, leading to increased plasma cholesterol and triglyceride levels concomitant with increased risk for atherosclerosis (40). The uptake of these remnants is mediated by apoE, one of their major protein constituents. The majority of FD patients have the E2E2 phenotype. In the normal population the frequency of the E2E2 phenotype is about 1% (40), whereas the prevalence of FD in the general population has been estimated as 1 to 4 in 10.000. Hence, only 1 to 4% of all individuals with the E2E2 phenotype develop FD. Utermann et al. (18) suggested that other genetic and/or environmental factors in addition to the E2E2 phenotype are required for the expression of FD. Mahley et al. (41) postulated a possible explanation for this recessive mode of inheritance with reduced penetrance of FD in case of E2E2 homozygosity (41). They showed that the conformation of the 130-150 α-helical region of apoE (the supposed receptor-binding domain) can be easily modulated, leading to a change in receptor-binding activity. It was demonstrated that increased receptor binding activity of apoE2(Arg158→Cys) can be obtained *in vitro* by cleavage with thrombin (42), by cysteamine treatment (42) or by changing the lipid composition of the lipoprotein particle (43). They suggested that the conformation of apoE2(Arg158→Cys) is sensitive to its environment and that the cysteine at residue 158 has a secondary, rather than a direct influence on the receptor binding activity. This was recently confirmed by Wilson et al. (44) who showed that the three- dimensional conformation of the amino-terminal part of apoE (which includes residue 158) can be disrupted by the introduction of a cysteine residue on this position. Mahley et al. (41) suggested that this property could explain the requirement for additional environmental and/or genetic factors for the expression of FD in the case of apoE2E2 homozygotes.

However, FD is sometimes also observed in individuals with heterozygosity for rare apoE variants (**Chapter 8 & 9**; see also Table 3 in the General Introduction). In most of these cases FD displays a dominant mode of inheritance. This suggests

that in these cases the presence of one normal APOE allele does not prevent the development of FD. Family studies indicated that FD is inherited in a dominant fashion in case of heterozygosity for apoE1(Lys146→Glu) (45,46), apoE2-(Lys146→Gln) (47-49), apoE3(Cys112→Arg;Arg142→Cys) (50,51), apoE3-Leiden (4,52-55) and apoE4(Glu13→Lys;Arg145→Cys) (56). In addition, in these cases the inheritance of FD displays high rates of penetrance, i.e. the majority of individuals carrying these mutant alleles will develop FD at later age.

The reason for this difference in mode of inheritance of FD between the common E2(Arg158→Cys) variant and the rare apoE variants is subject to much speculation. Again, Mahley et al. (41) suggested that the loss of any positive charged amino acid residue within the putative receptor-binding domain affects the binding of apoE by reducing the ionic interaction with the ligand binding domains of the LDL-receptor. It was also suggested that this reduced binding could not be influenced by environmental factors such as lipid composition of the lipoprotein particle. As a consequence any mutation in the 130-150 residue fragment of apoE should result in "permanent" receptor-binding defects. These assumptions were based on a number of binding experiments using isolated human apoE from various variants, associated with dimyristoylphosphatidylcholine (DMPC)-complexes and were sustained by a number of experiments using apoE variants made by site-directed mutagenesis (57). There are however indications that this hypothesis not always holds true. First, the findings of Chappel (58) are in sharp contrast with the above mentioned DMPC-complex binding experiments. He showed that isolated VLDL of three rare apoE variants including E2(Lys146→Gln), had even higher binding affinities for the LDL-receptor than apoE3. Second, we recently discovered two unrelated probands in The Netherlands exhibiting E2E2 homozygosity but differing in the molecular basis for this protein phenotype. They turned out to display the apoE2(Arg158→Cys)/E2(Arg134→Gln) genotype (AMJM van den Maagdenberg et al. in prep.). Despite the loss of an additional charge (Arg134) in the receptor-binding domain, these individuals were normolipidemic. Also family members of these two probandi carrying this apoE variant, did not show any signs or symptoms typical for FD. These data indicate that (i) experiments using DMPC-complexes not necessarily reflexes the *in vivo* situation (for as much this can be simulated using isolated VLDL-particles), and (ii) not all rare apoE variants leading to the loss of a positive charge in the receptor-binding domain of apoE are associated with the development of FD. This suggests that the hypothesis of Mahley et al. (41) only holds true for some of the residues within the positive cluster.

In case of the apoE2(Lys146→Gln), the reason for the dominant mode of inheritance is currently under investigation. Preliminary studies suggest that the

VLDL particles of these patients are poor substrates for LPL, resulting in the accumulation of β-VLDL particles with high relative amounts of triglycerides. We suggest that this, in addition to the loss of a positively charged amino acid in the putative LDL-receptor binding domain, might explain the dominant mode of inheritance of FD in E2(Lys-146→Gln) allele carriers (**Chapter 8**).

Also in the case of apoE3-Leiden we showed that FD was inherited in a dominant fashion, although with a variable degree of penetrance (**Chapter 9**). It was demonstrated that, as was also found for apoE2, the 22 kD NH_2-part of the protein displayed normal receptor binding activity (43,54). This suggests that both the cysteine residue at position 158 as well as the two extra α-helices in apoE3-Leiden formed by the tandem repeat at residues 120-126, change the conformation of the apoE molecule in such a way that the C-terminal part of the protein prevents an efficient interaction of the binding domain with the receptor. Preliminary studies show that β-VLDL particles from hyperlipidemic E3-Leiden subjects are less active in binding than β-VLDL particles from normolipidemic E3-Leiden subjects. This suggests that the binding activity of E3-Leiden depends on the lipid composition of the VLDL particle. In addition to this, we found VLDL particles of E3-Leiden heterozygotes are "almost" phenotypically homozygous for this variants because E3-Leiden shows a high preference for this lipid fraction, in contrast to apoE3 and apoE2 (55). Although we did not yet study the reason for this difference in distribution, this could explain the dominant mode of inheritance of FD in case of E3-Leiden allele carriers.

In conclusion, FD is a genetically heterogeneous disease. It displays a recessively inherited multifactorial disease in case of the common E2(Arg158→Cys) variant, and a dominantly inherited disease with a high rate of penetrance in case of some rare apoE variants. From a clinical point of view, we strongly recommend that all patients with elevated plasma cholesterol and triglyceride levels concomitant with increased cholesterol/triglyceride ratios in the VLDL fraction should be analyzed in detail for apoE phenotype and genotype. In case of E2E2 homozygosity, this will sustain the diagnosis of FD. However, when the suggested FD patients do not exhibit the common E2E2 homozygosity, the patients might carry a rare apoE variant, and thus family studies are indicated as in these cases early diagnosis of FD is feasible.

Following these strategies of FD diagnosis, we found that about 20 % of all patients diagnosed as FD in lipid clinics on lipid levels exhibit non-E2E2 homozygosity. After performing family studies we eventually found that about 50 % of all patients exhibited heterozygosity for rare apoE variants.

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Summary

This thesis describes various aspects of the apoE polymorphism in healthy populations (**Chapters 2-6**) and in individuals with hyperlipidaemia (**Chapters 7-9**). In **Chapter 2** we describe a new method to study the apoE polymorphism. This method is based on isoelectric focusing of serum or plasma followed by immunoblotting using anti-apoE antibodies as first antibody. Compared with conventional methods, this method is less laborious, suitable for large scale screening purposes and it needs only a few microliters of serum or plasma that could be stored at - 20 °C for long periods of time.

With this method, we screened a population of 2018 randomly selected 35-year-old males from three different areas in The Netherlands (**Chapter 3**). The APOE allele (E*2, E*3, and E*4) frequencies estimated in this study were comparable to those reported for other Caucasian populations. We found the E*4 allele to be associated with elevated plasma cholesterol and apoB levels and with decreased apoE concentrations, whereas the opposite was true for the E*2 allele. The APOE alleles primarily affected apoE concentrations which is sustained 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% which is relatively low as compared with that reported for other population samples.

In **Chapter 4** we describe the phenotype distributions and allele frequencies of the APOE gene and its influence on plasma lipid and (apo)lipoprotein levels in 160 Dutch twin families. Also in this population the apoE polymorphism primarily affected the plasma apoE concentrations. Using the group of monozygotic (MZ) twins we were able to show that the apoE polymorphism exhibited no influence on the within-pair differences of the measured lipoprotein parameters. This indicates that the APOE gene acts as a "level" gene rather than a "variability" gene, thereby supporting the hypothesis that the apoE polymorphism plays an independent role in determining plasma lipid levels.

In a sub-group of 303 parents of these twin families we studied the influence of the apoE polymorphism on plasma levels of lipoprotein(a) [Lp(a)], one of the major single risk factors for atherosclerosis (**Chapter 5**). Parallel to the effect of the apoE polymorphism on low density lipoprotein (LDL)-cholesterol levels, the APOE*2 allele was found to be associated with decreased plasma Lp(a) levels, whereas the APOE*4 allele increased the Lp(a) levels. For the Lp(a) levels, the genetic variance associated with the APOE locus contributed about 4% to the total phenotypic variance. Since the apoE polymorphism effects LDL-receptor activity, we

concluded that, at least in healthy normolipidemic individuals, plasma levels of Lp(a) are modulated by the LDL-receptor activity.

The Greenland Inuit population display an extremely low incidence of ischaemic heart disease when compared with Western populations. In **Chapter 6** we evaluated the influence of genetic- and environmental factors on levels of the measured lipid parameters, which could explain this low risk. In the present-day Inuits we found a relative high APOE*4 allele frequency (0.229) whereas the APOE*2 allele frequency was very low. The apoE polymorphism showed only little influence on the plasma lipid and (apo)lipoprotein levels. Smoking only showed a weak influence on the plasma apoB level. However, regular alcohol consumption showed a beneficial effect in the Inuit by decreasing the levels of very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL)-cholesterol, LDL-cholesterol and apoB and increasing the apoA1 and high density lipoprotein (HDL)-cholesterol levels. Our study shows that the Greenland Inuit at present display a high risk lipoprotein profile which is strongly influenced by alcohol consumption. In addition to this, we studied the fatty acids composition of the plasma cholesterol esters in this Inuit population. From this we learned that, although 13% of the Inuit claim to consume their traditional diet, they show almost the same relative fatty acids composition of the plasma cholesterol esters.

In a group of 120 patients with heterozygous familial hypercholesterolemia (FH) the influence of the apoE polymorphism on pre-treatment plasma lipid levels and on the response to treatment with simvastatin was studied (**Chapter 7**). The apoE phenotype distribution did not differ significantly between the FH group and a healthy sample group of the Dutch population. In the FH patient group a considerable interindividual variation in response to simvastatin was observed, but this variation could not be related to the apoE polymorphism. However, considering males and females separately, we found that female FH patients with the apoE3E3 phenotype responded better on simvastatin treatment with respect to LDL-cholesterol than male FH patients with the apoE3E3 phenotype. In contrast to what we found for healthy populations, with the exception of the Greenland Inuit, also in the FH patients group the apoE polymorphism did not influence the plasma lipid levels. As was first proposed by Utermann, the influence of the apoE polymorphism on the lipid levels is assumed to be the result of a more efficient catabolism of chylomicron- and VLDL remnants in individuals with the E*4 allele, and a less efficient catabolism of these lipoprotein particles in subjects exhibiting the E*2 allele due to a defect in binding of apoE2 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. We found that Inuit had very low levels of VLDL cholesterol and triglycerides. We suggest that these low levels are not sufficient to influence the hepatic demand for cholesterol and, as a consequence, the LDL-receptor activity, which directly influences the plasma cholesterol levels. In contrast to this are the FH patients. In their case we suggest that the high levels of LDL overrules the influence of the apoE polymorphism on the LDL-receptor activity, which again does not result in an influence on the plasma cholesterol levels.

In addition to the influence of the common apoE variants on the lipoprotein metabolism in healthy individuals, there are also a number of associations between apoE variants and various disorders in the lipoprotein metabolism. The best studied association is that between E2E2 homozygosity and familial dysbetalipoproteinemia (FD), a genetic disorder of the lipoprotein metabolism predisposing to premature atherosclerosis.

In the general population, FD is found with a frequency between 2-10 per 10,000. As determined by isoelectric focusing, most patients with FD exhibit the homozygous apoE2E2 phenotype. However, only 1 to 4% of the E2E2 homozygotes will develop FD. We wondered whether this reduced penetrance of FD in E2E2 homozygotes could be due to additional heterogeneity in the APOE*2 allele. In **Chapter 8** we studied the genetic heterogeneity of the APOE gene in 40 FD patients with the E2E2 phenotype and found that they all were homozygous for the apoE2(Arg158→Cys) isoform. Three unrelated individuals with the E3E2 phenotype were found to exhibit the rare E2(Lys146→Gln) mutation. This mutation was not found among normolipidemic individuals with either the E2E2 or the E3E2 phenotype selected from a random population sample. Family studies of the three probands heterozygous for the E*2(Lys146→Gln) allele showed that heterozygosity for this rare allele predisposes to FD with high penetrance.

In addition to this rare apoE2(Lys146→Gln) variant, by screening of FD patients, five probands showing heterozygosity for the APOE*3-Leiden allele were found (**Chapter 9**). Genealogical studies revealed that these probands share common ancestry in the seventeenth century. In a group of 128 family members, spanning three generations, 37 additional heterozygous APOE*3-Leiden gene carriers were detected. Multiple linear regression analysis revealed that most of the variability in expression of FD in APOE*3-Leiden allele carriers can be explained by age. Body mass index showed a less significant influence on the expression of FD. Gender had no effect on the expression in E*3-Leiden allele carriers, nor did it influence the

age of onset of FD. Isoelectric focusing of plasma and of isolated VLDL, IDL and HDL density fractions showed that in E*3-Leiden allele carriers the apoE3-Leiden variant largely predominates over its normal apoE counterpart, especially in the VLDL and IDL density fractions. We conclude that in APOE*3-Leiden allele carriers FD is dominantly inherited with a high rate of penetrance, i.e. the presence of normally functioning apoE molecules in the plasma does not prevent the age-related expression of this disease.

These results reinforce the concept that FD is a genetically heterogeneous disease entity with either a dominant mode of inheritance in case of heterozygosity for rare apoE variants or with a recessive mode of inheritance in case of homozygosity for apoE2(Arg158→Cys). It is shown that these dominantly inherited forms of FD display high rates of penetrance, making early diagnosis in these families feasible. From a clinical point of view, we strongly recommend that all patients with elevated plasma cholesterol and triglyceride levels concomitant with increased cholesterol/triglyceride ratios in the VLDL fraction should be analyzed in detail for apoE phenotype and genotype. In case of E2E2 homozygosity, this will sustain the diagnosis of FD. However, when the suggested FD patients do not exhibit the common E2E2 homozygosity, the patients might carry a rare apoE variant, and thus family studies are indicated.

Samenvatting

Dit proefschrift beschrijft de invloed van het apoE polymorfisme op het lipoproteïne metabolisme bij gezonde mensen (Hoofdstuk 2 t/m 6) en bij mensen met een erfelijke storing in het lipoproteïne metabolisme (Hoofdstuk 7 t/m 9).

Allereerst wordt een nieuwe methode beschreven om het apoE fenotype te bepalen (Hoofdstuk 2). Deze nieuwe methode is gebaseerd op isoelectrofocusing van serum of plasma, gevolgd door immunoblotting met een polyclonaal anti-apoE als eerste antilichaam. Deze nieuwe methode bleek, in vergelijking met de reeds bestaande technieken, veel minder arbeidsintensief, geschikt voor toepassing op grote schaal, en veel betrouwbaarder en gevoeliger. Een ander belangrijk voordeel was het feit dat slechts enkele microliters plasma of serum voldoende was voor fenotypering. Dit plasma of serum hoeft niet "vers" te zijn. Ook langdurig ingevroren materiaal bleek nog goed bruikbaar te zijn.

Met deze nieuwe techniek werd een groep van 2018 gezonde Nederlandse mannen van 35 jaar oud onderzocht (Hoofdstuk 3). De APOE allel (E*2, E*3, E*4) frequenties die bij deze mannen gevonden werden, bleken vergelijkbaar met frequenties die eerder waren gevonden bij Duitse en Schotse bevolkingsgroepen. Wel waren er duidelijke verschillen tussen de Nederlandse groep en Japanners, Finnen, en Noord-Amerikanen. Uit ons onderzoek bleek dat het APOE*4 allel duidelijk geassocieerd is met verhoogde plasma cholesterol en apoB concentraties. Daarentegen bleek het APOE*2 allel geassocieerd te zijn met verlaagde cholesterol en apoB concentraties. Naar schatting 18 % van de totale variantie in de plasma apoE concentratie werd door het apoE polymorfisme verklaard.

In Hoofdstuk 4 worden de apoE fenotype- en APOE allelfrequenties en hun invloed op plasma lipiden en (apo)lipoproteïnen concentraties bij 160 Nederlandse tweeling-families beschreven. Ook uit dit onderzoek bleek dat het apoE polymorfisme vooral de plasma apoE concentratie beïnvloedt. Bij een groep van monozygote tweelingen bleek het apoE polymorfisme de verschillen voor diverse gemeten parameters binnen de tweeling-paren niet te beïnvloeden. Hiermee gedraagt het APOE gen zich als een "niveau" gen, d.w.z. de invloed van het APOE gen op de lipiden concentraties wordt bij monozygote tweelingen niet beïnvloed door variatie in omgevingsfactoren. Dit ondersteunt de hypothese dat de invloed van het apoE polymorfisme op het lipiden metabolisme niet beïnvloed wordt door variatie in omgevingsfactoren (waaronder een verschil in voeding).

Bij 303 ouders van deze tweeling-families werd tevens de invloed van het apoE polymorfisme op de Lp(a) concentratie in plasma onderzocht (Hoofdstuk 5). Het door ons gevonden effect liep evenwijdig aan de invloed van apoE op LDL-

cholesterol. Bij personen met een APOE*2 allel vonden wij verlaagde Lp(a) concentraties, terwijl bij APOE*4 allel dragers hogere Lp(a) concentraties werden gemeten. Ca. 4% van de totale variantie in plasma Lp(a) concentratie bleek te kunnen worden verklaard door het apoE polymorfisme. Dit ondersteunt de hypothese dat, in ieder geval bij gezonde mensen, een gedeelte van het Lp(a) uit plasma wordt geklaard door een proces waarbij de LDL-receptor een rol speelt.

De Groenlandse Inuit (of Eskimos) vormen een zeer bijzondere bevolkingsgroep. Hun jaarlijkse sterfte ten gevolge van hart- en vaatziekten is nog steeds zeer laag, zeker in vergelijking met de westerse landen. In Hoofdstuk 6 beschrijven wij de invloed van genetische- en omgevingsfactoren, die dit lage risico zouden kunnen verklaren. De door ons bestudeerde Inuit vertoonden een hoge APOE*4 allele frequentie (0.229) en een zeer lage APOE*2 allel frequentie. In tegenstelling tot wat wij eerder vonden bij een tweetal Nederlandse bevolkingsgroepen, bleek het apoE polymorfisme bij de Inuit geen invloed uit te oefenen op de plasma lipiden en (apo)lipoproteïnen concentraties. De plasma apoB concentratie werd slechts in geringe mate door roken beïnvloed. Alcohol gebruik had echter wel een sterke invloed op de VLDL+IDL-cholesterol, LDL-cholesterol, apoB, apoA1 en HDL-cholesterol concentraties. Uit deze studie blijkt dat Groenlandse Inuit voor wat betreft hun patroon van lipoproteïne concentraties een even hoog risico voor hart- en vaatziekten hebben als personen in "westerse" landen. Dit risico wordt, bij de Inuit, mogelijk in sterke mate beïnvloed door alcohol gebruik. Ons vermoeden dat dit lipiden profiel bij Inuit wordt veroorzaakt door aanpassingen aan een meer "westerse" levensstijl, inclusief "westerse" voeding, werd bevestigd door onderzoek naar de vetzuur samenstelling van de plasma cholesterol esters. Wij vonden dat, ondanks het feit dat 13 % van de Inuit naar zeggen nog steeds uitsluitend een traditioneel Inuit voedingspatroon hebben, alle deelnemende Inuit een vetzuurprofiel hebben wat zeer sterk lijkt op het "westerse" profiel.

In samenwerking met een aantal lipiden poliklinieken (Nijmegen, Amsterdam en Utrecht) was het mogelijk om in een groep van 120 heterozygote Nederlandse patienten met familiaire hypercholesterolemie (FH) de invloed van het apoE polymorfisme te bestuderen op: (i) de basale lipiden concentraties, en (ii) de verandering van de plasma lipiden concentraties onder invloed van een behandeling met simvastatine (Hoofdstuk 7). Wij vonden geen verschil in de apoE fenotype verdeling tussen de FH patienten groep en een gezonde Nederlandse controle populatie. In deze FH patienten groep bleek het apoE polymorfisme de plasma lipiden concentraties, gemeten voordat met de simvastatine behandeling werd aangevangen, niet te beïnvloeden. In de totale groep van FH patienten bleek het apoE polymorfisme de verandering van de lipiden concentraties onder invloed van

simvastatine niet te beïnvloeden. Wel vonden wij dat vrouwelijke FH patienten met het E3E3 fenotype een sterkere LDL-cholesterol verlaging na simvastatine behandeling vertoonden dan de mannelijke FH patienten met het E3E3 fenotype.

Het effect van het apoE polymorfisme op de plasma lipiden concentraties bij gezonde personen is het gevolg van (i) een snellere klaring van chylomicronen- en VLDL-remnants bij individuen met een APOE*4 allel, en (ii) een tragere klaring van deze remnants bij individuen met een APOE*2 allel. Als reactie hierop wordt de LDL-receptor activiteit van de levercel beïnvloed, waardoor meer (bij APOE*2 allel dragers) of minder (bij APOE*4 allel dragers) LDL-cholesterol uit het bloed wordt opgenomen. Bij de Groenlandse Inuit vonden we zeer lage VLDL+IDL-cholesterol en triglyceriden concentraties. Wij vermoeden dat ongeacht het apoE fenotype, deze lage "remnant" concentraties onvoldoende zijn om de lever, via de opname van remnant deeltjes, met cholesterol te verzadigen. Dit zou kunnen verklaren waarom de LDL-cholesterol en totaal plasma cholesterol concentraties bij de Inuit niet worden beïnvloed door het apoE fenotype. Bij de FH patienten is waarschijnlijk precies het tegenovergestelde het geval. FH patienten hebben door hun LDL-receptor defect een zeer hoge LDL-cholesterol concentratie. Hierdoor wordt het "subtiele" apoE polymorfisme effect teniet gedaan, waardoor ook bij FH patienten het apoE polymorfisme geen duidelijke invloed op de LDL-cholesterol concentratie heeft.

Behalve de invloed van het normale apoE polymorfisme op het lipiden metabolisme bij gezonde mensen, zijn er ook een aantal storingen van het lipiden metabolisme bekend waarvan wordt aangenomen dat ze worden veroorzaakt door afwijkende vormen van apoE. Verreweg de meest bekende, aan apoE geassocieerde lipiden afwijking, is familiaire dysbetalipoproteinemie (FD).

FD is een aandoening die bij ca. 0.02-0.1 % van de bevolking voorkomt. Meer dan 90 % van de patienten met FD hebben het apoE2E2 fenotype. Echter slechts 1-4 % van alle E2E2 homozygoten krijgt FD. Daarom spreekt men van een recessieve overerving met gereduceerde penetrantie van FD bij E2E2 homozygoten. Naast omgevingsfactoren zoals voeding, hormonen en alcohol gebruik zijn waarschijnlijk andere genetische factoren noodzakelijk om FD bij deze personen tot expressie te laten komen. Wij waren daarom zeer geïnteresseerd in de mogelijke microheterogeniteit van apoE2.

In Hoofdstuk 8 wordt een onderzoek van het APOE gen bij 43 FD patienten beschreven. DNA onderzoek wees uit dat 40 van deze FD patienten homozygoot waren voor het APOE*2(Arg158→Cys) allel. Echter, drie FD patienten bleken het E3E2 fenotype te hebben. DNA sequentie onderzoek toonde aan dat het E*2 allel van deze patienten afweek van het normale E*2 allel. Alle drie waren zij drager

van het zeldzame APOE*2(Lys146→Gln) allel. Ter controle werden tevens 13 gezonde E2E2 homozygoten en 50 E3E2 heterozygoten onderzocht. Bij geen van deze mensen werd het APOE*2(Lys146→Gln) allel aangetroffen. Uit onderzoek bij familieleden van deze drie FD patienten met de E2(Lys146→Gln) mutatie bleek dat vrijwel alle dragers van dit zeldzame allel in meer of mindere mate tekenen van FD vertoonden. Dit maakt het aannemelijk dat FD geassocieerd met deze apoE variant dominant overerft (heterozygotie voor het "defecte" allel is voldoende om de ziekte te ontwikkelen), met een hoge penetrantie (vrijwel alle dragers van het "defecte" allel ontwikkelen uiteindelijk de ziekte).

Bij een volgende screening van FD patienten vonden wij, naast de boven beschreven E2(Lys146→Gln) variant, tevens 5 patienten die alle heterozygoot voor het apoE3-Leiden allel waren (Hoofdstuk 9). Uit uitgebreid genealogisch onderzoek bleek dat deze 5 patienten een gemeenschappelijk voorouder hebben in de 17de eeuw. Bij 128 familieleden van deze 5 patienten vonden wij nog eens 37 personen die heterozygoot waren voor apoE3-Leiden. Uit multiple regressie analyse bleek dat de ernst van de aandoening bij deze personen in sterke mate wordt beïnvloed door leeftijd, en in mindere mate door het relatieve lichaamsgewicht. Hierbij was er geen verschil tussen mannen en vrouwen. Ook was er geen duidelijk verband met de leeftijd waarop de ziekte zich voor het eerst openbaarde. Uit een experiment, waarbij plasma en de apolipoproteïnen van de geïsoleerde VLDL, IDL en HDL fracties werden geisoelectrofocuseerd, bleek dat apoE3-Leiden een duidelijke voorkeur vertoonde voor de VLDL en IDL fractie. In deze fracties was vrijwel geen "normaal" apoE aanwezig. Uit onze studie blijkt dat ook bij personen met apoE3-Leiden de overerving van FD dominant en hoog penetrant is.

Onze resultaten ondersteunen de stelling dat FD een genetisch heterogene aandoening is, die zowel een dominant overervingspatroon (zoals bij apoE2(Lys 146→Gln) en apoE3-Leiden) als een recessief overervingspatroon (bij apoE2E2 homozygoten) kan hebben.

Wij adviseren om bij alle patienten met een verhoogde plasma cholesterol-concentratie en een verhoogde plasma triglyceride concentratie, gepaard gaande met een verhoogde ratio van cholesterol/triglyceride in VLDL, zowel een apoE fenotypering als een apoE genotypering te laten doen. Indien de betreffende patiënt E2E2 homozygoot blijkt te zijn, ondersteunt dit de diagnose van de recessieve vorm van FD. Wordt echter een ander apoE fenotype/genotype gevonden, dan zal er vrijwel altijd sprake zijn van een zeldzame apoE variant met een dominant overervingspatroon van FD. Bij deze dominant overerfbare vormen van FD is de ziekte vrijwel altijd ook hoog penetrant, waardoor een vroegtijdige diagnose bij familieleden mogelijk is.

Abbreviations

ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
APO/apo	Apolipoprotein
ASO	Allele Specific Oligo
AS-RFLP	Allele Specific Restriction Fragment Length Polymorphism
BMI	Body Mass Index
CETP	Cholesteryl Ester Transfer Protein
DGGE	Denaturating Gradient Gel Electrophoresis
DMPC	Dimyristoylphosphatidylcholine
DZ	Dizygous/dizygotic
ELISA	Enzyme Linked Immunosorbent Assay
FCH	Familial Combined Hyperlipoproteinemia
FD	Familial Dysbetalipoproteinemia
FH	Familial Hypercholesterolemia
HDL	High Density Lipoprotein(s)
HL	Hepatic Lipase
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
HTG	Hypertriglyceridemia
IDL	Intermediate Density Lipoprotein(s)
IEF	Isoelectric Focusing
IHD	Ischaemic Heart Disease
INA	Immuno Nephelometry Assay
LCAT	Lecithin Cholesterol Acyltransferase
LDL	Low Density Lipoprotein(s)
LDLR	Low density Lipoprotein Receptor
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein Lipase
LRP	Low Density Lipoprotein Receptor-related Protein
mLDL	Modified Low Density Lipoprotein(s)
MZ	Monozygous/monozygotic
NS	Not Significant (P; probability >0.05)
PCR	Polymerase Chain Reaction
PUFA	Poly Unsaturated Fatty Acids
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SEM	Standard Error of the Mean
Type III	Type III Hyperlipoproteinemia
USA	United States of America
VLDL	Very Low Density Lipoprotein(s)

Curriculum Vitae

De auteur van dit proefschrift werd op 23 juni 1956 geboren te Leiden. In 1972 behaalde hij het MAVO-4 diploma aan de Immanuelschool voor Christelijke MAVO te Alphen aan den Rijn. In 1975 werd het MBO diploma voor analist Klinisch-Chemische Differentiatie en in 1977 werd het HBO-A diploma voor analist Microbiologische richting behaald aan de Laboratoriumschool Rijnland te Leiderdorp. Van november 1977 tot en met februari 1979 vervulde hij zijn militaire dienstplicht als dienstplichtig officier in opleiding (met de rang van Vaandrig) bij 121 Rayon Verbindingsdienst Compagnie te Ede. Gedurende de periode maart 1979 tot en met november 1991 vervulde hij met grote regelmaat tevens zijn taak als reserve-officier met de rang van Kapitein bij de Aan en Afvoertroepen (Koninklijke Landmacht). Van maart tot en met december 1979 was hij werkzaam als Analist op de afdeling Weefseltypering van het Centraal laboratorium van de Bloedtransfusiedienst te Amsterdam. Van januari 1980 tot en met juni 1985 was hij werkzaam als Analist-A op het Laboratorium voor Stralengenetica en Chemische Mutagenese van de Rijksuniversiteit Leiden, onder directe begeleiding van Prof. Dr. G.R. Mohn. Van juli 1985 tot november 1991 was hij als Research Analist in dienst van de afdeling Anthropogenetica, Rijksuniversiteit Leiden. Het onderzoek gedurende deze laatste periode is in hoofdlijnen beschreven in dit proefschrift, en geschiedde in een samenwerkingsverband met het voormalige Gaubius Instituut, waar hij voor het grootste gedeelte werkzaam was. De supervisie gedurende deze periode berustte bij Dr. E.C. Klasen (tot augustus 1988), Dr. R.R. Frants (vanaf augustus 1988) en Dr. Ir. L.M. Havekes (de gehele periode). Sinds 1 januari 1992 is hij werkzaam op de afdeling Fibrinolyse en Proteolyse van het IVVO-TNO Gaubius Laboratorium (sectiehoofd Dr. C. Kluft).

Nawoord

Bij het tot stand komen van dit proefschrift heb ik van een groot aantal personen op zeer uiteenlopende wijze steun ontvangen. Zonder iemand hierbij te kort te doen wil ik een aantal van hen met name noemen.

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Ook de bijdrage van Lenie van Maurik (het afnemen van de vele buizen bloed) wil ik hier niet vergeten.

Lars Johansen and Jorgen Jespersen trusted my technical skills, and allowed me to enter the "Inuit Research Project". Without their assistance, chapter 6 would never have been written. I sincerely hope our results will be beneficial for the Greenland Inuit.

Tijdens mijn onderzoek heb ruim 6 maanden doorgebracht op het laboratorium van Dr. Maryvonne Rosseneu, AZ St Jan, Brugge, Belgie. Ik weet zeker dat men in het begin moeite heeft gehad met die Hollandse indringer en zijn "monsters" en drop. Ik ben echter altijd zeer gastvrij ontvangen, en heb me daar echt thuis gevoeld. Hiervoor wil ik Christine, Gerda, Walter, Hans; Hans, Jo, Raf, en alle andere medewerkers van het "labo" bedanken.

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Tevens wil ik mijn moeder, broer, zusters en hun partners, mijn erkentelijk tonen voor de wijze waarop zij mij, vooral de laatste twee jaar, thuis hebben opgevangen.

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Selected List of Publications

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