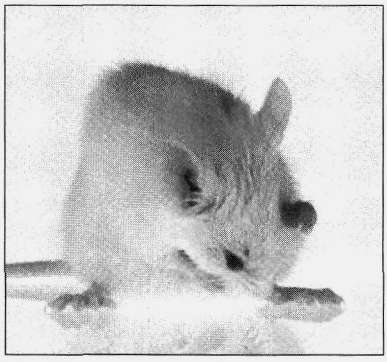
Chylomicron and Very Low Density Lipoprotein Metabolism in Transgenic Mice Carrying Mutant Alleles of APOE



Stellingen

behorende bij het proefschrift

Chylomicron and very low density lipoprotein metabolism in transgenic mice carrying mutant alleles of APOE

1. Een dominant overervende APOE mutatie betekent niet dat deze leidt tot een extremer fenotype dan een recessief overervende APOE mutatie (dit proefschrift).

2. Het expressie niveau van het APOE gen is een belangrijke factor voor de gevoeligheid voor dieet-geïnduceerde hyperlipidemie (van Ree et al., 1994, Atherosclerosis 111:25-37, dit proefschrift).

3. Alleen die studies met betrekking tot de *in vivo* klaring van lipoproteïnen uit plasma, waarbij rekening is gehouden met de concentratie van in het plasma reeds aanwezige lipoproteïnen, dragen bij aan het ophelderen van de betreffende mechanismen (dit proefschrift).

4. Juist de verschillen in lipoproteïne metabolisme van mens en (transgene) muis vergroten ons inzicht in het lipoproteïne metabolisme van de mens (dit proefschrift).

5. ApoE is een multifunctioneel eiwit.

6. Monofactoriële erfelijke ziekten van hart en vaten bestaan niet.

7. Lever lipase speelt een niet onmisbare rol in de opname van chylomicronen en VLDL remnant lipoproteïnen door de lever (Homanics et al., 1995, J. Biol. Chem. 270:2974-2980; Amar et al., 1996, Circulation 94:I-398).

8. Opname van remnant lipoproteïnen via het LDL receptor-gerelateerde eiwit (LRP) vereist door de lever geproduceerd en gebonden apoE (Fazio et al., 1996, Circulation, 94: I-679)

9. Psychiatrische ziekten onderscheiden zich van veel andere ziekten in het feit dat het onduidelijk is wie het meest onder de ziekte lijdt: de patiënt of zijn/haar geliefde.

10. Tegenstanders van genetische manipulatie realiseren zich vaak niet dat poedels en superbintjes ook produkten zijn van actief manipuleren van erfelijke eigenschappen.

11. Natuur in Nederland?. Weidevogels verblijven in ons land bij de gratie van het poldergemaal.

Leiden, 12 maart 1997 Bart van Vlijmen

Chylomicron and Very Low Density Lipoprotein Metabolism in Transgenic Mice Carrying Mutant Alleles of APOE

Proefschrift
ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College van Dekanen
te verdedigen op woensdag 12 maart 1997
te klokke 14.15 uur

door

Bart Jozef Maria van Vlijmen
geboren te Delft in 1967

Promotiecommissie:

Promotor:	Prof. dr. ir. L.M. Havekes
Copromotor:	Dr. M.H. Hofker
Referent:	Prof. dr. Th.J.C. van Berkel
Overige leden:	Prof. dr. R.R. Frants
	Prof. dr. A. van der Laarse
	Dr. J.M.G. Princen (TNO-PG, Gaubius Laboratorium, Leiden)
	Dr. P.H.E. Groot (SmithKline Beecham Pharmaceuticals, Welwyn, Engeland)

ISBN 90-9010254-X
Trefwoorden: apolipoprotein E, lipoprotein metabolism, transgenic mice

The studies presented in this thesis were performed at the Gaubius Laboratory of TNO-PG, Leiden and the MGC-Department of Human Genetics of Leiden University. This work was financially supported by the Netherlands Organisation for Scientific Research and the Netherlands Heart Foundation (NWO/NHS program 'Stimuleringsfonds' project #901-04-092)

Financial support by TNO Prevention and Health, and the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Aan mijn ouders
Voor Marieke

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Cardiovascular diseases are the leading cause of mortality in the industrialized world, together accounting for about one half of the total number of annual deaths. The main cause of cardiovascular diseases is atherosclerosis. An important risk factor in the development and acceleration of atherosclerotic disease is an elevated level of cholesterol and/or triglycerides in the plasma. Cholesterol and triglycerides are transported in the blood by lipoproteins. As a consequence, much effort in the field of research into cardiovascular disease is focused on the lipoprotein metabolism.

1.1. LIPOPROTEIN METABOLISM

Cholesterol is essential for the biosynthesis of cellular membranes, steroid hormones and bile acids, whereas triglycerides are important as an energy source for muscle and other tissue and energy storage in adipose tissue. Distribution of hydrophobic cholesterol and triglycerides over the body, through aqueous compartments like the blood, is performed by specific transportation vehicles called lipoproteins. Lipoproteins are spherical particles containing a central hydrophobic core filled with cholesterol (in esterified form) and triglycerides, and a surface monolayer consisting of phospholipids, unesterified cholesterol and proteins, called apolipoproteins. Polar groups of phospholipids and apolipoproteins interact with the aqueous plasma environment, and thus allow solubilization and transport of the particles through an aqueous environment. Several types of lipoproteins can be distinguished, which are classified according to their density as determined by gradient ultracentrifugation. The four major classes are: chylomicrons, very low density lipoproteins (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The density of a lipoprotein is inversely related to the size of the particle i.e the ratio of low-density, nonpolar core lipid and high density surface protein. Besides differing in density, lipoproteins also differ in electrophoretic mobility and apolipoprotein composition (for characteristics of lipoproteins see Table I).

According to the site of synthesis and function, lipoprotein metabolism can be divided into three pathways (Figure 1A-C): i) the exogenous pathway, which involves the metabolism of intestinally derived lipoproteins, ii) the endogenous pathway, which involves the fate of hepatically derived lipoproteins, and iii) reversed cholesterol pathway involving the transport of lipids from peripheral tissue to the liver and their subsequent transformation into bile acids. (For review see Mahley, 1984; Gotto, 1986; Breslow, 1988; Havel and Kane, 1989; Eisenberg, 1990).

Table I. Physical Properties and Composition of Human Plasma Lipoproteins (Gotto, 1986)

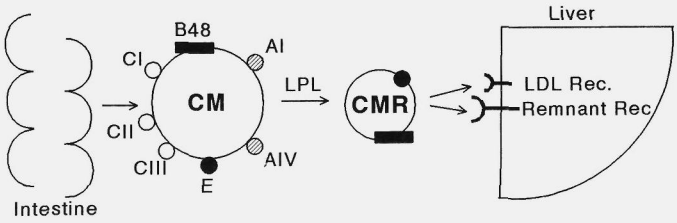
	Chylomicrons	VLDL	IDL	LDL	HDL
diameter (nm)	75-1200	30-80	25-35	19-25	5-12
density	<0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.210
mobility*	origin	pre-ß	pre-ß/ß	ß	α
protein	1-2	6-10	11	21	45-55
triglyceride	88	56	29	13	15
phospholipid	8	20	26	28	45
cholesteryl ester	3	15	34	48	30
free cholesterol	1	8	9	10	10
apolipoproteins	AI,AIV,B48, CI,CII,CIII,E	B100,CI, CII,CIII,E	B100,E	B100	AI,AII,AIV, CI,CIII,E

*According to the mobility of plasma α-and ß-globulins on agarose gelelectroforeses.
The values given for protein, triglyceride, phospholipid, cholesterylester, and free cholesterol are expressed as the percentage of total weight.

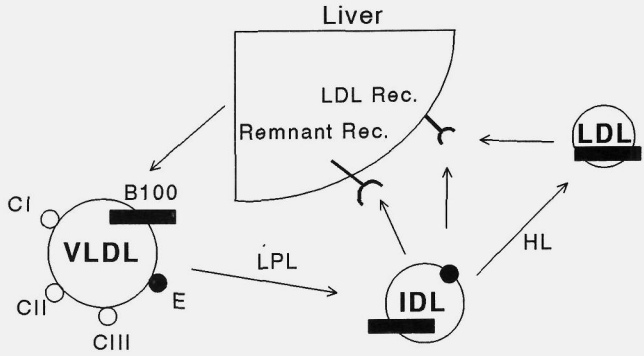
1.1.1 The exogenous pathway

Dietary cholesterol and triglycerides (in the form of fatty acids) are absorbed by the intestinal epithelium and are subsequently packaged into chylomicrons (see Figure 1A) (Mahley and Hussain, 1991). Nascent chylomicrons are very large triglyceride rich lipoproteins (> 1000 Å) and possess primairily apolipoprotein (apo) B48, apoAI and apoAIV. The chylomicrons are secreted from the enterocytes into the mesenteric lymph after which they enter the general circulation. In the general circulation chylomicrons undergo several modifications. First, chylomicrons acquire C apolipoproteins (apoCI, CII and CIII) and apoE from circulating lipoproteins. The C apolipoproteins modulate the metabolism of these lipoproteins. For example, apoCII serves as a cofactor for lipoprotein lipase (LPL), which catalyses the hydrolysis of triglycerides. The hydrolysis results in the liberation of free fatty acids, which are stored as triglycerides in adipose tissue or are used as an energy source in muscle and other tissues. Triglyceride hydrolysis occurs while the particles are bound to the surface of vascular endothelial cells (Olivecrona and Bengtsson-Olivecrona, 1990a;b). Upon hydrolysis the chylomicron particles become smaller, and sheets of surface phospholipids and apolipoproteins (especially apoAI) are shed from the particle and give rise to HDL. As the chylomicron particles are hydrolysed they loose their affinity for the C apolipoproteins, which are then transferred to HDL (Tall and Small, 1978). The residual particle is now called the chylomicron remnant. The chylomicron remnants acquire additional apoE from other plasma lipoproteins, which results in a rapid clearance of these particles from the circulation by the liver (Mahley, 1988; 1989a). The liver uptake of remnant lipoproteins is thought to involve a so-called secretion re-capture process (Brown, 1991; Mahley and Hussain, 1991; Shimano, 1994a): As the first step, remnant lipoproteins enter the space of

A. Exogenous pathway



B. Endogenous pathway



C. Reversed cholesterol pathway

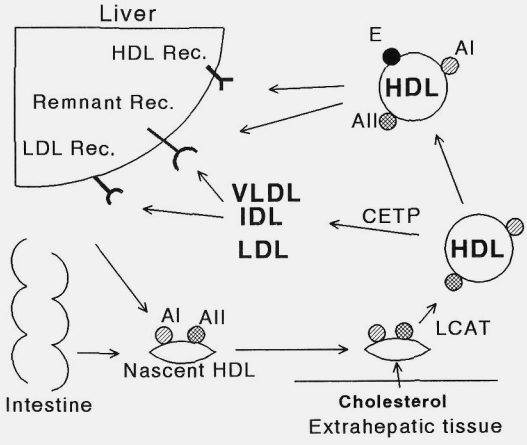


Figure 1. Schematic representation of the three pathways in human lipoprotein metabolism. CM, chylomicron; CMR, chylomicron remnant; LPL, lipoprotein lipase; HL hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; E, apoE, etc.

Disse and become enriched with surface bound apoE, derived from hepatocytes. The apoE-enriched remnants presumably bind to cell surface heparan sulphate proteoglycans (HSPG; Ji, 1993; 1994; 1995). As the second step, the apoE-enriched remnants become internalized via endocytotic receptors. Internalisation of the remnants may occur by the receptor alone or in a receptor-HSPG-complex (Ji, 1995). The hepatic lipoprotein receptors involved in remnant uptake are the LDL receptor, which is an apoB/E specific receptor (Windler, 1988; Choi, 1991, Mahley and Hussain, 1991), and a second putative remnant receptor. Studies by Herz (1988) and Kowall (1989) have indicated the LDL receptor-related protein (LRP) as a likely candidate for the remnant receptor. Furthermore, van Dijk (1992) postulated the existence of a specific remnant receptor distinct from LRP in rat parenchymal cells, with a binding site for ß-VLDL.

Recent *in vitro* studies showed that LPL and hepatic lipase (HL) may also play a role in mediating the uptake of remnants into the liver cells independent of their catalytic activity (Sultan, 1990; Beisiegel, 1991; Eisenberg, 1992; Shafi, 1994; Ji, 1994b, Diaro, 1994).

1.1.2 The endogenous pathway

In the endogenous pathway (see Figure 1B), hepatic cholesterol (derived from *de novo* synthesis or chylomicron remnants taken up from the blood) and triglycerides (derived from lipogenesis, from plasma fatty acids, or chylomicron remnant uptake from the blood) are secreted into the circulation in VLDL particles (Gibbons, 1990). The nascent triglyceride-rich VLDL particles contain apoB100 and small amounts of apoC and apoE. Upon entering the circulation, additional amounts of apoE and apoC are acquired. The initial metabolic fate of VLDL largely resembles that of chylomicrons; the VLDL-triglycerides are hydrolyzed by LPL leading to the formation of VLDL remnant lipoproteins or intermediate density lipoproteins (IDL). Some of the VLDL remnants are directly cleared from the plasma, via hepatic LDL and/or remnant receptors. The remaining VLDL remnants are further processed, probably by further lipolysis involving hepatic lipase (Rubinstein, 1985). The eventual formation of LDL out of VLDL remnants is accompanied by a further loss of triglycerides, phospholipids, C-apolipoproteins and apoE (Gotto, 1987; Marzetta, 1990). The resulting LDL is rich in cholesterol esters and contains apoB100 as the sole protein constituent. In humans, two-third of the plasma cholesterol is normally present in the LDL fraction. The plasma half-life of LDL is approximately 2.5 days in normal humans. Hepatic LDL receptors are responsible for the removal of about two-third of circulating LDL (Kita, 1982) and about one-third is removed via extrahepatic LDL receptors. ApoB100 serves as the ligand in the LDL receptor-mediated uptake. A small fraction of LDL is removed from circulation by LDL-receptor independent pathways, such as the scavenger receptor on cells of reticuloendothelial origin. This LDL is probably chemically and/or physically modified. High concentrations of (modified) LDL in the plasma, will result in an increased uptake of cholesterol esters by macrofages via the scavenger receptor pathway. As a result of the massive cholesterol desposition, these macrophages become so-called foam cells. This

process is assumed to be one of the initial steps in the development of an atherosclerotic plaque (Brown and Goldstein, 1983).

1.1.3 Reversed cholesterol pathway

Cholesterol in peripheral tissues can be transported back to the liver. This process, referred to as reversed cholesterol transport, and involves HDL as the means of transport (see Figure 1C). Cholesterol-loaded cells can release their cholesterol in the interstitial fluid, where HDL, containing apoAI and apoAII, serves as an acceptor. This cholesterol is converted in the circulation in an esterified form due to the action of the enzyme lecithin:cholesterol acyl transferase (LCAT), with apoAI as cofactor. The HDL-cholesterol may then be delivered to the liver via several pathways: i) cholesteryl ester transfer protein (CETP), present in humans and other mammals, transfers cholesterol esters from HDL to lower density lipoproteins like VLDL, LDL and IDL, which are then taken up by the liver, ii) HDL can acquire apoE upon circulating in the plasma, which allows direct uptake of HDL via hepatic receptors recognising apoE as a ligand, or iii) selective uptake of HDL associated cholesterolesters by the liver, via a process which has not yet been fully characterized, but may include scavenger receptor SR-BI (Acton, 1996).

1.1.4 Lipoproteins and atherosclerosis

Elevation of plasma LDL and/or remnant lipoproteins levels is considered to be highly unfavourable and predisposes to the development of atherosclerotic disease, which is the major cause of morbidity and mortality in the western society. Atherogenesis is a complex process in which the lumen of a blood vessel becomes narrowed by cellular and extracellular substances to the point of obstruction. Atherosclerotic lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the arterial wall (for review, Ross 1993). Injury causes dysfunction of the endothelium, leading to the trapping of lipoprotein, predominantly LDL and remnant lipoprotein, in the artery. Monocytes and T lymphocytes attach to the injured vessel wall and migrate between endothelial cells. The monocytes become macrophages, and accumulate the lipid and become foam cells and, together with the accompanying lymphocytes become the fatty streak. A continuous cell influx and proliferation lead to the more advanced lesion, distinguished by a fibrous character, and ultimately to a fibrous atherosclerotic plaque.

1.2. INHERITABLE DISORDERS OF CHYLOMICRON AND VLDL METABOLISM

1.2.1 General

Normally, chylomicrons, VLDL and their remnants are efficiently metabolized, leading to low fasting plasma levels of these lipoproteins. Elevated levels of chylomicrons, VLDL or their remnants can occur when a particular step in the metabolic pathway is hampered. Inappropriate metabolism of the lipoproteins may result in an impaired delivery of the lipids to the tissues and can consequently, lead to accumulation of these lipids in the plasma.

Elevation of plasma lipid levels is considered to be highly unfavourable and predisposes to the development of atherosclerosis. Impaired metabolism of lipoproteins may occur when a gene encoding for an apolipoprotein, processing protein or a lipoprotein receptor is mutated.

Many genetic defects associated with impaired metabolism of chylomicrons and VLDL have been described (for review see Havel and Kane, 1995). The presence of a dysfunctional apoB results in an impaired assembly or secretion of triglyceride-rich lipoproteins, leading to fat malabsorption, severe triglyceride accumulation in enterocytes, and the virtual absence of VLDL and LDL from plasma (Abetalipoproteinemia, for review see Kane and Havel, 1995). Patients homozygous for LPL or apoCII deficiency (functional LPL deficiency) show impaired lipolysis of VLDL and chylomicron triglycerides and consequently, extreme accumulation of triglycerides in the plasma. (Brunzell, 1995). Whereas ApoB, LPL and apoCII deficiencies are rare, LDL receptor gene deficiencies are more frequently observed. Although the LDL receptor is thought to play a major role in the clearance of chylomicron and VLDL remnant lipoproteins, individuals homozygous for LDL receptor deficiency are solely characterized by elevation of plasma LDL (Familial Hypercholesterolemia, for review see Goldstein, 1995). Normal levels of chylomicron or VLDL remnant lipoproteins in affected subjects suggest that an alternative receptor pathway for remnant removal should be present, as was proposed by Kita (1982).

Another, extensively characterized genetic disorder of chylomicron and VLDL metabolism is Familial Dysbetalipoproteinemia (FD). FD is characterized by the accumulation of chylomicron and VLDL remnant lipoproteins in the plasma due to a genetically defective apolipoprotein E. Since FD, and the role of apoE in FD are the major issues dealt with in this thesis, they will be discussed in more detail below.

1.2.2. Familial Dysbetalipoproteinemia

1.2.2.1 Etiology

Familial Dysbetalipoproteinemia (FD) is an inherited disorder of chylomicron and VLDL remnant lipoprotein metabolism characterized by the accumulation in the plasma of VLDL, showing characteristic ß-mobility upon agarose gel electrophoresis (for review see Brewer, 1983; Mahley and Rall, 1989; Fazio, 1993a). The primary genetic cause of FD is the presence of a defective form of apoE (Mahley and Rall, 1989) or complete apoE deficiency (Ghiseli, 1981, Shaefer, 1986), leading to an impaired clearance of chylomicron and VLDL remnant lipoproteins, and subsequent formation of the ß-VLDL. A small percentage of the individuals with FD develop overt hyperlipidaemia with elevated plasma cholesterol (>7.5 mmol/l), triglyceride (> 2 mmol/l) and are diagnosed as Type III Hyperlipoproteinemic. In addition, hyperlipidemic FD patients have high plasma apoE levels. The estimated frequency of FD associated with hyperlipidaemia, is about 0.01%-0.04% in Caucasian populations. The hyperlipidemic phenotype rarely manifests itself before adulthood. Hyperlipidemia is much more prevalent in men than in women, and occurs earlier in men (Morganroth, 1975). Women do usually not develop hyperlipidemia before the menopause. There seems to be a

correlation between obesity and age of onset, and the presence of other clinical disorders, such as diabetes mellitus or hypothyroidism, which may also result in an earlier onset.

A characteristic clinical feature of FD is the frequent occurrence of pathognomonic xanthomatas (lipid deposits). These particular xanthomatas, termed xanthomata striata palmaris, occur as yellowish lipid deposits of cholestrol in the palmar creases. In addition, individuals with FD often have tuberous and tubero-eruptive xanthomas, although these lesions are not unique to FD. These tuberous lesions appear mostly on the elbows and knees (Polano, 1973).

1.2.2.2 Atherosclerosis

The accumulation of chylomicron and VLDL remnant lipoproteins predisposes individuals with FD to premature or accelerated atherosclerosis (Morganroth, 1975; Hazzard, 1975; Mishkel, 1975; Stuyt, 1983). The vascular disease in FD, which occurs in one third to more than the half of hyperlipidemic subjects has an unusual distribution. Peripheral vascular disease involving the lower extremities is almost as common as coronary artery disease. This is strikingly different from the distribution of vascular disease seen in familial hypercholesterolemia (excessive LDL concentrations), in which there is less involvement of the lower extremities. Although the mechanism underlying the predisposition for atherosclerosis of peripheral vessels in FD is unknown, it is noteworthy that certain cholesterol-fed animals that have high levels of ß-VLDL also have much more peripheral vascular disease than coronary atherosclerosis (Mahley, 1979; 1984).

1.2.2.3 The role of apolipoprotein E

Investigators first described apoE as a lipoprotein constituent in 1973 (Shore and Shore, 1973) and was designated as the arginine-rich protein. ApoE is present in plasma at concentrations of 3-5 mg/dl, and is equally distributed among VLDL, IDL and HDL (Rall, 1982). Around 90% of plasma apoE is produced by the liver, where it is secreted from hepatocytes either in association with VLDL (Elshourbagy, 1985; Lin, 1986) or in association with small discoidal particles (Dolphin, 1986; Hussain, 1989). The second largest site of apoE synthesis is the brain, where apoE is produced by astrocytes (Elshourbagy, 1985; Boyles, 1985). In the brain apoE is the major apolipoprotein present in the cerebrospinal fluid, where it is associated with small discoidal particles, and may play a role in cholesterol and phospholipid transport. Macrophages also produce large quantities of apoE in repair response to tissue injury, especially nerve injury, suggesting a role for apoE in nerve repair (Mahley, 1988). Remarkably, apoE is not synthesized in the epithelium of intestines, in spite of the fact that the intestine is a major site for lipoprotein biosynthesis (Mahley, 1988).

The human apoE gene is 3.6 kb in length (Das, 1985, Paik, 1985), contains four exons and is located on chromosome 19q13 (Das, 1985), within a cluster of apoCI, apoCII and the apoCI pseudogene (Lauer, 1988; Davison, 1986). Tissue specific expression is controlled by

an array of elements found in the immediate 5'-flanking region of the APOE gene throughout the whole APOE-CI-CII gene cluster (Taylor, 1991; Zannis, 1992). The apoE mRNA encodes for a protein composed of 317 amino acids, with an 18-amino acid signal peptide sequence. Cleavage generates the 299-amino acid mature apoE of 34 kDa (Zannis, 1984).

The apoE protein consists of two functional domains (Wetterau, 1988): A carboxy-terminal domain (10 kDa), which is a typical apolipoprotein domain, having strong amphipatic α-helical character and represents the lipid binding domain of the molecule (Aggerbeck, 1988). This carboxy-terminal domain also contains at least one heparin binding domain, around residue 214-236, and may be involved in the binding of lipoprotein particles to heparan sulphate proteoglycans on the vessel wall, enabling LPL to hydrolyse the particle (Weisgraber, 1986a). The amino-terminal domain (22 kDa) has a globular structure (Wilson, 1991) and contains the LDL receptor binding domain, located in the arginine, lysine and histidine-rich segment between amino acids 136-150 (Mahley, 1988).

Human ApoE gene is polymorphic (Zannis and Breslow, 1981; Utermann, 1982). There are three common alleles (E*2,E*3,E*4). E*3 is the most frequent allele (70% - 85%), which codes for a cysteine at residue 112 and arginine at residue 158 (Weisgraber, 1981; Rall, 1982). E*4 is the second most frequent allele (12%-18%); apoE4 differs from apoE3 in having arginine rather than cysteine at position 112. The other major allele is E*2 (3%-12%); apoE2 differs from apoE3 in having cysteine instead of arginine at residue 158. The apoE2 isoform is defective in binding to the LDL receptor (Havel, 1980; Zannis, 1980; Gregg, 1981; Schneider, 1981; Weisgraber, 1982; Rall, 1983a), and as a consequence of this reduced receptor binding affinity, subjects homozygous for the E*2 allele have an impaired hepatic uptake of remnant lipoprotein and develop FD (Mahley and Rall, 1989). Even though ß-VLDLs are detectable in the plasma of all apoE2 homozygotes, only a small percentage ($<5\%$) eventually develop overt hyperlipidaemia (Utermann, 1985; Mahley and Rall, 1989). Heterozygous subjects never show accumulation of ß-VLDL or overt dyslipidemia, indicating that hyperlipidemic phenotype induced by the apoE2 variant is transmitted as a recessive trait with very low penetrance. The disease shows a large clinical variation both intraindividually, depending on body weight, age, sex and the nutritional status of the patient. An explanation for the highly variable expression of hyperlipidemia among E2E2 individuals is that the defective binding of apoE2 may be reversible (Innerarity, 1984). *In vitro* studies showed that LDL receptor binding activity dramatically improved after cysteamine treatment, which modifies cysteine into positively charged lysine analogue. The positive charge at residue 158 is not necessary for normal binding; however it appears to be necessary initially for the alignment of other residues within the apoE molecule, such that these exist in a receptor-binding conformation (Wilson, 1991). It has been reported that the binding activity of $d<1.006$ lipoproteins isolated from the hyperlipidemic E2E2 subject increased upon weight loss and drug treatment, leading to a decreased cholesterol to triglyceride ratio in the VLDL particles (Innerarity, 1986). Thus, the binding activity may be modulated to some extent, depending on the lipid composition of the particle, which may explain the highly variable

expression level of the disease among E2E2 subjects.

Besides the apoE2 variant several other naturally occurring rare variants of apoE have been found in association with FD (table II). These substitutions in apoE disrupt LDL receptor-binding to some extent. Many of these variants are able to produce the clinical phenotype in the heterozygous carriers. In fact, APOE*3(Arg142→Cys), APOE*2(Arg145→Cys), APOE*1(Lys146→Glu), APOE*2(Lys146→Gln) and the APOE*3-Leiden mutation (a 7-amino acid tandem repeat of residues 120-126) have been shown to cause dominant FD (Havel, 1983; Rall, 1989; Emi, 1988; Mann, 1988; Rall, 1983; Smit, 1987; 1990; de Knijff, 1994; Havekes, 1984; 1986; de Knijff, 1991). Some of these dominant variants are located in the 131-150 receptor-binding domain and would, in contrast to apoE2, lead to irreversible LDL receptor-binding defect and thus causing the dominant expression of FD (Mahley, 1990). The mutation in apoE3-Leiden is located outside the binding domain between residue 120-126 (Wardell, 1989; van den Maagdenberg, 1989). However, this large insertion would cause a dramatic change in the conformation of the receptor-binding domain leading to an irreversible binding defect of the protein. When complexed to phospholipid vesicles the dominant variant displayed a paradoxically high binding (20-40%) to the LDL receptor when compared to the recessive apoE2 variant (<2%) (Chappel, 1989). Therefore, the accumulation of $d < 1.006$ lipoproteins in dominant carriers cannot be ascribed simply to the low affinity of the mutant apoE to the LDL receptor. Ji (1994c) reported that dominant apoE variants and not recessive variants have reduced binding affinity for heparan sulphate proteoglycans. The reduced affinity for HSPG would result in the reduced trapping of remnant lipoproteins in the space of Disse thus causing an impaired uptake from plasma by the liver.

The mechanisms of the way in which a single defective allele can disrupt normal clearance of plasma lipoproteins cannot be explained by low affinity for hepatic binding sites

Table II. Apolipoprotein E Isoforms and Some Rare Variants Associated with FD

		HLP	Inheritance	LDL-receptor binding activity
				%
Normal isoforms	apoE3	No	-	100
	apo4(Cys112→Arg)	No	-	100
Defective isoform	apoE2(Arg158→Cys)	Yes	Recessive	2
Defective rare variant	apoE3(Cys112→Arg,Arg142→Cys)	Yes	Dominant	20
	apoE2(Arg145→Cys)	Yes	Dominant	-
	apoE4(Gly13→Lys,Arg145→Cys)	Yes	Dominant	-
	apoE1(Lys146→Glu)	Yes	Dominant	<5
	apoE2(Lys146→Gln)	Yes	Dominant	40
	apoE3-Leiden(Cys112→Arg, 7aa insertion)	Yes	Dominant	25

HLP, hyperlipoproteinemia

solely. Why does the normal apoE allele not protect against hyperlipidemia? VLDL isolated from heterozygotes for the dominant apoE3-Leiden and the apoE3(Arg142→Cys) variant but not heterozygotes for the recessive variant are enriched in defective apoE (de Knijff, 1991, Horie, 1992). It is suggested that the effective concentration of good functioning apoE may be reduced and become rate limiting. *In vitro* studies by Fazio (1993b) showed that some dominant variants prefer to bind to VLDL, and thereby produce a high ratio of mutant to normal apoE on the surface of the particle and creating apparent local homozygosity for the binding-defective variants.

1.2.2.4 The role of additional genetic and environmental factors in the expression of hyperlipidemia

The variable expression of FD in E2E2 subjects, and also in carriers of the dominant apoE variants, indicate that other genetic and/or environmental factors modulate the expression of the disease. Utermann (1985) hypothesised that the development of hyperlipidemia in E2E2 also required the simultaneous but independent inheritance of other genetic defects that produce hyperlipidaemia. Mahley (1989) proposed that the hepatic LDL receptor expression level may have a profound modulating effect on the expression of hyperlipidemia in FD. Sex, age and nutrition which are aggravating factors in FD are known to effect the expression level of hepatic LDL receptors at least in certain animals, consequently modulating hepatic uptake of remnant lipoproteins and thereby the level of hyperlipidemia. Another mechanism that may contribute to remnant accumulation is overproduction of lipoproteins. Increased caloric intake would stimulate intestinal lipoprotein production, and probably hepatic VLDL as well.

In FD the expression of the hyperlipidemia is possibly modulated by combinations of multiple genetic and environmental factors. A major challenge for the future will be to elucidate the interaction of environmental and genetic variations that contribute to the hyperlipidemic phenotype. In general, identifying new genes and environmental factors involved in chylomicron and VLDL metabolism, will help in defining in detail the molecular mechanisms underlying the complex expression patterns of hyperlipidemia in FD patients in particular and in the general population at large.

1.3. ANIMAL MODELS IN LIPOPROTEIN AND ATHEROSCLEROSIS RESERACH

1.3.1 General

The large number of genes involved, the genetic heterogeneity and the complex interaction with variable environmental factors hamper the identification of the individual genetic and environmental factors involved in lipoprotein metabolism and atherosclerosis development in man. Studying lipoprotein metabolism and atherosclerosis development in an suitable animal model, facilitates the study of these factors under well-defined genetic and well-controlled environmental conditions. However, differences between man and animal models has to be considered, such as quantitative differences, and differences in metabolic

pathways. The objective of most animal studies is not to duplicate precisely genetic variations that occur in humans but rather to understand better the biochemical mechanisms that underlie heritable variations of lipoprotein metabolism. Animal models may provide a means of identifying new genes and environmental factors involved in this process.

The major species which serve as models for the study of lipoprotein metabolism as well as atherosclerosis include rabbits, rats, mice, pigs, and non-human primates (for review, see Reue, 1990; Overturf and Loose-Mitchel, 1992). Cholesterol-fed rabbits represent the classical model for the study of lipoproteins and atherosclerosis. There is an extensive body of information about lipoprotein metabolism and pathology. The best characterized rabbit model is the Watanabe-heritable-hyperlipidemic (WHHL) rabbit (Yamamoto, 1986). These animals have a severely reduced number of LDL receptors, due to mutation in the LDL receptor gene. Like its human counterpart (Familial Hypercholesterolemia patients), the WHHL rabbit exhibits elevated serum cholesterol levels and spontaneously occurring atherosclerosis due to the defect in the LDL receptor. Studies using this model have contributed to our understanding of biochemical and pathological consequences associated with LDL receptor defects. Unfortunately, WHHL rabbits are difficult to breed, and inbred stocks are therefore expensive and not readily available.

For rats, much information about lipoprotein metabolism, nutrition, histology, and endocrinology is available. Inbred strains are available, and genetically determined variations affecting hypertension, obesity, diabetes, and lipid metabolism have been identified (Gill, 1989). However, rats differ from humans in quantitative expression of lipoproteins (for example, LDL levels are low) as well as in certain biochemical pathways (for example, apoB48 is produced by the liver and CETP activity in the plasma is very low).

The lipoprotein metabolism of the pig closely resembles that of humans. A number of genetic hyperlipidemias, as well as other variations have been identified and partially characterized. Pigs have been widely used for studies of atherosclerosis, and the lesions closely resembles those of man (Rapacz and Hasler-Rapacz, 1989). Lipoprotein metabolism of non-human primates is also very similar to that of humans and has been well characterised (Laber-Laird and Rudel, 1989). The similarity to humans makes non-human primates particularly useful for drug testing. However, both pigs and non-human primates are expensive to maintain and genetic studies are difficult.

1.3.2 The mouse as a model system for lipoprotein metabolism

For many years the mouse was not widely used as an experimental model for lipoprotein and atherosclerosis research. The distribution of cholesterol among the lipoprotein classes differs from that of humans. In the mouse most plasma cholesterol (80%-90%) is present in the HDL fraction. When the mouse is fed a diet of about 30% fat, non-HDL fractions do increase, but the distribution of cholesterol among the lipoprotein classes is still different from humans: in the mouse the increase in plasma cholesterol is mainly confined to VLDL, whereas in human it is LDL (Ishida, 1991). The reason for this difference may be that the

mouse edits the trunctated apoB48 in both intestine and liver, whereas humans edit only in the intestine (Higuchi, 1992). Other important differences in the lipoprotein system of mice and humans include the fact that the mouse lacks cholesteryl ester transfer protein (CETP) as well as lipoprotein (a). In addition, mice can be highly resistant to the development of atherosclerosis (Jokinen, 1985).

Despite these disadvantages, the mouse has become the most powerful animal model in the study of lipoprotein metabolism and atherosclerosis development: The mouse is the mammal of choice for genetic studies (Paigen, 1995). The mouse offers a variety of advantages when compared with other animal models. Hundreds of inbred strains are available, each strain representing a unique gene pool in which naturally occurring polymorphisms have been fixed by inbreeding. Inbred strains of mice form an infinite source of genetically identical individuals. Breeding patterns are controllable, and the time to produce successive generations is very short (3 months). Another major advantage is that the genetic map of the mouse is well-defined (Lusis and Sparks, 1989). Therefore, the mouse provides an attractive animal model system for examining the heritable variation of lipoprotein metabolism since genetic and biochemical analyses are greatly simplified and environmental influences can be controlled.

Recent advances in dietary regiments contributed to the use of the mouse in studies on atherosclerosis. Atherogenic diets have been developed that are tolerated by mice for the extended time required for lesion formation. These diets contain app. 15% fat (w/w), 1.25% cholesterol, and 0.5% sodium cholate (Nishina, 1990). Development of such diets has enabled investigators to study susceptibility to diet-induced atherosclerosis in genetically defined inbred lines of mice. Surveys of inbred lines have demonstrated that the animals from each line respond reproducibly to atherogenic diets. Certain inbred strains (for example *C57BL/6*) are susceptible to diet-induced atherosclerosis, developing fatty streaks after a 14-week exposure to an atherogenic diet, whereas other strains (for instance C3H) are resistant, remaining free of fatty streaks even after a one-year exposure to the atherogenic diet (Ishida, 1991, Nishina, 1993). The use of recombinant inbred strains and backcrosses has led to the discovery of several genes affecting atherosclerosis susceptibility such as ath-1, ath-2, ath-3, and ath-4. These loci probably represent single gene mutants that affect the levels of HDL cholesterol and atherosclerosis susceptibility (Paigen, 1987; 1989; Steward-Philips, 1989).

However, the major advantage of the mouse among mammals is the availability of technology to manipulate its genome. By means of conventional transgenesis technology, a DNA fragment containing the gene of interest can be added to the mouse genome (for review see Palmiter and Brinsyer, 1986; Jaenisch, 1988; Hogan, 1989). Thus, gene-function can be studied by overexpression in a homogenous environmental and genetic background. By including tissue-specific regulatory elements into the transgene, gene-function can also be studied at tissue specific level. Since, conventional transgenesis implies the addition of extra copies to the genome, only dominant effects can be studied.

Gene-targeting technology using embryonic stem (ES) cells endogenous murine genes can

be modified to create mice with a lack of function or with subtle mutations in a specific gene (for review see Capecchi, 1989a; 1989b; Baribault and Kemeer, 1989; Frohman and Martin, 1989; Joyner, 1993). By the disruption of a gene its function can be deduced. However, studies on gene function may be hampered when other genes take over the function of the disrupted gene (back-up system). In addition, disruption of vital genes may lead to early lethality which may preclude any analysis of *in vivo* function. Gene-targeting technology also facilitates the replacement of the endogenous murine gene by the (human) mutant gene and thus expresses mutant genes under control of the mouse regulatory elements. Combinations of transgenic and/or gene-targeted mice facilitates the study of gene-gene interactions. In addition, cross-breeding transgenic mice expressing recessive alleles with mice with the respective gene knock-out, allows studying the effects of recessive alleles in mice as well.

With the unique availability of technology to manipulate its genome, the mouse provides a powerful investigatory tool for studying gene function and thus also the function of the genes that are involved in lipoprotein metabolism.

1.3.3 Transgenic and gene-targeting technology in studying genes involved in remnant lipoprotein metabolism.

Transgenic mouse technology provides a powerful tool for studying the role of genes encoding for proteins involved in lipoprotein metabolism. Most of the genes encoding for apolipoprotein, processing proteins and lipoprotein receptors have been overexpressed or disrupted in mice (for review, see Breslow, 1993; 1994abc; 1996; Stolzfus and Rubin, 1993; Lusis, 1993; Rubin, 1994; Paigen and Smith, 1994). A brief overview of the phenotypic characteristics of these mice is given in table III. Several of these mice lack or overexpress genes that are supposed to have an important role in remnant lipoprotein metabolism, such as the apoE, LDL receptor, LRP, C apolipoprotein and lipase genes. Studies in these mice have revealed many insights into the *in vivo* role of these genes.

Transgenic mice overexpressing apoE (Simonet, 1990, Smith, 1990; Shimano, 1991) demonstrated that apoE can reduce plasma cholesterol levels by increasing the clearance rate of VLDL and LDL lipoproteins (Shimano, 1992a). In addition, apoE overexpression protected against diet-induced hypercholesterolemia (Shimano, 1992b) and atherosclerosis (Spangler, 1993). In contrast to apoE overexpression, ApoE deficiency in mice (Zhang, 1992; Plump, 1992) resulted in extreme serum cholesterol levels due to a severe defect in clearance of remnant lipoproteins from the plasma (Zhang, 1992; Plump, 1992). ApoE knockout mice spontaneously develop atherosclerotic lesions when just on a normal chow diet. These studies unequivocally provided *in vivo* evidence that apoE plays a central role in remnant lipoprotein metabolism.

The LDL receptor was thought to play an important role in apoE-mediated remnant removal. *In vivo* evidence in transgenic mice confirmed this, revealing that overexpression

Table III. Transgenic and Knock-out Mouse Models For Studying Lipoprotein Metabolism and Atherosclerosis

		overexpression	deficiency	references
Apolipoproteins	A-I	HDL-C↑↑, lesions↓↓	HDL-C↓, lesions=	Walsh,1989; Rubin,1991 Williamson,1992; Li,1993
	A-II	small HDL, lesions↑	HDL-C↓	Schultz,1992; Warden,1993 Weng, 1995
	A-IV	VLDL-TG↑		Aalto Setala ,1994
	B	IDL/LDL-C↑, lesions↑	lethal	Xiong,1991; Chiesa,1993; Purcell Huynh,1995 Homanics,1993; Farese,1995
	CI	VLDL-TG↑	VLDL-C↑(diet)	Simonet,1991; Shachter,1996; Jong,1996 van Ree,1995
	CII	VLDL-TG↑		Shachter,1994
	CIII	VLDL-TG↑↑	VLDL-TG↓	Ito,1990 Maeda,1994
	E	VLDL/LDL-C↓(diet), lesions↓	VLDL/IDL-C↑↑↑, VLDL-TG↓, lesions↑↑↑	Simonet,1990; Smith,1990; Shimano,1991; Sprangler,1991 Zhang,1992; Plump,1992; van Ree,1995
	apo(a)	normal, lesions↑		Chiesa,1992; Lawn,1992
Processing proteins	LPL	VLDL-TG↓, HDL-C↑	lethal	Shimada,1993; Liu,1994; Zsigmond,1994 Coleman,1995
	HL	VLDL-TG↑, HDC-C↑	HDL-C↑	Schultz,1996 Homanics,1995
	LCAT	HDL-C↑		Francone,1995
	CETP	HDL-C↓, lesions↑		Agellon,1991; Hayek,1995
Receptors	LDLR	LDL-C↓	IDL/LDL-C↑, lesions↑	Hofmann,1988; Yokode,1990 Ishibashi,1993;1994
	LRP		lethal	Herz,1993
	ScR	VLDL/LDL-C↓,HDL-C↑(diet)	normal	Wolle,1995 Suzuki,1995
	VLDLR		normal	Fryman,1995

leads to increased clearance of apoB and apoE lipoproteins, consequently leading todecreased levels of these plasma lipoproteins both on a normal and high/fat cholesterol diet((Hofmann, 1988; Yokode, 1991). In addition, Ishibashi (1993) showed that in LDL receptor knockout mice a second pathway exists for remnant lipoprotein removal, as was earlier proposed by Kita (1982).

To study the role of the LRP in remnant lipoprotein metabolism mice were generated that lack the LRP. Unfortunately, mouse embryos that were homozygous for the disrupted LRP allele died *in utero* (Herz, 1993). To circumvent this problem, Willnow (1994) created animals transiently overexpressing RAP, a dominant negative regulator of LRP function, using adenovirus technology. These studies demonstrated that LRP provides an important backup mechanism when the LDL receptor pathway is blocked either by genetic deficiency, down-regulated or lack of appropriate ligands, like mutant apoE.

The *in vivo* role of C apolipoproteins in chylomicron and VLDL remnant lipoprotein metabolism has been studied using transgenic and knock-out mouse technology. Studies on apoCI transgenic mice (Simonet, 1991; Shachter, 1996; Jong, 1996) with mild hypertriglyceridemia, suggest that apoCI mediates inhibition of lipoprotein clearance via a pathway other than the LDL receptor pathway, presumably the LRP (Jong, 1996). In addition, data obtained in APOCI-deficient mice suggest that apoCI modulates receptor-mediated clearance of remnant lipoproteins (van Ree, 1995). In addition to apoCI transgenic, APOCII and APOCIII transgenic mice (Shachter, 1994; Ito, 1990; Aalto-Setala, 1992) have also been created. All these mice display hypertriglyceridemia, although to a different extent. This is not caused by defective VLDL-triglyceride lipolysis, but rather by effects on apoE-mediated clearance of lipoproteins, although this has only been studied for the APOCIII transgenic mice (de Silva, 1994).

Lipoprotein lipase is supposed to have an important role in the initial step chylomicron and VLDL metabolism i.e. triglyceride hydrolysis. The role of LPL in triglyceride catabolism was confirmed in LPL transgenic mice (Shimada, 1994; Liu, 1994, Zsigmond, 1994) and heterozygous LPL-deficient mice (homozygosity appeared to be lethal) (Coleman, 1995). Hepatic lipase (HL) is thought to play an important role in the apoE-mediated sequestration and internalisation of remnant lipoproteins by hepatic cells. HL-deficient mice (Homanics, 1995) had increased plasma cholesterol (30%), phospholipids and HDL cholesterol, but plasma triglycerides were not altered. Upon dietary stress HL-deficient mice display an increase in HDL. These data suggest a role for HL in HDL remodelling, and not the expected role for HL in the metabolism of triglyceride-rich particles, since HL-deficient mice display a normal clearance rate of exogenously introduced chylomicrons, and tolerate fat load (Homanics, 1995). Possibly other lipase may bind to sites in the liver normally occupied by HL and facilitate the clearance of triglyceride-rich particles in these mice.

1.4. OUTLINE OF THIS THESIS

Increased plasma levels of chylomicron and VLDL remnant lipoproteins predispose to

atherosclerosis. This increase can be caused by disease such as FD. However, also a Western lifestyle may lead to increased plasma levels of these atherogenic lipoproteins. In the present thesis we investigated the environmental and genetic factors that influence remnant lipoprotein metabolism. Therefore, transgenic mice were generated that express human APOE mutants that are associated with FD. These mice offer the possiblity of studying these APOE mutants in a homogenous environmental and genetic background. Moreover, these mice can be used to identify and characterize the genetic and/or envrironmental factors that modulate remnant lipoprotein metabolism in general.

Transgenic mice expressing the human APOE*3-Leiden mutation were used to study the effect of different cholesterol-containing diets on the hyperlipidemic phenotype and on the possible concurrent development of atherosclerotic plaques (chapter 2). In a second study (chapter 3), we studied in more detail the effect of diet composition and duration of feeding on serum cholesterol and atherosclerosis development in the aortic sinus, using quantitative image analysis. Hyperlipoproteinemia in APOE*3-Leiden transgenic mice appeared to be highly dependent on age-and gender-related factors. In chapter 4, mechanisms underlying the age-and gender-dependent hyperlipoproteinemia were dissected in detail. The role of the endogenous apoE gene in determining the expression level of hyperlipoproteinemia in APOE*3-Leiden transgenic mice was investigated in detail in chapter 5. The responsiveness of plasma lipid levels to diets and endogenous hormone-related factors, suggested that APOE*3-Leiden transgenic may provide a sensitive model for testing hypolipidemic drugs. This was investigated in chapter 6. The strong modulating effect of n-3 fish oils on remnant lipoprotein levels was investigated in chapter 7. Finally, transgenic mice expressing the common apoE2 variant became available. These mice were compared with APOE*3-Leiden mice both in the presence and absence of the endogenous apoE gene. In chapter 8, we studied the mechanisms underlying the differential expression pattern of FD between these two apoE variants.

1.5 REFERENCES

Aalto Setala, K., E. A. Fisher, X. Chen, T. Chajek Shaul, T. Hayek, R. Zechner, A. Walsh, R. Ramakrishnan, H. N. Ginsberg, and J. L. Breslow. 1992. Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles. *J. Clin. Invest.* 90:1889-1900.

Aalto Setala, K. , C. L. Bisgaier, A. Ho, K. A. Kieft, M. G. Traber, H. J. Kayden, R. Ramakrishnan, A. Walsh, A. D. Essenburg, and J. L. Breslow. 1994. Intestinal expression of human apolipoprotein A-IV in transgenic mice fails to influence dietary lipid absorption or feeding behavior. *J. Clin. Invest.* 93:1776-1786.

Acton, S, A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 271:518-520.

Agellon, L. B., A. Walsh, T. Hayek, P. Moulin, X. C. Jiang, S. A. Shelanski, and A. R. Tall. 1991. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J. Biol. Chem.* 266:10796-10801.

Aggerbeck, L. P., J. R. Wetterau, K. H. Weisgraber, C. S. Wu, and F. T. Lindgren. 1988. Human apolipoprotein E3 in aqueous solution. II. Properties of the amino- and carboxyl-terminal domains. *J. Biol.*

Chem. 263:6249-6258.

Baribault, H., and R. Kemler. 1989. Embryonic stem cell culture and gene targeting in transgenic mice. *Mol. Biol. Med.* 6:481-492.

Beisiegel, U., W. Weber, and G. Bengtsson-Olivecrona. 1991. Lipoprotein lipase enhances the binding of chylomicronns to low density lipoprotein receptor related protein. *Proc. Natl. Acad. Sci. U. S. A.* 88:8342-8346.

Boyles, J. K., R. E. Pitas, E. Wilson, R. W. Mahley, and J. M. Taylor. 1985. Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest.* 76:1501-1513.

Breslow, J. 1988. Apolipoprotein genetic variation and human disease. *Physiol. Rev.* 68:85-132.

Breslow, J. L. 1993. Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc. Natl. Acad. Sci.* 90:8314-8318.

Breslow, J. L. 1994a. Lipoprotein metabolism and atherosclerosis susceptibility in transgenic mice. *Curr. Opin. Lipidol.* 5:175-184.

Breslow, J. L. 1994b. Insights into lipoprotein metabolism from studies in transgenic mice. *Annu. Rev. Physiol.* 56:797-810.

Breslow, J . 1994c. Lipoproteins and heart disease. Transgenic mice models are helping in the search for new therapies. *Biotechnology. N. Y.* 12:365-7, 369.

Breslow, J. L. 1996. Mouse models of atherosclerosis. *Science* 272:685-688.

Brewer, H. B., L. A. Zech, R. E. Gregg, D. Schwartz, amd E. J. Schaefer. 1983. Type III hyperlipoproteinemia: diagnosis, molecular defects, pathology nd treatment. *Ann. Intern. Med.* 98:623-640.

Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implicationsfor cholesterol deposition in atherosclerois. *Ann. Rev. Biochem.* 52:223-261.

Brown, M. S., J. Herz, R. C. Kowal, and J. L. Goldstein. 1991. The low-density lipoprotein receptor related protein: double agent or decoy? *Curr. Opin. Lipidol.* 2:65-72.

Brunzell, J. D. 1995. Familial lipoprotein lipase deficiency and other causes of the chylomicronemia syndrome. *In:* The metabolic basis of inherited disease, 7th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. and Valle, Eds. New York: McGraw-Hill, pp. 1913-1932.

Capecchi, M. R. 1989a. Altering the genome by homologous recombination. *Science.* 244:1288-1292.

Capecchi, M. R. 1989b. The new mouse genetics: altering the genome by gene targeting. *Trends Genet.* 5:70-76.

Chappel, D. A. 1989. High receptor binding affinity of lipoproteins in atypical dysbetalipoproteinemia (Type III hyperlipoproteinemia). *J. Clin. Invest.* 84:1906-1915.

Chiesa, G., H. H. Hobbs, M. L. Koschinsky, R. M. Lawn, S. D. Maika, and R. E. Hammer. 1992. Reconstitution of lipoprotein(a) by infusion of human low density lipoprotein into transgenic mice expressing human apolipoprotein(a). *J. Biol. Chem.* 267:24369-24374.

Chiesa, G., D. F. Johnson, Z. Yao, T. L. Innerarity, R. W. Mahley, S. G. Young, R. H. Hammer, and H. H. Hobbs. 1993. Expression of human apolipoprotein B100 in transgenic mice. Editing of human apolipoprotein B100 mRNA. *J. Biol. Chem.* 268:23747-23750.

Choi, S. Y., L. G. Fong, M. J. Kirven, and A. D. Cooper. 1991. Use of an anti-low density lipoprotein receptor antibody to quantify the riole of the LDL receptor in removal of chylomicron remnants in the mouse *in vivo*. *J. Clin. Invest.* 88:1173-1181.

Coleman, T., R. L. Seip, J. M. Gimble, D. Lee, N. Maeda, and C. F. Semenkovich. 1995. COOH-terminal disruption of lipoprotein lipase in mice is lethal in homozygotes, but heterozygotes have elevated triglycerides and impaired enzyme activity. *J. Biol. Chem.* 270:12518-12525.

Das, H. K., J. McPherson, G. A. Bruns, S. K. Karathanasis, and J. L. Breslow. 1985. Isolation, characterization, and mapping to chromosome 19 of the human apolipoprotein E gene. *J. Biol. Chem.* 260:6240-6247.

Davignon, J., R. E. gregg, and C. F. Sing. 1988. Apolipoprotein E polymorfism and atherosclerosis. *Atherosclerosis* 8:1-21.

Davison, P. J., P. Norton, S. C. Wallis, L. Gil, M. Cook, R. Williamson, and S. E. Humphries. 1986. There two gene sequences for human apolipoprotein CI (APOCI) on chromosome 19, one of which is 4 kb from the gene for APOE. *Biochem. Biophys. Res. Commun.* 136:876-884.

de Silva, H. V., S. J. Lauer, J. Wang, W. S. Simonet, K. H. Weisgraber, R. W. Mahley, and J. M. Taylor. 1994. Overexpression of human apolipoprotein C-III in transgenic mice results in an accumulation of

apolipoprotein B48 remnants that is corrected by excess apolipoprotein E. *J. Biol. Chem.* 269:2324-2335.

de Knijff, P., A. M. J. M. van den Maagdenberg, A. F. H. Stalenhoef, J. A. Gevers Leuven, P. N. M. Demacker, L. P. Kuyt, R. R. Frants, and L. M. Havekes, L. M. 1991. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *J. Clin. Invest.* 88:643-655.

de Knijff, P., A. M. van den Maagdenberg, D. I. Boomsma, A. F. Stalenhoef, A. H. Smelt, J. J. Kastelein, A. D. Marais, R. R. Frants, and L. M. Havekes. 1994. Variable expression of familial dysbetalipoproteinemia in apolipoprotein E2 (Lys146→Gln) Allele carriers. J. Clin. Invest. 94:1252-1262.

Diaro, P., M-I. Malewiak, D. Lagrange, and S. Griglio. 1994. Hepatic lipase may act as a ligand in the uptake of artificial chylomicron remnant-like particles by isolated rat hepatocytes. *Biochem. J.* 299:889-894.

Dolphin, P. J., S. J. Forsyt, and E. S. Krul. 1986. Post-secretory acquisition of apolipoprotein E by nascent rat hepatic very low density lipoproteins in the absence of cholesteryl ester transfer. *Biochim. Biophys. Acta* 875:21-30.

Eisenberg, S. 1990. Metabolism of apolipoproteins and lipoproteins. *Curr. Opin. Lipidol.* 1:205-215.

Eisenberg, S. E. Sehayek, T. Olivecrona, and I. Vlodavsky. 1992. Lipoprotein lipase enhances binding of of lipoproteins to heparan sulphate on cell surfaces and extracellular matrix. *J. Clin. Invest.* 90:2013-2021.

Elshourbagy, N. A., W. S. Liao, R. W. Mahley, and J. M. Taylor. 1985. Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. *Proc. Natl. Acad. Sci. U. S. A.* 82:203-207.

Emi, M. L. L. Wu, M. A. Robertson, R. L. Meyers, R. A. Hegele, R. R. Williams, R. White, and J.-M. Lalouele. 1988. Genotyping and sequence analysis of apolipoprotein E isoforms. *Genomics* 3:373-379.

Farese, R. V., Jr., D. L. Ruland, L. M. Flynn, R. P. Stokowski, and S. G. Young. 1994. Knockout of the mouse apolipoprotein B gene results in embrynic lethalithy in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. U. S. A.* 92:1774-1778.

Fazio, S. 1993a. Recent insights into the pathogenesis of type III hyperlipoproteinemia. *Trends Cardiovasc. Med.* 3: 191-196.

Fazio, S., Y. Horie, K. H. Weisgraber, L. M. Havekes, and S. C. Rall, Jr. 1993b. Preferential association of apolipoprotein E Leiden with very low density lipoproteins of human plasma. *J. Lipid Res.* 34: 447-453.

Francone, O. L., E. L. Gong, D. S. Ng, C. J. Fielding, and E. M. Rubin. 1995. Expression of human lecithin-cholesterol acyltransferase in transgenic mice. Effect of human apolipoprotein AI and human apolipoprotein all on plasma lipoprotein cholesterol metabolism. *J. Clin. Invest.* 96:1440-1448.

Frohman, M. A., and G. R. Martin. 1989. Cut, paste, and save: new approaches to altering specific genes in mice. *Cell* 56:145-147.

Fryman, P. K., M. S. Brown, T. Yamamoto, J. L. Goldstein, and J. Herz. *1995.* Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor. *Proc. Acad. Sci. U. S. A.* 92:8453-8457.

Ghiselli, G., E. J. Schaefer, P. Gascon, and H. B. Brewer Jr. 1981. Type III hyperlipoproteinemia associated with apolipoprotein E deficiency. *Science.* 214:1239-1241.

Gibbons, G. F. 1990. Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem. J.* 268:1-13.

Gill, T. J., G. J. Smith, R. W. Wissler, and H. W. Kunz. 1989. The rat as an experimental animal. *Science.* 245:269-276

Goldstein, J. L., H. H. Hobbs, and M. S. Brown. Familial hypercholesterolemia. *In:* The metabolic basis of inherited disease, 7th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. and Valle, Eds. New York: McGraw-Hill, pp. 1981-2030.

Gotto, A. M., H. J. Pownall, and J. R. Havel. 1986. Introduction to the plasma lipoproteins. *Methods Enzymol.* 128:3-40.

Gotto, A. M. 1987 *In:* Plasma lipoproteins. Gotto, A. M. ed. Vol. 14, chapter I.

Gregg, R. E., L. A. Zech, E. J. Schaefer, and H. B. Brewer. 1981. Type III hyperlipoproteinemia: Defective metabolism of an abnormal apolipoprotein E. *Science* 211:584-586.

Havekes, L. M., J. A. Gevers Leuven, E van Corven, E. de Wit, and J. J. Emeis. 1984. Functionally inactive apolipoprotein E3 in a type III hyperlipoproteinaemic patient. *Eur. J. Clin. Invest.* 14:7-11.

Havekes, L., E. de Wit, J. Gevers Leuven, E. Klasen, G. Utermann, W. Weber, and U. Beisiegel. 1986. Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. *Hum. Genet.* 73:157-163.

Havel, R. J., Y. -S. Chao, E. Windler, L. Kotite, and L. S. S. Guo. 1980. Isoprotein specificity in the hepatic

uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia. *Proc. Natl. Acad. Sci. USA.* 77:4349-4353.

Havel, R. J., L. Kotite, J. P. Kane, P. Tun, and T. Bersot. 1983. Atypical familial dysbetalipoproteinemia associated with apolipoprotein phenotype E33. *J. Clin. Invest.* 72:379-387.

Havel, R. J., and J. P. Kane. 1989. Introduction: Structure and metabolism of plasma lipoproteins. *In:* The metabolic basis of inherited disease, 6th ed. Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds. New York: McGraw-Hill, pp. 1129-1138.

Havel, R.J. and J. P. Kane. 1995. Introduction: structure and metabolism of plasma lipoproteins. Familial hypercholesterolemia. *In:* The metabolic basis of inherited disease, 7th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. and Valle, Eds. New York: McGraw-Hill, pp. 1981-2030.

Hayek, T., L. Masucci Magoulas, X. Jiang, A. Walsh, E. Rubin, J. L. Breslow, and A. R. Tall. 1995. Decreased early atherosclerotic lesions in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *J. Clin. Invest.* 96:2071-2074.

Hazzard, W. R., T. F. O'Donnell, and Y. L. Lee. 1975. Broad-ß disease (type III hyperlipoproteinemia) in a large kindred. Evidence for a monogenic mechanism. *Ann. Intern. Med.* 82:141-149.

Herz, J., U. Hamman, S. Rogne, O. Myklebost, H. Gausepohl, and K. K. Stanley. 1988. Surface location and high affinity for calcium of a 500 kD membrane closely related to the LDL receptor suggest a physiological role as lipoprotein receptor. *EMBO J.* 7:4119-4127.

Herz, J., D. E. Clouthier, and R. E. Hammer. 1993. LDL receptor-related protein internalizes and degrades uPA/PAI-1 complexes and is essential for embryo implantation. *Cell* 71:411-421.

Higuchi, K., K. Kitagawa, K. Kogishi, and T. Takeda. 1992. Developmental and age-related changes in apolipoprotein B mRNA editing in mice. 1992. *J. Lipid. Res.* 33:1753-1764.

Hofmann, S. L., D. W. Russell, M. S. Brown, J. L. Goldstein, and R. E. Hammer. 1988. Overexpression of low density lipoprotein (LDL) receptor eliminates LDL from plasma in transgenic mice. *Science* 239:1277-1281.

Hogan, B., F. Constantini, and E. Lacey. 1986. *Manipulating the Mouse Embryo: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Homanics, G. E., T. J. Smith, S. H. Zhang, D. Lee, S. G. Young, and N. Maeda. 1993. Targeted modification of the apolipoprotein B gene results in hypobetalipoproteinemia and developmental abnormalities in mice. *Proc. Acad. Sci. U. S. A.* 90:2389-2393.

Homanics, G. E., H. V. de Silva, J. Osada, S. H. Zhang, H. Wong, J. Borensztajn, and N. Maeda. 1995. Mild dyslipidemia in mice following targeted inactivation of the hepatic lipase gene. *J. Biol. Chem.* 270:2974-2980.

Horie, Y., S. Fazio, J. R. Westerlund, K. H. Weisgraber, and S. C. Rall Jr. 1992. The functional charateristics of a human apolipoprotein E variant (cysteine at residue 142) may explain its association with the expression of type III hyperlipoproteinemia. *J. Biol. Chem.* 267:1962-1968.

Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1984. Defective hepatic lipoprotein receptor binding of beta-very low density lipoproteins from type III hyperlipoproteinemic patients. Importance of apolipoprotein E. *J. Biol. Chem.* 259:860-869.

Hussain, M. M., E. E. Zannis, M. Kelly, and V. I. Zannis. 1989. Synthesis modification and flotation properties of rat hepatocyte apolipoproteins. *Biochim. Biophys. Acta.* 1001:90-101.

Innerarity, T. L., E. J. Friedlander, S. C. Rall,Jr., K. H. Weisgraber, and R. W. Mahley. 1983. The receptor-binding domain of human apolipoprotein E. Binding of apolipoprotein E fragments. *J. Biol. Chem.* 258:12341-12347.

Innerarity, T. L., K. H. Weisgraber, K. S. Arnold, S. C. Rall,Jr., and R. W. Mahley. 1984. Normalization of receptor binding of apolipoprotein E2. Evidence for modulation of the binding site conformation. *J. Biol. Chem.* 259:7261-7267.

Innerarity, T. L., D. Y. Hui, T. P. Bersot, and R. W. Mahley. 1986. Type III hyperlipoproteinemia: a focus on lipoprotein receptor-apolipoprotein E2 interactions. *In:* Lipoprotein deficiency syndromes. A. Angel, and J. Frohlich, editors. Plenum, New York. p 273-288.

Ishibashi, S., M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* 92:883-893.

Ishibashi, S., J. L. Goldstein, M. S. Brown, J. Herz, and D. K. Burns. 1994. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J. Clin. Invest.*

93:1885-1893.

Ishida, B. Y., and B. Paigen. 1989. Atherosclerosis in the mouse. *In* Genetic Factors in Atherosclerosis: Approaches and Model Systems. A. J. Lusis and S. R. Sparkes, editors. Karger, Basel. 189-222.

Ishida, B. Y., P. J. Blanche, A. V. Nichols, M. Yashar, and B. Paigen. 1991. Effects of atherogenic diet consumption on lipoproteins in mouse strains C57BL6 and C3H. *J. Lipid. res.* 32:559-568.

Ito, Y., N. Azrolan, A. O'Connell, A. Walsh, and J. L. Breslow. 1990. Hypertriglyceridemia as a result of human apo CIII gene expression in transgenic mice. *Science* 249:790-793.

Jaenissch, R. 1988. Transgenic animals. *Science* 240:1468-1474.

Ji, Z. S., W. J. Brecht, R. D. Miranda, M. M. Hussain, T. L. Innerarity, and R. W. Mahley. 1993. Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J. Biol. Chem.* 268:10160-10167.

Ji, Z-S, S. Fazio, Y-L Lee, and R. W. Mahley. 1994a. Secretion-Capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 269:2764-2772.

Ji, Z-S., S. J. Lauer, S. Fazio, A. Bensadoun, J. M. Taylor, and R. W. Mahley. 1994b. Enhanced binding of remnant lipoprotein by hepatic lipase-secreting hepatoma cells in culture. *J. Biol. Chem.* 269:13429-13436.

Ji, Z. S., S. Fazio, and R. W. Mahley. 1994c. Variable heparan sulfate proteoglycan binding of apolipoprotein E variants may modulate the expression of type III hyperlipoproteinemia. *J. Biol. Chem.* 269:13421-13428.

Ji, Z-S, D. A. Sana, and R. W. Mahley. 1995. Intravenous heparinase inhibits remnant lipoprotein clearance from the plasma and uptake by the liver: in-vivo role of heparan sulphate proteoglycans. *J. Lipid. Res.* 36:583-592.

Jokinnen, M., T. Carlsson, and R. Prichard. 1985. Animal models in atherosclerosis research. *Exp. Mol. Path.* 42:1-28.

Joyner, A. L. 1993. Gene-targeting, a practical approach. Oford: Oxford University Press.

Kane, J. P., and R. J. Havel. 1995. Disorders of the biogenesis and secretion of lipoproteins containing the B apolipoproteins. *In:* The metabolic basis of inherited disease, 7th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. and Valle, Eds. New York: McGraw-Hill, pp. 1853-1885.

Kita, T., M. S. Brown, D. W. Bilheimer, and J. L. Goldstein. 1982a. Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. *Proc. Natl. Acad. Sci. USA.* 79:5693-5697.

Kita, T., J. L. Goldstein, M. S. Brown, Y. Watanabe, C. Hornick, and R. J. Havel. 1982b. Hepatic uptake of chylomicron remnants in WHHL rabbits. A mechanism genetically distinct from the low density lipoprotein receptor. *Proc. Natl. Acad. Sci. U. S. A.* 79:3623-3627.

Kowall, R.C., J. Herz, J. L. Goldstein, V. Esser, and M. S. Brown. 1989. Low density lipoprotein receptor-related mediates uptake of cholesterolesters derived from apoE-enriched lipoproteins. *Proc. Natl. Acad. Sci. U. S. A.* 86:5810-5814.

Laber-Laird and L. Rudel. 1989 *In:* Genetic factors in atherosclerosis: Approaches and model systems. Lusis, A. J. and R. S. Sparkes, Eds. Karger AG. pp 170-188.

Lauer, S. J., D. Walker, N. A. Elshourbagy, C. A. Reardon, B. Levy Wilson, and J. M. Taylor. 1988. Two copies of the human apolipoprotein C-I gene are linked closely to the apolipoprotein E gene. *J. Biol. Chem.* 263:7277-7286.

Lawn, R. M., D. P. Wade, R. E. Hammer, G. Chiesa, J. G. Verstuyft, and E. M. Rubin. 1992. Atherogenesis in transgenic mice expressing human apolipoprotein(a). *Nature.* 360:670-672.

Li, H., R. L. Reddick, and N. Maeda. 1993. Lack of apoA-I is not associated with increased susceptibility to atherosclerosis in mice. *Arterioscler. Thromb.* 13:1814-1821.

Lin, C-T., Y. Xu, J. Y. Xu, and L. Chan. 1986. Immunoreactive apolipoprotein E is awidely distributed cellular protein. Immunohistochemical localization of apolipoprotein E in baboon tissues. *J. Clin. Invest.* 78:947-958.

Liu, M. S., F. R. Jirik, R. C. LeBoeuf, H. Henderson, L. W. Castellani, A. J. Lusis, Y. Ma, I. J. Forsythe, H. Zhang, E. Kirk, and et al. 1994. Alteration of lipid profiles in plasma of transgenic mice expressing human lipoprotein lipase. *J. Biol. Chem.* 269:11417-11424.

Lusis, A. J. 1988. Genetic factors affecting blood lipoproteins: the candidate gene approch. *J. Lipid. Res.* 29:397-429.

Lusis, A. J., and R. S. Sparks. 1989. Chromosomal organisation of genes involved in plasma lipoprotein metabolism: human and mouse "fat maps". *In:* Genetic factors in atherosclerosis: approaches and model systems (Lusis, A. J., and S. R. Sparks, eds). Monogr Hum. Genet, Basel Karger 12:79-94.

Luis, A. J. 1993. The mouse model for atherosclerosis. *Trends Cardiovasc. Med.* 3:135-143.

Maeda, N., H. Li, D. Lee, P. Oliver, S. H. Quarfordt, and J. Osada. 1994. Targeted disruption of the apolipoprotein C-III gene in mice results in hypotriglyceridemia and protection from postprandial hypertriglyceridemia. *J. Biol. Chem.* 269:23610-23616.

Mahley, R. W. 1979. Dietary fat, cholesterol, and accelerated atherosclerosis. *In:* Atherosclerosis Reviews. R. Paoletti, and A. M. J. Gotto, editors. Raven Press, New York. 1-25.

Mahley, R. W., T. L. Innerarity, S. C. Rall Jr., and K. H. Weisgraber. 1984a. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipd. Res.* 25:1277-1294.

Mahley, R. W., and B. Angelin. 1984b. Type III hyperlipoproteinemia: Recent insights into the genetics defect of familial dysbetalipoproteinemia. *Adv. Intern. Med.* 29:385-411.

Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 240:622-630.

Mahley, R. W., D. Y. Hui, T. L. Innerarity, and U. Beisiegel. 1989a. Chylomicron remnant metabolism: Role of hepatic lipoprotein receptors in mediating uptake. *Aterioscleroses* 9 (suppl I):114-118.

Mahley, R. W., and S. C. Rall Jr. 1989b. Type III hyperlipoproteinemia (dysbetalipoproteinemia): The role of apolipoprotein E in normal and abnormal metabolism. *In:* The metabolic basis of inherited disease, 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. and Valle, Eds. New York: McGraw-Hill, pp. 1195-1213.

Mahley, R. W., T. L. Innerarity, S. C. Rall Jr., K. H. Weisgraber and J. M. Taylor. 1990. Apolipoprotein E: genetic variants provide insight into its structure and function. *Curr. Opin. Lipid.* 1:87-95.

Mahley, R. W., and and M. M. Hussain. 1991. Chylomicron and chylomicron remnant metabolism. *Curr. Opin. Lipidol.* 2:170-176.

Mann, W. A., R. E. Gregg, D. L. Sprecher, and H. B. J. Brewer. 1989. Apolipoprotein E-1Harrisburg: a new variant of apolipoprotein E dominantly associated with type III hyperlipoproteinemia. *Biochim. Biophys. Acta.* 1005:239-244.

Marzetta, C. A., D. M. Foster, and J. D. Brunzell. 1990. Conversion of VLDL and IDL precursors into various LDL subpopulations using density gradient ultracentrifugation. *J. Lipid. Res.* 31:975-984.

Mishkel, M. A., D. J. Nazir, and S. Crowther. 1975. A longitudinal assesment of lipid ratios in the diagnosis of type III hyperlipoproteinemia. *Clin. Chim. acta.* 58:121-136.

Morganroth, J., R. I. Levi, and D. S. Frederickson. 1975. The biochemical, clinical, and genetic features of type III hyperlipoproteinemia. *Ann. Intern. Med.* 82: 158-174.

Nishina, P. M., J. Verstuyft, and B. Paigen. 1990. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* 31:859-869.

Nishina, P. M., J. Wang, W. Toyofuku, F. A. Kuypers, B. Y. Ishida, and B. Paigen. 1993. Atherosclerosis and plasma and liver lipids in nine inbred strains of mice. *Lipids.* 28:599-605.

Olivecrona, T. and G. Bengtsson-olivecrona. 1990a. Lipases involved in lipoprotein metabolism. *Curr. Opin. Lipidol.* 1:116-121.

Olivecrona, T. and G. Bengtsson-olivecrona. 1990b. Lipoprotein lipase and hepatic lipase. *Curr. Opin. Lipidol.* 1:220-230.

Overturf, M. L., and D. S. Loose-Mitchel. 1992. *In vivo* model systems: the choice of the expreimental animal models for the analysis of lipoproteins and atheroscleosis. *Curr. Opin. Lipidol.* 3: 179-185.

Paigen, K. 1995. A miracle enough: the power of mice. *Nature Medicine* 1:215-220.

Paigen, B., D. Mitchell, P. A. Holmes, and D. Albee. 1987. Genetic analysis of strains C57BL6J and BALBcJ for Ath-1, a gene determining atherosclerosis susceptibility in mice. *Biochem. Genet.* 25:881-892.

Paigen, B., M. N. Nesbitt, D. Mitchell, D. Albee, and R. C. LeBoeuf. 1989. Ath-2, a second gene determining atherosclerosis susceptibility and high density lipoprotein levels in mice. *Genetics.* 122:163-168.

Paigen, B., A. S. Plump, and R. rubin. 1994. The muse as a model for human cardivascular disease and hyperlipidemia. *Curr. Opin. Lipidol.* 5:258-264.

Paik, Y. K., D. J. Chang, C. A. Reardon, G. E. Davies, R. W. Mahley, and J. M. Taylor. 1985. Nucleotide sequence and structure of the human apolipoprotein E gene. *Proc. Natl. Acad. Sci. U. S. A.* 82:3445-3449.

Palmiter, R. D., and R. L. Brinster. 1986. Germ-line transformation of mice. *Ann. Rev. Genet.* 20:465-499.

Plump, A. S., J. D. Smith, T. Hayek, K. Aalto Setala, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* 71:343-353.

Polano, M. K. 1973. Xanthoma types in relation to the type of hyperlipoproteinemia. *Nutr. Metabol.* 15:107-

118

Purcell Huynh, D. A., R. V. Farese,Jr., D. F. Johnson, L. M. Flynn, V. Pierotti, D. L. Newland, M. F. Linton, D. A. Sanan, and S. G. Young. 1995. Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J. Clin. Invest.* 95:2246-2257.

Rall Jr, S. C., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E: the complete amino acid sequence. *J. Biol. Chem.* 257:4171-4178.

Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1983a. Identical structural and receptor binding defects in apolipoprotein E2 in hypo-, normo-, and hypercholesterolemic dysbetalipoproteinemia. *J. Clin. Invest.* 71:1023-1031.

Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, T. P. Bersot, R. W. Mahley, and C. B. Blum. 1983b. Identification of a new structural variant of human apolipoprotein E, E2(Lys146-Gln), in a type III hyperlipoproteinemic subject with the E32 phenotype. *J. Clin. Invest.* 72:1288-1297.

Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1986. Isolation and characterization of apolipoprotein E. *Methods. Enzymol.* 128:273-287.

Rall, S. C., Jr, Y. M. Newhouse, H. R. G. Clarke, K. H. Weisgraber, B. J. McCarthy, R. W. Mahley, and T. Bersot. 1989. Type III hyperlipoproteinemia associated with a apolipoprotein Ephenotype E33. Structure and genetics of an apolipoprotein E3 variant. *J. Clin. Invest.* 83:1095-1101.

Rapacz, J. and J. Hasler-Rapacz. 1989 *In:* Genetic factors in atherosclerosis: Approaches and model systems. Lusis, A. J. and R. S. Sparkes, Eds. Karger AG. pp 139-169

Reue, K. L., C. H. Warden, and A. J. Lusis. 1990. Animal models for genetic lipoprotein disorders. *Curr. Opim. Lipidol.* 1:143-150.

Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801-809

Rubin, E. M., R. M. Krauss, E. A. Spangler, J. G. Verstuyft, and S. M. Clift. 1991. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature.* 353:265-267.

Rubin, E. M. and D. J. Smith. 1994. Atherosclerosis in mice: getting to the heart of a polygenic disorder. *Trends. Genet.* 10:199-203.

Rubinstein, A., J. C. Gibson, J. R. Paterniti, G. Kakis, A. Little, H. N. Ginsberg, and W. V. Brown. 1985. Effect of heparin-induced lipoplysis on the distribution of apolipoprotein E among lipoprotein subclasses. Studies with patients deficient in hepatic triglyceride lipase and lipoprotein lipase. *J. Clin. Invest.* 75:710-721.

Schaefer, E. J., R. E. Gregg, G. Ghiselli, T. M. Forte, J. M. Ordovas, L. A. Zech, and H. B. Brewer, Jr. 1986. Familial apolipoprotein E deficiency. *J. Clin. Invest.* 78:1206-1219.

Schneider, W. J., P. T. Kovanen, M. S. Brown, J. L. Goldstein, G. Utermann, W. Weber, R. J. Havel, L. Koite, J. P. Kane, T. L. Inneraity, and R. W. Mahley. 1981. Familial Dysbetalipoprotenemia. Abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes of liver and adrenal of rats, rabbits and cows. *J. Clin. Invest.* 68:1075-1085.

Schultz, J. R., E. L. Gong, M. R. McCall, A. V. Nichols, S. M. Clift, and E. M. Rubin. 1992. Expression of human apolipoprotein A-II and its effect on high density lipoproteins in transgenic mice. *J. Biol. Chem.* 267:21630-21636.

Schultz, J. R., A. Gambarotta, N. M. Couture, C. R. Coffill, and T. A-M. Neville, 1996. Mild hyperlipidemia in chow-fed human and mouse hepatic lipase transgenic mice. *Circulation* 94: 2314.

Shachter, N. S., T. Hayek, T. Leff, J. D. Smith, D. W. Rosenberg, A. Walsh, R. Ramakrishnan, I. J. Goldberg, H. N. Ginsberg, and J. L. Breslow. 1994. Overexpression of apolipoprotein CII causes hypertriglyceridemia in transgenic mice. *J. Clin. Invest.* 93:1683-1690.

Shafi, S., S. E. Brady, A. Bensadoun, and R. J. Havel. 1994. Role of hepatic lipase in the uptake and processing of chylomicron remnants in rat liver. *J. Lipid. Res.* 35:709-720.

Shimada, M., H. Shimano, T. Gotoda, K. Yamamoto, M. Kawamura, T. Inaba, Y. Yazaki, and N. Yamada. 1994. Overexpression of human lipoprotein lipase in transgenic mice. Resistance to diet-induced hypertriglyceridemia and hypercholesterolemia. *J. Biol. Chem.* 269:11673.

Shimano, H., N. Yamada, M. Shimada, N. Ohsawa, C. Fukazawa, Y. Yazaki, F. Takaku, and M. Katsuki. 1991. Hepatic and renal expression of rat apolipoprotein E under control of the metallothionein promoter in transgenic mice. *Biochim. Biophys. Acta.* 1090:91-94.

Shimano, H., N. Yamada, M. Katsuki, K. Yamamoto, T. Gotoda, K. Harada, M. Shimada, and Y. Yazaki. 1992a. Plasma lipoprotein metabolism in transgenic mice overexpressing apolipoprotein E. Accelerated

clearance of lipoproteins containing apolipoprotein B. *J. Clin. Invest.* 90:2084-2091.

Shimano, H., N. Yamada, M. Katsuki, M. Shimada, T. Gotoda, K. Harada, T. Murase, C. Fukazawa, F. Takaku, and Y. Yazaki. 1992b. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet- induced hypercholesterolemia. *Proc. Natl. Acad. Sci. USA* 89: 1750-1754.

Shimano, H., Y. Namba, J. Ohsuga, M. Kawamura, K. Yamamoto, M. Shimada, T. Gotoda, K. Harada, Y. Yazaki, and N. Yamada. 1994. Secretion-recapture process of apolipoprotein E in hepatic uptake of chylomicron remnants in transgenic mice. *J. Clin. Invest.* 93:2215-2223.

Shore, V. G., and B. Shore. 1973. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry* 12:502-507..

Simonet, W. S., N. Bucay, R. E. Pitas, S. J. Lauer, and J. M. Taylor. 1991. Multiple tissue-specific elements control the apolipoprotein E/C-I gene locus in transgenic mice. *J. Biol. Chem.* 266:8651-8654.

Smit, M., P. de Knijff, R. R. Frants, E. C. Klasen, and L. M. Havekes. 1987. Familial Dysbetalipoproteinemic subjects with the E3E2 phenotype exhibit and E2 isoform with only one cysteine residue. *Clin. Genet.* 32:335-341.

Smit, M., P. de Knijf, E. van der Kooij-Meijs, C. Groenendijk, A. M. J. M. van den Maagdenberg, J. A. Gevers Leuven, A. F. H. Stalenhoef, P. M. J. Stuyt, R. R. Frants, and L. M. Havekes. 1990. The E2(Lys146→Gln) variant results in a dominant mode of inheritance. *J. Lipid Res.* 31:45-53.

Smith, J. D., A. S. Plump, T. Hayek, A. Walsh, and J. L. Breslow. 1990. Accumulation of human apolipoprotein E in the plasma of transgenic mice. *J. Biol. Chem.* 265:14709-14712.

Spangler, E. A., J. G. Verstuyft, and E. M. Rubin. 1991. Effects of apolipoprotein E on plasma lipoproteins and atherosclerosis in transgenic mice [abst] *Circulation* 84:1030

Steward-Philips, J. L., J. Lough, and E. Skamene. 1989. Ath-3 a new gene for succeptibility to atherosclerosis in mice. *Cli. Invest. Med.* 12:121-126.

Stolzfus, L., and E. M. Rubin. Atherogenesis: Issights from the study of transgenic mice and gene-targeted mice. *Trends Cardiovasc. Med.* 3:130-142.

Stuyt, P. M. J., and A. van 't Laar. 1983. Clinical features of type III hyperlipoproteinemia. *Neth. J. Med.* 26:104-110.

Sultan, F., D. Lagrange, H. Jansen and S. Griglio. 1990. Inhibition of hepatic lipase activity impairs chylomicron remnant-removal in rats. *Biochim. Biophys. Acta* 1042:150-152.

Suzuki, H. Y. Kurihara, Y. Wada, N. Kamada, Y. Kawabe, H. Kurihara, Y. Yazaki, T. Doi, M. Takeya, K. Takahashi, E. Araki, S. Horiuchi, M. Nishijima, I. Frazer, D. Hughes, S. Gordon, and T. Kodama. 1995. Establishment and analysis of scavenger receptor knockout mice. *Circulation [supplement]* 92:2043.

Tall, A. R., and D. M. Small. 1978. Plasma high density lipoproteins. *N. Engl. J. Med.* 299:1232-1236.

Taylor, J. M., W. S. Simonet, N. Bucay, S. J. Lauer, and H. S. de Silva. 1991. expression of the human apolipoprotein Eapolipoprotein C-I locus in transgenic mice. *Curr. Opin. Lipodol.* 2:73-80.

Utermann, G., A. Steinmetz, and W. Weber. 1982. Genetic control of human apolipoprotein E polymorfism. comparison of one- and two-dimensional techniques of isoprotein analysis. *Human Genet.* 60:344-351.

Utermann, G. 1985. Morgani Lecture. Genetic polymorphism of apolipoprotein E - Impact on plasma lipoprotein metabolism. *In:* Diabetes, obesity, and hyperlipidemias. G. Crepaldi, A. Tiego, G. Baggio. Editors. Elsevier, New York. 3:1-28.

van Dijk, M. C. M., J. K. Kruijt, W. Boers, C. Linthorst, and T. J. C. van Berkel. 1992. Distinct properties of the recognition sites for ß-very low density lipoprotein (remnant receptor) and α2-macroglobulin (low density lipoproprotein-related protein) on rat parenchymal cells. *J. Biol. Chem.* 267:17732-17737.

van den Maagdenberg, A. M. J. M., P. de Knijff, A. F. Stalenhoef, J. A. Gevers Leuven, L. M. Havekes, and R. R. Frants. 1989. Apolipoprotein E3-Leiden allele results from a partial gene duplication in exon 4. *Biochem. Biophys. Res. Commun.* 165:851-857.

van Ree, J. H., J. A. A. van den Broek, V. E. H. Dahlmans, P. H. E. Groot, M. Vidgeon-Hart, R. R. Frants, B. Wierenga, L. M. Havekes, and M. H. Hofker. 1994. Diet-Induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.* 111:25-37.

van Ree, J. H., M. H. Hofker, W. J. van den Broek, J. M. van Deursen, H. van der Boom, R. R. Frants, B. Wieringa, and L. M. Havekes. 1995. Increased response to cholesterol feeding in apolipoprotein C1-deficient mice. *Biochem. J.* 305:905-911.

Walsh, A., Y. Ito, and J. L. Breslow. 1989. High levels of human apolipoprotein A-I in transgenic mice result in increased plasma levels of small high density lipoprotein (HDL) particles comparable to human HDL3.

J. Biol. Chem. 264:6488-6494.

Wardell, M. R., K. H. Weisgraber, L. M. Havekes, and S. C. J. Rall. 1989. Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121-127. *J. Biol. Chem.* 264:21205-21210.

Warden, C. H., C. C. Hedrick, J. H. Qiao, L. W. Castellani, and A. J. Lusis. 1993. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science.* 261:469-472.

Weisgraber, K. H., S. C. Rall Jr, and R. W. Mahley. 1981. Human E apoprotein heterogeneity. Cysteine-argenine interchanges in the amino acid sequence of the apo-E isoforms. *J. Biol. Chem.* 256:9077-9083.

Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor binding activity of the human E apoprotein due to cysteine-argine interchange at a single site. *J. Biol. Chem.* 257:2518-2521.

Weisgraber, K. H., S. C. Rall,Jr., R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1986. Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. *J. Biol. Chem.* 261:2068-2076.

Wetterau, J. R., L. P. Aggerbeck, S. C. Rall,Jr., and K. H. Weisgraber. 1988. Human apolipoprotein E3 in aqueous solution. I. Evidence for two structural domains. *J. Biol. Chem.* 263:6240-6248.

Williamson, R., D. Lee, J. Hagaman, and N. Maeda. 1992. Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. *Proc. Acad. Sci. U. S. A.* 89:7134-7138.

Willnow, T. E., Z. Sheng, S. Ishibashi, and J. Herz. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor anatagonist. *Science.* 264: 1471-1474.

Wilson, C., M. R. Wardell, K. H. Weisgraber, R. W. Mahley, and D. A. Agard. 1991. Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science* 252:1817-1822.

Windler, E. E. E., J. Greeve, W. H. Daerr and H. Greten. 1988. Binding of rat chylomicrons and their remnants to the hepatic low-density-lipoprotein receptor and its role inremnant removal. *Biochem. J.* 252:553-561.

Weng, W, T. Hayek, and J. L. Breslow. 1995. Dramatic reduction of high density lipoprotein in apolipoprotein A-II deficient mice created by homologous recombination in ES cells. *Circulation [supplement]* 92:492

Wolle, S., D. P. Via, L. Chan, J. A. Cornicelli, and C. L. Bisgaier. 1995. Hepatic overexpression of bovine scavenger receptor type I in transgenic mice prevents diet-induced hyperbetalipoproteinemia. *J. Clin. Invest.* 96:260-272.

Xiong, W., E. Zsigmond, A. M. Gotto,Jr., L. W. Reneker, and L. Chan. 1992. Transgenic mice expressing full-length human apolipoprotein B-100. Full-length human apolipoprotein B mRNA is essentially not edited in mouse intestine or liver [retracted in J Biol Chem 1993 Aug 15;268(23):17647]. *J. Biol. Chem.* 267:21412-21420.

Yamamoto, T., R. W. Bischop, M. S. Brown, J. L. Goldstein, and D. W. Russel. 1986. Deletion in custeine-rich regio of LDL receptor impedes transport to cell surface in WHHL rabbit. *Science* 232:1230-1237.

Yokode, M., R. E. Hammer, S. Ishibashi, M. S. Brown, and J. L. Goldstein. 1990. Diet-induced hypercholesterolemia in mice: prevention by overexpression of LDL receptors. *Science* 250:1273-1275.

Zannis, V. I., and J. L. Breslow. 1980. Characterization of aunique human apolipoprotein E variant associated with type III hyperlipoproteinemia. *J. Biol. Chem.* 255:1759-1762.

Zannis, V. I., and J. L. Breslow. 1981. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation aand posttranlational modification. *Biochemistry* 20:1033-1041.

Zannis, V. I., J. McPherson, G. Goldberger, S. K. Krathanasis, and J. L. Breslow. 1984. Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. *J. Biol. Chem.* 259:5495-5499.

Zannis, V. I., D. Kardassis, P. ardot, M. Hadzopoulou-Cladaras, E. E. Zani, and C. Cladaras. 1992. Molecular biology of the human apolipoprotein genes: gene regulation and structure/function relationship. *Curr. Opin. Lipidol.* 3:96-113.

Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258:468-471.

Zsigmond, E., E. Scheffler, T. M. Forte, R. Potenz, W. Wu, and L. Chan. 1994. Transgenic mice expressing human lipoprotein lipase driven by the mouse metallothionein promoter. A phenotype associated with increased perinatal mortality and reduced plasma very low density lipoprotein of normal size. *J. Biol. Chem.* 269:18757-18766.

CHAPTER 2

DIET-INDUCED HYPERLIPOPROTEINEMIA AND ATHEROSCLEROSIS IN APOLIPOPROTEIN E3-LEIDEN TRANSGENIC MICE

Bart J. M. van Vlijmen[1], Arn M. J. M. van den Maagdenberg[2], Marion J.J. Gijbels[1], Hans van der Boom[1], Harm HogenEsch[1], Rune R. Frants[2], Marten H. Hofker[2], and Louis M. Havekes[1]

[1]TNO-PG, Gaubius Laboratory, Leiden, The Netherlands, and [2]MGC-Department of Human Genetics, Leiden University, Leiden, The Netherlands

Reproduced from
The Journal of Clinical Investigation, 1994, 93:1403-1410
by copyright permission of
The American Society for Clinical Investigation

ABSTRACT

Apolipoprotein E3-Leiden (APOE*3-Leiden) transgenic mice have been used to study the effect of different cholesterol-containing diets on the remnant lipoprotein levels and composition and on the possible concurrent development of atherosclerotic plaques. On high fat / high cholesterol (HFC) diet, the high expressing lines #2 and #181 developed severe hypercholesterolemia (up to 40 and 60 mmol/l, respectively), whereas triglyceride levels remained almost normal when compared to regular mouse diet. The addition of cholate increased the hypercholesterolemic effect of this diet. In lines #2 and #181 serum levels of apoE3-Leiden also increased dramatically upon cholesterol feeding (up to 107 and 300 mg/dl, respectively). In these high expressing APOE*3-Leiden transgenic mice the increase in both serum cholesterol and apoE3-Leiden occurred mainly in the VLDL/LDL-sized fractions, whereas a considerable increase in large, apoE-rich HDL particles also occurred. In contrast to the high expressing lines, the low expressing line #195 reacted only mildly upon HFC diet.

On HFC diets, the high expressor APOE*3-Leiden mice developed atherosclerotic lesions in the aortic arch, the descending aorta and the carotid arteries, varying from fatty streaks containing foam cells to severe atherosclerotic plaques containing cholesterol crystals, fibrosis and necrotic calcified tissue. Quantitative evaluation revealed that the atherogenesis is positively correlated with the serum level of cholesterol-rich VLDL/LDL particles. In conclusion, with APOE*3-Leiden transgenic mice, factors can be studied that influence the metabolism of VLDL remnant lipoproteins and the development of atherosclerosis.

INTRODUCTION

Apolipoprotein E functions as a ligand for the receptor-mediated uptake of chylomicron and very low density lipoprotein (VLDL) remnants by hepatic lipoprotein receptors (1-3). Normally, these remnant particles are rapidly removed from the circulation. However, in humans, mutations in the structural gene for apoE can lead to a binding- defective apoE and, consequently, to a reduced clearance rate of remnant particles, as has been described for patients with familial dysbetalipoproteinemia (FD) (4,5). The accumulation of atherogenic remnant particles in the plasma of FD patients results in a predisposition for coronary and/or peripheral atherosclerosis (5).

In most cases, FD is associated with homozygosity for the APOE*2(Arg158→Cys) allele (5). However, heterozygosity for the rare APOE*3-Leiden allele is also associated with FD (6). Thus in the case of APOE*3-Leiden heterozygosity, FD is inherited in a dominant fashion. Among all homozygous APOE*2(Arg158→Cys) subjects (1% in the general population), only a small proportion (about 4%) develops FD at a later age. Hence, additional factors are required for the expression of the disease. Known factors that strongly influence the severity of FD are age, gender, nutritional status and body mass index (BMI) (5).

Although almost all heterozygous APOE*3-Leiden subjects display FD, also in these subjects the severity of the disease is largely dependent on additional environmental and/or genetic factors (6).

Studying the (subtle) environmental and genetic factors influencing remnant metabolism and atherosclerosis in humans, numerous limitations are encountered: relevant environmental variables are often hard to control; detailed biochemical studies in humans are frequently not allowed; and humans exhibit a considerable heterogeneity in genetic background whereas the number of subjects carrying specific mutations or combinations of specific mutations are usually very limited.

For the study of genetic and environmental factors which modulate the lipoprotein remnant metabolism and the development of atherosclerosis *in vivo* in a more standardized way, we studied in this paper the feasibility of transgenic mice. Since conventional transgenesis implies the addition of extra copies of genes to the genome, only dominant effects can be studied using this approach. Therefore, we chose the dominant APOE*3-Leiden gene as a transgene. Recently, we reported the generation of three different APOE*3-Leiden/APOC1 transgenic mouse lines (#2, #181, and #195) with different levels of expression of the APOE*3-Leiden gene (7). It appeared that these transgenic mice developed hyperlipidemia, which was positively correlated with the level of expression of the transgene, even on a regular mouse diet. Furthermore, the high expressing line #181 appeared to be highly responsive to cholesterol-rich diets with regard to serum cholesterol levels in the VLDL/LDL-sized fractions. The human APOC1 gene was also included in the transgene in order to be able to introduce simultaneously the endogenous DNA sequence that is responsible for the liver-specific expression of the APOE gene. The potential contribution of the APOC1 gene to the phenotypic expression of these lines can be excluded as transgenic mice with a high level expression of a similar but wild type APOE-APOC1 transgene do not express hyperlipidemia (8,9).

In the present paper, we used these APOE*3-Leiden transgenic mice to study the effect of different semi-synthetic cholesterol-containing diets on the remnant lipoprotein levels and composition and on the possible concurrent development of atherosclerotic plaques. These mice develop atherosclerotic plaques of which the severity is positively correlated with the serum levels of cholesterol-rich VLDL/LDL-sized lipoproteins and the level of expression of the APOE*3-Leiden transgene. We can conclude that the APOE*3-Leiden transgenic mice offer a suitable animal model system for studying the environmental factors involved in remnant metabolism and concurrent atherogenesis.

METHODS

Animals

Transgenic mice of lines # 2, # 181, and # 195, expressing human APOE*3-Leiden and human APOC1 genes were generated previously (7). Micro-injection has been performed in eggs from (C57BL/6J x CBA/J) F1 females that had been mated to males of the same genetic background. Transgenic and non-transgenic littermates were obtained by breeding with C57BL/6J mice. Mice of F2 generation have been used for the experiment. Identification of transgenic mice was done by PCR analysis on genomic tail DNA, as described before (7). Mice of the F2 generation, 8-10 weeks of age, were included in the experiments. Males and females were equally distributed among the various groups. Prior to the diet administration, mice were housed under standard conditions in conventional cages and given free excess to water and regular mouse diet. Diets were administered to groups of at least five mice of each line. Blood samples were taken before and after 6 weeks of feeding. Also, during diet feeding, mice were given free access to food and water. Animal weights were not significantly different between diets after 6 weeks of diet feeding. None of the transgenic or non-transgenic mice were lost during the entire study of 14 weeks.

Composition of the diets

Before starting the dietary treatment, mice were fed regular mouse diet (SRM-A: Hope Farms, Woerden, The Netherlands). The three semi-synthetic diets were composed essentially according to Nishina et al. (10), and were purchased from Hope Farms. The compositions of the different diets are given in Table I. The basic diet, containing only sucrose and basic nutrients, is designated as the low fat/cholesterol (LFC) diet. Two diets consist of the basic diet supplemented with cocoa butter (15%, by weight) and cholesterol (1%, by weight), and represent the high fat/cholesterol (HFC) diets. These two diets differ in amounts of added cholate (0.1% and 0.5%, by weight) and are designated as HFC/0.1% and HFC/0.5%, respectively. Cholate was added to facilitate intestinal uptake of fat and cholesterol.

Lipid and lipoprotein analysis

After a 12 hour fasting period, approximately 300 μl of blood was obtained from each individual mouse through tail-bleeding. Total serum cholesterol and triglyceride levels (without free glycerol) were measured enzymatically using commercially available kits: No. 236691 and No. 877557, respectively (Boehringer Mannheim, Germany).

Table I. Composition of the Semi-Synthetic Diets

	Type of diet		
Diet Components	LFC	HFC/0.1%	HFC/0.5%
		g/100 g diet	
Cocoa butter	-	15	15
Cholate	-	0.1	0.5
Cholesterol	-	1	1
Sucrose	50.5	40.5	40.5
Cornstarch	12.2	10	10
Corn oil	5	1	1
Cellulose	5	5.1	4.7

In addition, all diets contained 20% casein, 1% choline chloride, 0.2% methionine, and 5.1% mineral mixture. All percentages are in weight/weight. Energy contents of the three diets was 3883 kcal/kg for the LFC diet, and 4356 kcal/kg for both HFC diets.

For size fractionation of lipoproteins, 150 μl of pooled serum (from at least three mice per group), was applied to a 25 ml Superose 6B column (Pharmacia, Uppsala, Sweden) connected to a HPLC pump system and eluted at a constant rate of 0.5 ml/min with phosphate-buffered saline (pH 7.4). The effluent was collected in 0.5 ml fractions. Cholesterol and triglyceride concentrations in lipoprotein fractions were measured enzymatically, as described above.

ApoB/E containing lipoproteins were precipitated by mixing 200 μl of pooled serum (from three mice per group) with 20 μl of 4% sodium phoshotungstic acid (11). Subsequently, 10 μl of 1M $MgCl_2$ was added, and the mixture was kept at room temperature for 30 min. Thereafter, samples were centrifuged for 30 min in a minifuge. Some 150 μl of the supernatant was applied to a Superose 6B column, as described above. The amount of precipitable HDL-cholesterol was calculated as the difference of the total area under the curve of HDL fractions 31-41 of lipoprotein profiles before and after precipitation, and corrected for sample dilution in case of precipitation.

Quantitation of human apoE

Human apoE concentrations were measured by sandwich ELISA. Affinity purified polyclonal goat anti-human apoE antibodies were used for coating. Affinity purified polyclonal rabbit anti-human apoE antibodies were used as second antibodies. Thereafter, the plates were incubated with swine anti-rabbit IgG antibodies conjugated with horse radish peroxidase (HRP). Finally, HRP detection was performed using HRP substrate tetramethylbenzidine. Pooled plasma from healthy human subjects with known apoE level, was used as a standard.

Histological assessment of atherosclerosis

After 14 weeks of diet feeding, mice were sacrificed: a complete gross necropsy was performed and the entire aorta from the aortic valves to the iliac bifurcation was dissected. In addition, parathymic fat tissue was sampled to screen for the presence of arterial lesions in the carotid arteries. After phosphate-buffered formalin fixation, the heart was trimmed according to a modified method of Paigen et al. (12). In brief, the heart and the ascending aorta were cut perpendicular to the heart axis at four levels: a) just beneath the atrial tips, b) through the aorta and just above the atria, c) at the level of the aortic sinus and the aortic valves, and d) between aortic sinus and level a. In total, four heart sections were microscopically examined. The proximal 0.5 cm of aorta ascendens and aortic arch were divided into two parts for transverse sections. The remainder of the thoracic and abdominal aorta was divided into two parts for transverse and longitudinal sections. All tissue samples were dehydrated and paraffin embedded. Three micron sections were routinely stained with hematoxylin-phloxine-saffron (HPS). Additional stains used were Verhoeff's Van Gieson and von Kossa's silver stain. The total arterial endothelial surface examined was comparable for all animals.

For semi-quantitative assessment, the arterial lesions were classified into five categories: 1) early fatty streak: per section upto 10 foam cells present in the intima, 2) regular fatty streak: more than 10 foam cells present in the intima, 3) mild plaque: extension of foam cells into the media and mild fibrosis of the media without loss of architecture, 4) moderate plaque: foam cells in the media, fibrosis, cholesterol clefts, mineralisation and/or necrosis of the media and 5) severe plaque: as 4 but more extensive and deeper into the media. Every individual arterial lesion was classified accordingly. Within a group the total number of both mild lesions (category 1-3) and moderate plus severe lesions (category 4-5) of all mice were counted. After dividing the total number of lesions (mild and moderate plus severe, separately) by the number of mice per group, two separate mean scores for each group of animals were obtained.

Statistical analysis

Number Cruncher Statistical System (NCSS), version 5.02 (Kaysville, UT) was used for statistical analysis. Since the transgenic and non-transgenic mice were littermates and measured lipid parameters showed non-Gaussian distributions, we used non-parametric tests for statistical analysis. Mann-Whitney rank sum test was used when two groups were compared. *P* values less than 0.05 were

Table II. Serum Lipid and Lipoprotein Concentrations after 6 Wk of Dietary Treatment

Mouse line		Diet											
		SRM-A			LFC			HFC/0.1%			HFC/0.5%		
		TC	TG	apoE	TC	TG	apoE	TC	TG	apoE	TC	TG	apoE
		mmol/l		*mg/dl*	*mmol/l*		*mg/dl*	*mmol/l*		*mg/dl*	*mmol/l*		*mg/dl*
#2	Total	3.2 ± 0.7*	2.7 ± 0.7*	36 ± 18	6.4 ± 0.4*‡	5.4 ± 1.1*‡	54 ± 13	26.2 ± 1.5*‡	3.3 ± 0.4*	107 ± 42‡	39.9 ± 9.0*‡	1.6 ± 0.1*	68 ± 14‡
	VLDL	1.4	2.1	ND	2.3	3.9	1.9	13.0	2.0	6.9	21.1	1.1	12.3
	IDL/LDL	0.5	0.5	ND	1.4	0.9	12.4	7.0	0.6	43.7	10.7	0.3	28.1
	HDL	1.2	0.2	36.0	2.8	0.5	39.7	6.3	0.7	56.5	8.1	0.2	27.6
#181	Total	4.8 ± 1.3*	2.7 ± 1.0*	91 ± 28	13.3 ± 1.9*‡	29.4 ± 9.4*‡	147 ± 9‡	43.7 ± 13.2*‡	10.5 ± 3.7*‡	300 ± 78‡	59.1 ± 9.8*‡	4.5 ± 2.3*‡	189 ± 71‡
	VLDL	1.8	2.3	0.2	8.1	23.2	21.7	27.1	8.5	44.4	39.2	3.6	38.9
	IDL/LDL	0.5	0.3	ND	1.7	3.7	7.3	8.2	1.4	80.0	11.3	0.5	73.9
	HDL	2.4	0.1	90.8	3.5	2.4	117.8	8.1	0.6	175.6	8.6	0.3	76.2
#195	Total	2.5 ± 0.4	0.7 ± 0.2	3 ± 1	3.1 ± 1.0	0.5 ± 0.3	4 ± 1‡	4.1 ± 0.7*‡	0.3 ± 0.2	2 ± 1	8.1 ± 1.7*‡	0.3 ± 0.4*‡	2 ± 1
	VLDL	0.1	0.3	ND	0.3	0.2	ND	0.9	0.1	ND	3.9	0.1	0.6
	IDL/LDL	0.2	0.2	ND	0.4	0.1	0.3	0.7	0.1	0.3	1.5	0.1	0.3
	HDL	2.2	0.3	3.0	2.4	0.2	3.7	2.5	0.1	1.8	2.7	0.1	1.1
Control	Total	2.1 ± 0.3	0.5 ± 0.3	-	2.9 ± 0.2‡	0.6 ± 0.2	-	2.8 ± 0.5‡	0.1 ± 0.1‡	-	5.7 ± 1.6‡	0.1 ± 0.1‡	-
	VLDL	0.2	0.2	-	0.3	0.4	-	0.3	0.1	-	2.5	0.1	-
	IDL/LDL	0.1	0.1	-	0.4	0.1	-	0.3	0.1	-	1.2	0.1	-
	HDL	1.8	0.2	-	2.2	0.1	-	2.2	0.1	-	1.9	0.1	-

TC: total cholesterol (free + esterified), TG: triglyceride, apoE: ApoE3-Leiden. Total cholesterol, triglyceride and ApoE3-Leiden values are the mean serum levels ± S.D. of five mice per group. *$P<0.05$, indicating the difference between transgenic and non-transgenic groups of mice on the same diet, using non-parametric Mann-Whitney tests. ‡$P<0.05$, indicating the difference between semi-synthetic diet and regular SRM-A within each line, using non-parametric Mann-Whitney. The cholesterol, triglycerides and apoE in VLDL, IDL/LDL and HDL were calculated from the serum total cholesterol, triglycerides and apoE and the area under the curve in the lipoprotein profile (as determined by gelfiltration chromatography using a Superose 6B column). As observed in control mice on SRM-A fraction, number 20-25, 26-30 and 31-41 correspond to VLDL, IDL/LDL and HDL, respectively. ND: not detectable.

regarded as significant.

RESULTS

Effect of different diets on serum lipid and apoE3-Leiden levels

When kept on regular mouse diet (SRM-A), serum cholesterol and triglycerides levels were significantly higher in transgenic line #2 and #181 mice than in non-transgenic littermates (Table II). In transgenic line #195 mice elevations of serum lipid levels did not reach statistical significance. The serum levels of apoE3-Leiden correlated well with the extent of hyperlipidemia in the transgenic mice.

After 6 weeks on the LFC diet, line #2 and #181 mice showed a strong increase in serum cholesterol (2 and 3-fold, respectively) and serum triglycerides levels (2 and 11-fold, respectively). Mean apoE3-Leiden levels in these transgenic mice also increased significantly (up to 54 and 147 mg/dl, respectively). Line #195 mice did not show a strong increase in serum lipid levels on LFC diet compared to the control mice (Table II).

Feeding transgenic line #2 and #181 mice the HFC/0.1% diet resulted in a dramatic rise in serum cholesterol levels, while serum triglyceride levels decreased relative to the LFC diet (Table II). The ApoE3-Leiden level in line #2 and #181 mice on HFC/0.1% diet also increased to extremely high levels: 107 and 300 mg/dl, respectively. Further increasing the cholate content up to 0.5% (HFC/0.5% diet) resulted in a further increase in cholesterol levels, whereas the opposite effect was observed for serum triglycerides and serum apoE3-Leiden levels. Again, only a moderate response on serum lipid levels during these diets was observed for the low expressing line #195 and control mice. In the transgenic line #195 mice, apoE3-Leiden levels remained low during all three diet regimes.

ApoE3-Leiden and serum lipid levels

The results presented in Table II suggest a differential effect on serum apoE3-Leiden and serum cholesterol levels in line #2 and #181 mice when being fed different diets. To further demonstrate this, for each line separately, the mean level of apoE3-Leiden is plotted against the corresponding mean serum cholesterol level obtained per diet, as indicated (Figure 1). Strikingly, for both lines #2 and #181 the highest apoE3-Leiden levels were found following the HFC/0.1% diet rather than following the most severe hypercholesterolemic HFC/0.5% diet. In contrast to these observations, in line #195 mice no such relationship was observed.

Distribution of lipids and apoE3-Leiden among lipoprotein fractions following different diets

Because of the differential effect of the three diets on cholesterol and triglyceride levels (Table II) and the absence of a linear correlation between mean serum cholesterol and mean apoE3-Leiden levels as shown in Figure 1, we evaluated the distribution of cholesterol, triglyceride and apoE3-Leiden among the various lipoprotein fractions. Therefore, serum

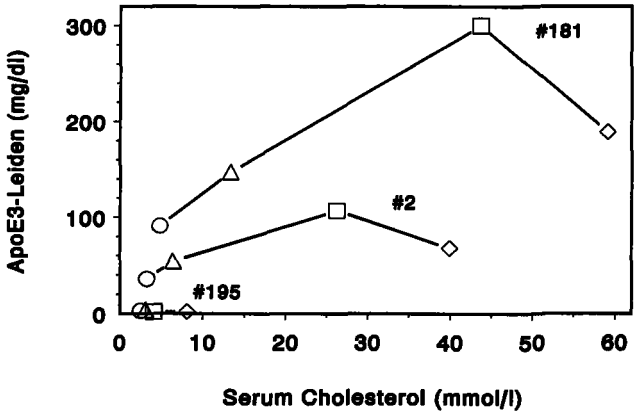


Figure 1. Correlation between mean serum cholesterol and human apoE3-Leiden levels in transgenic mice of line #181, #2 and #195. The values are obtained from Table II and represent mean levels after feeding transgenic mice SRM-A (○), LFC (Δ), HFC/0.1% (□) or HFC/0.5% (◇) for six weeks.

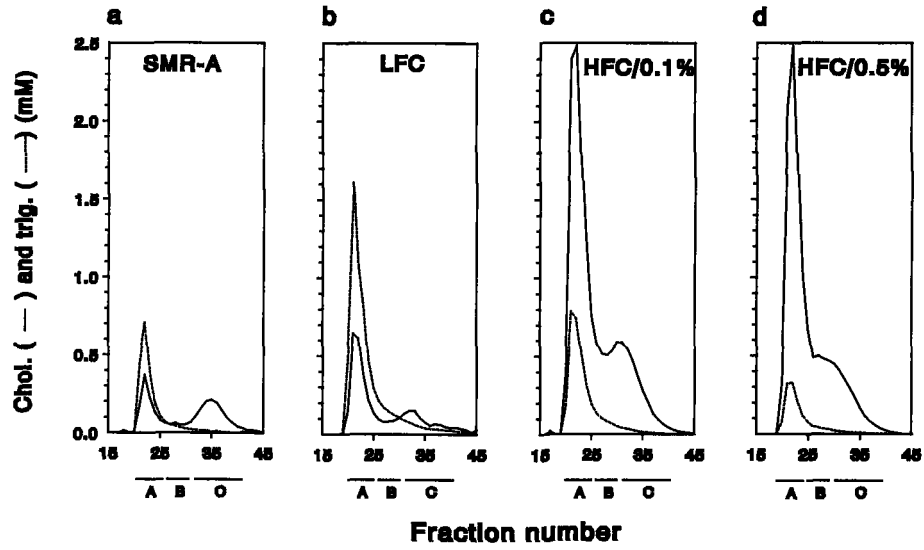


Figure 2. Distribution of serum cholesterol and triglycerides among lipoprotein fractions. Lipoprotein fractions were separated by gelfiltration chromatography using a 25 ml Superose 6B column as described in Methods section. Lipoprotein profiles are shown for transgenic line #181 mice fed SRM-A, or one of the specified diets (LFC, HFC/0.1% and HFC/0.5%) for six weeks. Each run is performed with a pool of serum of at least three mice of the same group. Fractions corresponding to VLDL, IDL/LDL and HDL (as observed in non-transgenic mice on SRM-A) are indicated by bar A, B and C, respectively.

lipoproteins were size-fractionated, using Superose 6B gel permeation chromatography. In Figure 2 lipoprotein profiles are shown for transgenic line #181 mice following six weeks of SRM-A diet (*panel A*) and following six weeks of three semi-synthetic diets (*panels B, C and D*). For all three semi-synthetic diets, the increase in cholesterol was most prominent in

the VLDL and IDL/LDL-sized fractions. In addition, triglycerides were almost exclusively confined to these fractions. Strikingly, as compared to the LFC diet, feeding the HFC diets leads to VLDL and IDL/LDL-sized fractions that are relatively rich in cholesterol, in particular with higher percentages of cholate (compare panel B with C and C with D). A similar dietary response was found for line #2 mice (results not shown).

A quantification of the lipid distribution among the various lipoprotein fractions is presented in Table II for all three lines and control mice. In addition to a cholesterol increase in the VLDL and IDL/LDL-sized fractions, a cholesterol increase was also observed in the HDL-sized fractions of the transgenic lines #2 and #181 with the HFC diets. On the contrary, in line #195 and in the non-transgenic littermates, HDL cholesterol was not influenced by cholesterol feeding. As shown for line #181 in Figure 2, the increase in HDL cholesterol is mainly due to an increase in the fraction of larger HDL particles. This suggests the generation of apoE-rich HDL particles with cholesterol feeding. To investigate this further, lipoprotein gel permeation profiles were made before and after precipitation of apoB/apoE-containing lipoproteins in serum with sodium phosphotungstic acid (Figure 3). In non-transgenic littermates (control mice) only a small proportion of the HDL fraction is precipitable, irrespective of cholesterol feeding. On the contrary, in line #181 a major proportion of the HDL cholesterol could be precipitated together with the VLDL and

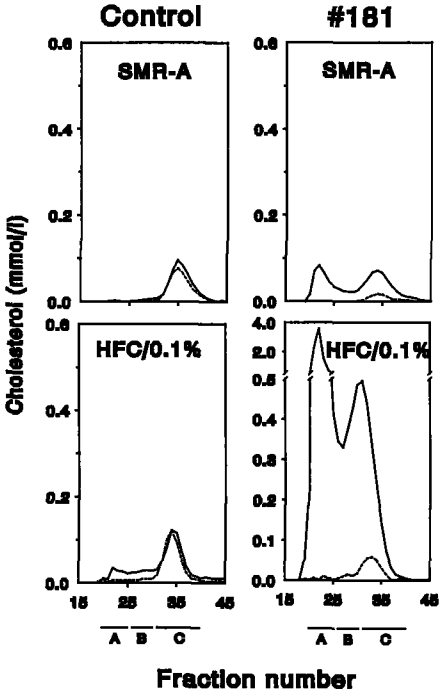


Figure 3. Distribution of serum cholesterol among lipoprotein fractions before (solid line) and after (dotted line) precipitation of apoB/E-containing lipoproteins. Apo B/E containing lipoproteins are precipitated with sodium phosphotungstic acid as described in Methods section. Cholesterol profiles for non-transgenic mice and transgenic line #181 mice are given as indicated after feeding SRM-A or the HC/0.1% diet. Fractions corresponding to VLDL, IDL/LDL and HDL (as observed in non-transgenic mice on SRM-A) are indicated by bar A, B and C, respectively.

IDL/LDL-sized fractions, both after cholesterol feeding and following a regular SRM-A diet. Similar experiments have been carried out for lines #2 and #195 and control mice. We calculated that on HFC/0.1% diet, the major proportion of HDL cholesterol is precipitable in lines #181 and #2 (85 and 80%, respectively), whereas in line #195 and in non-transgenic littermates this proportion is much lower (40 and 8%, respectively).

Table II also presents the quantitative distribution of apoE3-Leiden protein among the various lipoprotein fractions. For all three lines the HDL fraction is the major apoE3-Leiden containing lipoprotein fraction, following the application of each diet regime. In addition, in lines #2 and #181 cholesterol feeding also resulted in a dramatic increase in the amount of apoE3-Leiden present in the VLDL and IDL/LDL-sized fractions. In line #195 only very low amounts of apoE3-Leiden appear in the VLDL and IDL/LDL-sized fraction when being fed cholesterol.

Histological assessment of atherosclerosis

The results presented above indicate that in mice of lines # 2 and # 181, when fed either the HFC/0.1% or HFC/0.5% diet, an accumulation in the plasma of cholesterol-rich remnant lipoproteins occured to an extent which can be considered as atherogenic.
Atherogenesis in mice, following administration of the different diets, was assessed by histological analyses. After feeding the diets for a period of 14 weeks, each group of transgenic mice was analyzed for development of atherosclerotic lesions in the aortic arch, descending aorta and in the carotid arteries. The atherosclerotic lesions are classified in five categories as described in the Methods section. Examples of early and regular fatty streaks and mild, moderate and severe plaques are shown in Figure 4.

In Table III the mean number per mouse of early + regular fatty streaks + mild plaques on the one hand (Figure 4b-d) and the mean number per mouse of moderate + severe

*Table III. Atherosclerotic Lesions in ApoE*3-Leiden Transgenic Mice on Different Diets*

Mouse Line	SRM-A	Diet		
		LFC	HFC/0.1%	HFC/0.5%
		*Number of fatty steaks / plaques per mouse**		
Control	- / -	- / -	- / -	- / -
#195	- / -	- / -	- / -	- / -
#2	- / -	1.0/ -	2.8/0.8	2.0/3.0
#181	- / -	- / -	1.0/2.5	1.3/5.7

Semiquantative analysis of atherosclerosis in groups of transgenic and nontransgenic mice was determined after 14 Wks of diet feeding. The values are the mean of at least three mice and represent the mean number of fatty streaks and mean number of plaques per mouse as observed during histological assessment as decribed in Methods section. A dash means no lesions observed. *Fatty streaks represent mild lesions of category 1-3 (see Fig. 4, *b-d*), plaques represent severe lesions of category 4-5 (see Fig. 4, *e-f*).

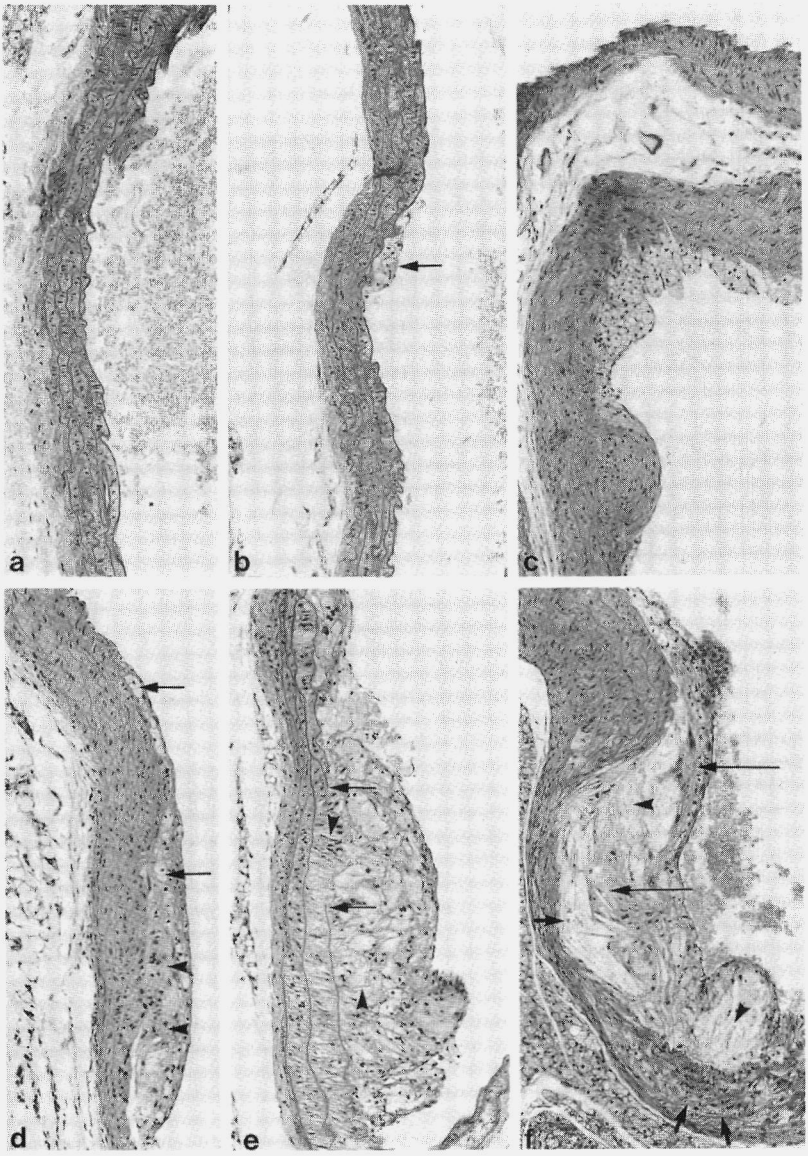


*Figure 4. Representative photographs of various stages of the atherosclerotic process observed in aorta and carotid arteries of transgenic APOE*3-Leiden mice. a.* Aorta without lesions. *b.* Aorta with small fatty streak (arrow). *c.* Aorta with regular fatty streak; media not affected. *d.* Aorta with mild plaque. Extension of foam cells into the media (arrow) and early fibrosis (arrowhead). Structure of the media intact. *e.* Moderate plaque in a carotid artery with foamcells and cholesterol clefts (arrowhead). Media moderately affected. Note the interrupted internal elastic lamina (arrow). *f.* Severe plaque in aorta showing extensive destruction of the media. Note foamcells, cholesterol clefts, fibrosis (long arrow), multifocal necrosis (arrowhead) and mild mineralisation (short arrow). All pictures represent HPS stained slides at a magnification of 170X.

plaques on the other hand (Figure 4e and f) are presented. With either diet used, no signs of atherosclerosis were observed in non-transgenic littermates and in transgenic line #195 mice. With the LFC diet, causing severe hypertriglyceridemia but less severe hypercholesterolemia (Table II), mice of line #2 and #181 developed no or only very mild lesions. On the contrary, following HFC diets these lines developed severe atherosclerosis. An increase in the amount of cholate from 0.1% to 0.5% resulted in relatively more severe plaques per mouse. Line #181 mice, with the most severely elevated levels of cholesterol-rich VLDL and IDL/LDL-sized lipoproteins on both HFC diets, were more susceptible to the formation of severe atherosclerotic plaques than line #2 mice. No differences were observed between males and females with respect to atherosclerosis susceptibility.

DISCUSSION

Previously, we reported the generation of three different APOE*3-Leiden transgenic mouse lines (7). In the present study the effect of different semi-synthetic cholesterol-containing diets on lipoprotein profiles is evaluated, with special reference to both the level and composition of VLDL/LDL-sized lipoproteins, and on the development of atherosclerotic lesions. All three transgenic lines studied are more responsive to high fat/cholesterol feeding than to non-transgenic littermates (Table II). This responsiveness is positively correlated with the basal level of apoE3-Leiden, despite the presence of endogenous mouse apoE. The observation that the low expressor line #195 responded much less obviously to the different diets, indicates that a threshold level of human apoE3-Leiden is required for developing hyperlipidemia with cholesterol feeding. There is some evidence that the amount of apoE may become rate-limiting in the clearance of remnant lipoproteins: (i) the injection of apoE in rabbits has been shown to transiently reduce lipid levels (13); and (ii) overexpression of rat apoE in transgenic mice resulted in reduced lipid levels because of an increased clearance of VLDL and, consequently, resistance to diet-induced hypercholesterolemia (14,15). These facts taken together with our finding that overexpression of apoE3-Leiden leads to strongly elevated lipid levels, support our former conclusion that apoE3-Leiden behaves like a dominant trait in the expression of hyperlipoproteinemia in these transgenic mice, like in human beings. We hypothesize that on the surface of the remnant particles, apoE3-Leiden affects the proper conformation of the effective endogenous mouse apoE protein, providing the relative quantity of the apoE3-Leiden protein is high enough.

The semi-synthetic diets used in this study are comparable to the diets defined by Nishina et al. (10) and are the first synthetic mouse diets that lead, like some non-defined diets, to the development of atherosclerotic lesions in inbred mice. In addition, these diets resulted in minimal lipid accumulation in the liver and formation of gallstones, which is a prerequisite for proper studies regarding diet-induced hyperlipidemia. These diets contain sucrose (up to 50%, Table I) which has been shown before to increase, in man and some other animals, the production and secretion of VLDL triglyceride by the liver, resulting in hypertriglyceridemia

(16,17). This increased hepatic lipogenesis occurs can occur since fructose bypasses phosphofructokinase which is the rate-limiting enzyme in the glycolytic pathway (10). From the present study, it is obvious that the overproduction of VLDL having been fed sucrose also leads to hypertriglyceridemia in the APOE*3-Leiden transgenic mice, provided the transgene is expressed sufficiently (Table II).

Feeding high fat/cholesterol (HFC) diets resulted in severe hypercholesterolemia in high-expressing transgenic APOE*3-Leiden mice, whereas serum triglyceride decreased as compared to the levels obtained following the LFC diet (Table II). A lowering of triglyceride levels concomitant with an increase in serum cholesterol having been fed HFC feeding has been described before by LeBoeuf et al. (18,19) and Paigen et al. (20). This effect was more pronounced with higher concentrations of cholate in the diets (Table II), which is in agreement with the results reported by Nishina et al. (10). Cholate facilitates the emulsification of cholesterol and triglyceride and thereby their intestinal absorption. However, whether cholate itself is the direct cause of lowering the triglyceride levels is at present subject to speculation.

In the high-expressing lines #2 and #181 but not in the low-expressing line #195, cholesterol increments having been fed cholesterol also occurred in the HDL fraction, which is the result of the generation of relatively large and apoE-rich HDL particles (Figures 2 and 3 and Table II). The increase in these lipoproteins might be due to the fact that mice do not have cholesterol ester transfer protein (CETP) activity (21,22). Presumably, in normal mice and in line #195 these apoE-rich HDL particles will be cleared efficiently from the circulation through the LDL receptor with the endogenous mouse apoE as ligand, whereas in the high-expressing APOE*3-Leiden mice this receptor-mediated clearance is disturbed.

The increase in serum cholesterol mainly occurred in the VLDL and IDL/LDL-sized fractions, and correlated positively with the level of expression of the transgene (Table II). In addition, in the VLDL and IDL/LDL-sized fractions the cholesterol to triglyceride ratio increased following a HFC diet, especially at higher cholate concentrations in the diet, and concomitant with a redistribution of the apoE3-Leiden protein from HDL to this particular lipoprotein fraction (Figure 2, Table II). Thus, cholesterol and apoE-rich VLDL remnant lipoproteins accumulate in the serum with cholesterol feeding.

The accumulation of cholesterol- and apoE-rich remnant lipoproteins in the plasma also occurs in patients with familial dysbetalipoproteinemia and is considered to be an atherogenic condition (5). Although the number of examined animals per group was small and the method of measuring atherosclerotic lesions was only semi-quantitative, the development of atherosclerotic lesions in the APOE*3-Leiden transgenic mice appeared to be positively correlated with the serum level of cholesterol-rich VLDL/LDL-sized lipoproteins.

Advanced human atherosclerotic lesions (plaques) are characterized by (i) a fibrous cap, composed mostly of smooth muscle cells and connective tissue, (ii) a cellular area beneath the cap consisting of a mixture of macrophages and smooth muscle cells containing lipid droplets and (iii) a deeper necrotic core which contains cellular debris, extracellular lipid

droplets, cholesterol crystals and calcium deposits (23,24). Recently, it has been reported that homozygous *Apoe* deficient mice also develop extensive hyperlipidemia and atherosclerosis, even on a regular mouse diet (25-27). However, no evidence has yet been presented of cholesterol clefts, necrosis and mineralization in these mice following four weeks of dietary treatment. It is possible that this period of dietary treatment is too short for the development of progressed atherosclerotic lesions. However, the possibility that the absence of apoE protein on the surface of remnant particles leads to a different mechanism of generation of the lipid laden macrophages in the fatty streaks and, eventually, to a different plaque histology, may also not be excluded.

Our results clearly show that, after 14 weeks of cholesterol feeding, the advanced atherosclerotic lesions in the APOE*3-Leiden transgenic mice do resemble their human counterparts: in these mice the plaques contain lipid-filled foam cells, fibrosis, necrotic areas, cholesterol crystals and mineralization (Figure 4). In these mice plaques developed not only near the sinus valves but also in the carotid arteries and in other parts of the aorta, especially near sites of arterial bifurcations. We did not find any atherosclerotic lesion in non-transgenic littermates having been fed cholesterol. In contrast, Stewart-Philips and Lough (28) did observe atherosclerosis in non-transgenic C57BL/6J mice having been fed cholesterol. The discrepancy between their results and our findings could be due to: (i) the length of dietary treatment (35 versus 14 weeks), (ii) the fact that our non-transgenic littermates are not fully C57BL/6J inbred (F2 generation), and (iii) the fact that the lesions have been quantified using the hematoxyllin-phloxine-saffron (HPS) staining method. Although this method allows histological characterization of the lesions, it might be less suitable for detecting small fatty streaks as compared to the common lipid staining method with oil-red O.

We also did not find sex differences regarding the susceptibility to develop atherosclerotic lesions. This is in conflict with the results reported by Paigen et al (29) and Warden et al (30), showing that females are more susceptible to atherosclerosis than males. The absence of sex differences in our study might also be due to the rather semi-quantitative method of measuring atherosclerotic lesions and to the relatively small numbers of mice analysed.

We conclude that the APOE*3-Leiden transgenic mice present a suitable animal model system for studying the factors influencing the hepatic VLDL synthesis and their effect on the plasma level of atherogenic remnant lipoproteins. Moreover, since in these mice the plaque formation is reproducible and easy modifiable by administration of different diets and since plaque histology resembles that in humans, these mice may also be used for testing anti-atherosclerotic drugs.

ACKNOWLEDGEMENTS

We are grateful to Mr. Jaap van Rijn for animal care and Miss Inge de Bruijn for analyses of tail DNA. Drs. Chris Zurcher and Ko Willems van Dijk are gratefully thanked for helpful discussions. This research was supported by the Netherlands Heart Foundation (project #

88.086), the Netherlands Organisation for Scientific Research (project #900-504-092 and #900-539-117) and the Netherlands Royal Academy of Arts and Science (to M.H.H.).

REFERENCES

1. Havel, R.J., Y.-S. Chao, E. Windler, L. Kotite, and L.S.S. Guo. 1980. Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia. *Proc. Natl. Acad. Sci. USA.* 77:4349-4353.
2. Sherill, B.C., T.L. Innerarity, and R.W. Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. *J. Biol. Chem.* 255:1804-1807.
3. Mahley, R.W. 1988. Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science.* 240:622-630.
4. Mahley, R.W., T.L. Innerarity, S.C. Rall Jr., K.H. Weisgraber, and J.M. Taylor. 1990. Apolipoprotein E: genetic variants provide insight into its structure and function. *Curr. Opin. Lipid.* 1:87-95.
5. Mahley, R.W. and S.C. Rall Jr. 1989. Type III hyperlipoproteinemia (dysbetalipoproteinemia): The role of apolipoprotein E in normal and abnormal metabolism. *In* The Metabolic Basis of Inherited Disease. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1195-1213.
6. de Knijff, P., A.M.J.M. van den Maagdenberg, A.F.H. Stalenhoef, J.A. Gevers Leuven, P.N.M. Demacker, L.P. Kuyt, R.R. Frants, and L.M. Havekes. 1991. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *J. Clin. Invest.* 88:643-655.
7. van den Maagdenberg, A.M.J.M., M.H. Hofker, P.J.A. Krimpenfort, I.H. de Bruijn, B. van Vlijmen, H. van der Boom, L.M. Havekes, and R.R. Frants. 1993. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 268:10540-10545.
8. Simonet, W.S., N. Bucay, S.J. Lauer, D.O. Wirak, M.E. Stevens, K.H. Weisgraber, R.E. Pitas, and J.M. Taylor. 1990. In the absence of a downstream element, the apolipoprotein E gene is expressed at high levels in kidneys of transgenic mice. *J. Biol. Chem.* 265:10809-10812.
9. Simonet, W.S., N. Bucay, R.E. Pitas, S.J. Lauer, and J.M. Taylor. 1991. Multiple tissue-specific elements control the apolipoprotein E/C-1 gene locus in transgenic mice. *J. Biol. Chem.* 266:8651-8654.
10. Nishina, P.M., J. Verstuyft, and B. Paigen. 1990. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* 31:859-869.
11. Lopez-Virella, M.F., P. Stone, S. Ellis, and J.A. Colwell. 1977. Cholesterol determination in high-density lipoproteins separated by three different methods. *Clin. Chem.* 23:882-884.
12. Paigen, B., P.A. Holmes, D. Mitchell, and R.A. Williams. 1987. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 68:231-240.
13. Mahley, R.W., K.H. Weisgraber, M.M. Hussain, B. Greenman, M. Fisher, T. Vogel, and M. Gorecki. 1989. Intravenous infusion of apolipoprotein E accelerates clearance of plasma lipoproteins in rabbits. *J. Clin. Invest.* 83:2125-2130.
14. Shimano, H., N. Yamada, M. Katsuki, M. Shimada, T. Gotoda, K. Harada, T. Murase, C. Fukazawa, F. Takaku, and Y. Yakazi. 1992. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* 89:1750-1754.
15. Shimano, H., N. Yamada, M. Katsuki, K. Yamamoto, T. Gotoda, K. Harada, M. Shimada, and Y. Yazaki. 1992. Plasma lipoprotein metabolism in transgenic mice overexpressing apolipoprotein E. Accelerated clearance of lipoproteins containing apolipoprotein B. *J. Clin. Invest.* 90:2084-2091.
16. Grundy, S.M. and M.A. Denke. 1990. Dietary influences on serum lipids and lipoproteins. *J. Lipid Res.* 31:1149-1172.
17. Witztum, J.L. and G. Schonfeld. 1978. Carbohydrate diet induced changes in very low density lipoprotein composition and structure. *Diabetes.* 27:1215-1229.
18. Lusis, A.J., B.A. Taylor, D. Quon, S. Zollman, and R.C. LeBoeuf. 1987. Genetic factors controlling

structure and expression of apolipoproteins B and E in mice. *J. Biol. Chem.* 262:7594-7604.

19. LeBoeuf, R., D. Puppione, V. Schumaker, and A. Lusis. 1983. Genetic control of lipid transport in mice. I. Structural properties and polymorphisms of plasma lipoproteins. *J. Biol. Chem.* 258:5063-5070.
20. Paigen, B., A. Morrow, C. Brandon, D. Mitchell, P. Holms. 1985. Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis.* 57:65-73.
21. Agellon, L.B., A. Walsh, T. Hayek, P. Moulin, X.C. Jiang, S.A. Shelanski, and A.R. Tall. 1991. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J. Biol. Chem.* 266:10796-10801.
22. Jiang, X.C., L.B. Agellon, A. Walsh, J.L. Breslow, and A. Tall. 1992. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Depending on natural flanking sequences. *J. Clin. Invest.* 90:1290-1295.
23. Munro, J.M., and R.S. Cotran. 1988. Biology of disease. The pathogenesis of atherosclerosis: Atherogenesis and inflammation. *Lab. Invest.* 58:249-261.
24. Ishida, B.Y., and B. Paigen. 1989. Atherosclerosis in the mouse. *In* Genetic Factors in Atherosclerosis: Approaches and Model Systems. A.J. Lusis and S.R. Sparkes, editors. Karger, Basel. 189-222.
25. Plump, A.S., J.D. Smith, T. Hayek, K. Aalto-Setälä, A. Walsh, J.G. Verstuyft, E.M. Rubin, and J.L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* 71:343-353.
26. Zhang, S.H., R.L. Reddick, J.A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* 258:468-471.
27. Piedrahita, J.A., S.H. Zhang, J.R. Hagaman, P.M. Oliver, and N. Maeda. 1992. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc. Natl. Acad. Sci. USA.* 89:4471-4475.
28. Stewart-Phillips, J.L., and J. Lough. 1991. Pathology of atherosclerosis in cholesterol-fed, susceptible mice. *Atherosclerosis.* 90:211-218.
29. Paigen, B., P.A. Holmes, D. Mitchell, and D. Albee. 1987. Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALP/c, and C3H. *Atherosclerosis.* 64:215-221.
30. Warden, C.H., C.C. Hedrick, J.H. Qiao, L.W. Castellani, and A.J. Lusis. 1993. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science.* 261:469-472.

CHAPTER 3

QUANTITATIVE ASSESSMENT OF AORTIC ATHEROSCLEROSIS IN APOE*3-LEIDEN TRANSGENIC MICE AND ITS RELATIONSHIP TO SERUM CHOLESTEROL EXPOSURE.

Pieter H.E. Groot[1], Bart J.M. van Vlijmen[2], G. Martin Benson[1], Marten H. Hofker[3], Raymond Schiffelers[3], Martin Vidgeon-Hart[3], and Louis M. Havekes[1]

[1]Department of Vascular Biology, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn (Herts), United Kingdom, [2]TNO-Prevention and Health, Gaubius Laboratory, Leiden, and [3]Department of Human Genetics, Leiden, The Netherlands.

Reproduced from
Arteriosclerosis, Thrombosis and Vascular Biology, 1996, 16:926-933
by copyright permission of The American Heart Association

ABSTRACT

Transgenic mice, overexpressing the human dysfunctional apolipoprotein E variant, apoE3- Leiden develop hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. In the present study we investigated the the effects of diet composition and feeding period on serum cholesterol exposure and the amount of atherosclerosis in the aortic sinus in these mice, using quantitative image analysis. On each of the three diets tested -a low-fat diet, a high saturated fat plus cholesterol diet and a high saturated fat/high cholesterol/0.5% cholate diet- transgenic animals showed a marked hyperlipidemia as compared to non-transgenic littermates. Measurement of the atherosclerotic lesion areas in cross sections of the aortic sinus in animals exposed to these 3 diets for up to 6 months showed a 5 to 10 times greater lesion area in transgenic mice as compared to non-transgenic controls. Highly significant positive correlations were found between the log transformed data on lesion area and serum cholesterol exposure (r=0.82-0.85 for the 1, 2 and 3 months treatment groups), indicating that the hyperlipidemia is likely to be a major determinant in lesion formation. Based on these findings we suggest that the apo E3 Leiden mouse represents a promising model for intervention studies with hypolipidemic and anti-atherosclerotic drugs.

INTRODUCTION

Apolipoprotein E (apo E) is a major regulator of plasma lipoprotein metabolism functioning as a ligand for the receptor-mediated uptake of chylomicron- and very low density lipoprotein (VLDL) remnants as well as apoE-rich high density lipoprotein (HDL) (1,2). The importance of apo E is well illustrated in familial dyslipoproteinemia whose underlying cause is one of several mutations in the apo E gene, resulting in the production of dysfunctional apo E or its absense from plasma (3). One of those variants, APOE*3-Leiden is a tandem duplication of codons 120-126 in the apo E gene and the presence of a single allele for this mutation results in the expression of familial dyslipoproteinemia (4-6). The dominance of APOE*3-Leiden trait in expressing of this disease is probably due to the high affinity of the dysfunctional apo E3 Leiden over wild type apo E for triglyceride-rich lipoproteins (7). Recently we have described the development of a transgenic mouse carrying a transgene containing the APOE*3-Leiden and APO C-I genes plus the downstream regulatory elements for enhanced liver expression (8). APOE*3-Leiden mice were shown to develop hyperlipidemia which could be greatly enhanced by feeding a diet enriched in saturated fat, cholesterol and sodium cholate. In a subsequent study (9) an increased susceptibility for diet induced atherosclerosis was also demonstrated. This mouse model of hyperlipidaemia and premature atherosclerosis is of interest for investigating the influence of environmental and genetic factors on disease progression and for testing candidate hypolipidemic and anti-atherosclerotic drugs. The present report describes a detailed study

on the effect of diet composition and duration of exposure to these diets on the hyperlipidemia and on lesion area in the aortic root using color video image analysis.

METHODS

Mice

All animal procedures were approved by the institutional committees on animal experimentation of TNO-Prevention and Health and SmithKline Beecham. Transgenic mice expressing human APOE*3-Leiden and APOCI genes (line 2), were generated as described by van den Maagdenberg et al. (8). Microinjection of the transgene was performed in eggs from (C57BL/6JxCBA/J) F1 females that had been mated to males of the same genetic background and transgenic founder mice were bred with C57BL/6J mice to establish a transgenic strain. Subsequent generations were produced by mating male transgene carriers (identified with an ELISA for human apo E, (9)) with C57BL/6J females. Non-transgenic littermates were used routinely as controls. In the present study 132 females (66 trangenic and 66 non-transgenic) from the F7 generation were used. At the start of the study animals aged 8-10 weeks were allocated randomly to one of the experimental groups on the basis of age and litter and were housed in groups of up to 8 . Animals had free access to water and food.

Diets

Prior to the study, animals were kept on standard rat/mouse chow (SRM-A, Hope Farms, Woerden, The Netherlands). During the experimental period, animals were fed one of three semi-synthetic diets containing sucrose as the main energy source. These diets were essentially composed as described by Nishina et al (10,11) and were a low fat and cholesterol control diet (LFC), a cocoa butter (15%) and cholesterol (0.25%) enriched high fat/cholesterol diet (HFC) and a cocoa butter (15%), cholesterol (1%) and cholate (0.5%) enriched high fat/cholesterol diet (HFC/0.5%cholate) and were all formulated by Hope Farms. The composition is shown in Table I. Experimental diets were fed for up to 3 months (LFC and HFC/0.5% cholate diets) or 6 months (HFC diet) and all diets were well tolerated by the animals. Body weights were not significantly

Table I. Composition of the Semi-Synthetic Diets

Diet Components	Type of diet		
	LFC	HFC	HFC/0.5%
		g/100 g diet	
Cocoa butter	-	15	15
Cholate	-	-	0.5
Cholesterol	-	0.25	1
Sucrose	50.5	40.5	40.5
Cornstarch	12.2	10	10
Corn oil	5	1	1
Cellulose	5	5.95	4.7

In addition to the constituents given in the Table all diets contained 20% casein, 1% choline chloride (50% w/v), 0.2% methionine, and 5.1% vitamin and mineral mixture. Digestible energy contents of the diets were: low fat and cholesterol (LFC) diet, 3883 kcal/kg; high fat and cholesterol (HFC) diet, 4356 kcal/kg; high fat and cholesterol plus cholic acid (HFC/0.5% cholate) diet, 4356 kcal/kg.

different between diets at any of the time points tested. None of the animals was lost during the study.

Lipid and lipoprotein analysis.

Blood samples were taken from the tail under light diethylether anaesthesia after an overnight fast. Total serum cholesterol and triglyceride concentrations (without free glycerol) were measured enzymatically using commercial test kits 236691 from Boehringer Mannheim GmbH, Mannheim, Germany and and test kit 337-B from Sigma, St Louis , USA, respectively. For a more detailed analysis of the serum lipoprotein profiles Superose 6B column chromatography was used. Equal volumes of serum from all the animals in a group were pooled and 200 μl aliquots were analysed on a 25 ml Superose 6B column (Pharmacia AB, Uppsala, Sweden) eluted at a constant rate of 0.5 ml/min with PBS buffer (pH=7.4) using a HPLC pump system. Cholesterol concentrations in the lipoprotein fractions were analysed as described above.

Perfusion of hearts and aortas.

Mice were sacrificed by Nembutal anaesthesia and the thorax was opened. The heart and vascular tree were perfused in situ with oxygenated Krebs Ringer bicarbonate buffer at 37 °C under a pressure of approximately 120 cm of water through a cannula positioned into the left ventricle and an outlet created by cutting the lower vena cava. After 30 min, the buffer was replaced by neutral buffered formalin (3.7% formaldehyde, Formal-fix, Shandon Scientific Ltd, U.K.) at (37 °C) and the perfusion fixation continued for a further 30 min. Finally, the hearts and aortas were dissected out, cleaned of extravascular fat, and stored in neutral buffered formalin until they were processed.

Tissue preparation and sectioning of the aortic root.

The hearts were bisected just below the atria and the base of the hearts plus aortic root were taken for analysis. The tissue was washed three times over a period of 8 hours in fresh OCT liquid (Bayer Diagnostics, Germany) and then left overnight in OCT liquid. The following day, hearts were placed in fresh OCT liquid on a cryostat chuck (Bright Instrument Company Ltd., UK) with the aorta facing the chuck and frozen using dry ice. The hearts were then sectioned perpendicular to the axis of the aorta, starting within the heart and working in the direction of the aortic arch as described by Paigen et al (12). Once the aortic root was identified by the appearance of aortic valve leaflets, alternate 10 mm sections were taken and mounted on gelatinised slides. Sections were air dried for 1 hour and rinsed briefly in 60% isopropyl alcohol. Sections were then staining with oil red O, counter stained with Mayer's haematoxylin stain, cover slipped with glycerol gelatine and sealed with nail varnish. These sections were used for quantification of aortic atherosclerosis, as detailed below.

In some animals the perfused-fixed aortas were used to visualise and document atherosclerosis over the whole length of the blood vessel. To this end, aortas were cleaned of extravascular fat and stained for lipids with oil-red O. The presence of oil-red O positive atherosclerotic lesions was studied under a stereo microscope (Olympus, SZH-10) fitted with a 3 chip colour video camera (model HV-C10, Hitachi, Japan) and a video colour printer (Mavigraph, Sony, Japan) for documentation. Aortas were also opened longitudinally in order to study lesion morphology en face.

Quantification of atherosclerosis in sections of the aortic root.

Up to 40 sections of the aortic root per animal were imaged using an Olympus BH-2 microscope equipped with an 4x objective, neutral density (ND-6) and blue filters (KB-4) and a video camera (Hitachi, HV-C10). Twenty-four bit full colour images were acquired using a PC fitted with a framegrabbing board (MFG/3M/V, Data Cell, Ltd, Maidenhead, Berks, U.K.) and running Optimas software (version 4.02, Bioscan Inc. USA). The images were all captured under identical lighting, microscope, camera and PC conditions and were stored on optical discs (Panasonic, Matsushita, Japan) in tiff format. Quantification of atherosclerotic lesion areas in these cross sections (from here on referred to as lesion area) was performed using the Optimas software. We initially selected threshold values (red, green and blue) that discriminated between lesion and non-lesion areas using

the lesions in approximately 12 histological sections. Then, as we continued with the area measurements, we checked that the thresholds we had selected were suitable for each section by getting the image analysis program to lay a mask over the areas within our thresholds. The threshold values we had selected were considered to be satisfactory because the mask covered all of the lesioned areas. Extra vascular fat and other non-lesion areas also identified by the threshold setting were edited out before lesion areas were computed. Absolute values for the surface area of lesions were obtained by calibration of the software using the image of the grid on a haemocytometer slide. After the initial analysis of all sections in some animals (see Results section), we analysed routinely the first 10 sections in the direction of the aortic arch from the point where all three aortic valve leaflets first appeared.

Estimation of vascular exposure to serum cholesterol.

To investigate the possible relationship between lesion area and exposure of the arterial wall to increased concentrations of plasma cholesterol, cholesterol exposures were calculated for each animal. Exposures were defined as the areas under the curve (AUC's) in serum cholesterol verus time plots. All animals used in the study were bled only once. This was done at the time that the animals were sacrificed. To calculate the cholesterol exposure we made best estimates of serum cholesterol concentration for the preceeding month(s) for each individual animal. For this calculation it was assumed that an animal with a relatively high serum cholesterol at sacrifice at say 3 months in comparison with the other animals in its experimental group sacrificed at 3 months would be likely to have had a similarly high relative serum cholesterol concentration at 1 and 2 months. As mean serum cholesterols concentrations of groups of animals on the same diet at 1 and 2 months were available (from the animals that were killed at 1 and 2 months for lesion analysis), 1 and 2 months serum cholesterol concentrations in the 3 months group could be estimated for each individual animal and plots of serum cholesterol against period on diet for each animal constructed. From those plots the AUC's for each animal were calculated.

Statistical analysis.

Serum lipid data sets were analysed and as some showed a non-Gaussian distribution, comparisons between groups were routinely made by a non-parametric Mann-Whitney test, using RS/1 statistical software (BBN Software Products Corp., MA, USA).The statistical significance of differences in lesion area between control and transgenic mice was determined by a two-tailed unpaired Student t-tests.To ascertain whether there was any relationship between the cholesterol exposures and lesion areas, data were analysed after taking the possible differences between diets and the periods of killing into consideration. The Analysis of Variance technique requires that data be normally distributed and that variability be similar across groups. A plot of standard deviation versus arithmetic mean derived for each subgroup of 6 animals strongly indicated that for both cholesterol exposure and lesion area variability increased as the mean value increased. On a logarithmic scale, however, this relationship disappeared, hence both variables were transformed to logarithms. Residual plots performed following the Analysis of Variance further validated the above two assumptions. To explore the possible relationships between the log10(lesion area) data and log10(cholesterol exposure), period and diet, a full model with all possible interaction terms was first fitted and an analysis of variance was performed. Since all the interactions were highly insignificant (*P*-values $>> 0.05$), the data were then fitted with the main effects being diet and period and the covariate log10(cholesterol exposure). In this analysis the effect of diet was insignificant and hence diet was removed from the model and the data from all the dietary groups were combined. All these tests were performed without considering the data in the 6 months (period 6) group, because these animals were all fed with the same diet.An analysis of variance was again performed to investigate the interaction between $\log_{10}$ (cholesterol exposure) and period. The data from period 6 were included for this analysis. Here, the interaction term would test for the parallelism of the slopes of the linear associations for the periods, if any. The interaction was not statistically significant at 5% level of significance ($p > 0.05$) and so

it was dropped from the model. This result justified considering equal slopes for the four periods. The analysis of variance was then repeated without the interaction term. Here, the log10(cholesterol exposure) term indicated a statistical significance (P=0.0001) and there were statistically significant differences between the four periods (P=0.0230). The significant effect for period signifies different intercepts for the regression lines for the four periods. Pairwise tests between periods showed that the 1 month group was significantly different from 2, 3 and 6 month groups, P=0.0094, 0.0075, 0.0094 respectively. However, there was no statistical significance for the differences among periods 2, 3 and 6 months. Therefore, the 2, 3 and 6 months groups were pooled and tested against period 1. The results of this ANOVA again showed significant difference (P=0.0036) thus justifying fitting lines with same slope but with different intercepts for the one month group and the combined 2,3 and 6 month group.

RESULTS

Effects of different diets on plasma lipoprotein concentrations

The effects of a switch from normal mouse diet to one of three semi-synthetic diets on serum cholesterol and triglyceride concentrations in female APOE*3-Leiden mice and their non-transgenic control littermates are shown in Figures 1 and 2 respectively. Experimental diets were chosen to simulate a Western type diet (HFC) or a more severely atherogenic diet (HFC/0.5% cholate) and compared with the control diet (LFC). All diets contain sucrose as

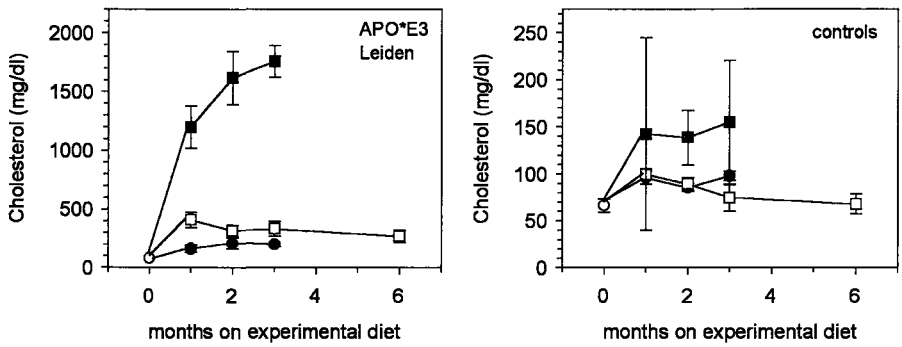


*Figure 1. Effects of diet and feeding period on serum cholesterol concentrations in APOE*3-Leiden mice and non-transgenic controls.* Prior to the study all mice were fed a standard chow diet (SRM-A diet). APOE*3-Leiden mice (Fig. 1A) and non-transgenic controls (Fig. 1B) were then switched to one of 3 semi-synthetic diets as specified in Table I and fed these diets for periods of up to 3 months (LFC and HFC/0.5% cholate diets) or 6 months (HFC diet). At the timepoints indicated in the Figure, animals were bled and sacrificed for lesion analysis. Data points represent the mean serum cholesterol concentration ± S.D. for 6 animals. The statistical significance of differences in serum cholesterol between control animals and apo E3 Leiden transgenics as well as between animals before and one month after the switch to the experimental diets were determined by the non-parametric Mann-Whitney tests. Serum cholesterol concentrations in transgenic animals (Fig 1A) were all higher than those in the corresponding groups of non-transgenic controls (Fig. 1B, P<0.005 for all corresponding data points). Following the switch from SRM-A diet to any of the 3 experimental diets (comparison of the 0 month data with the 1 month data) serum cholesterol in all groups increased significantly (P<0.005). Symbols: Symbols: ○, SRM-A diet; ●, LFC diet; □, HFC diet; ■, HFC/0.5% cholate diet. Note the difference in scale between Fig 1A and B. Abbreviations: SRM-A diet: standard rat/mouse chow; LFC: low fat and cholesterol diet; HFC: high fat plus cholesterol diet; HFC/0.5%:cholate, high fat and high cholesterol diet, supplemented with 0.5% cholate.

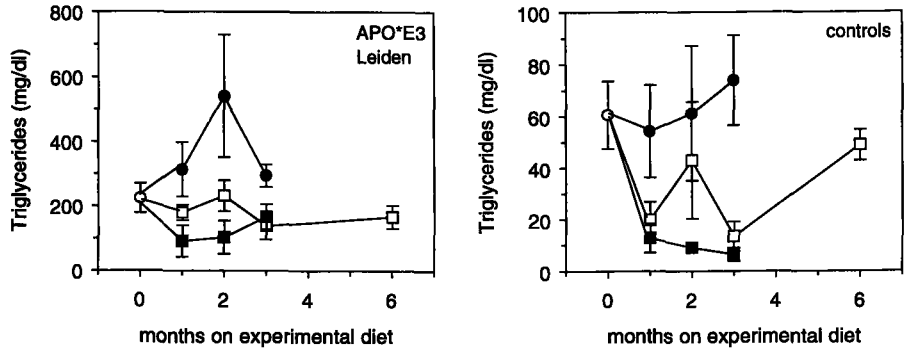


*Figure 2. Effects of diet and feeding period on serum triglycerides concentrations in APOE*3-Leiden mice and non-transgenic controls.* For details of the dietary treatments and statistics see Fig 1. Fig 2A, data from APOE*3-Leiden mice; Fig. 2B, data from non-transgenic controls. Serum triglyceride concentrations in transgenic animals (Fig. 2A) were all higher than those in the corresponding controls (Fig 2B) with $P<0.005$ for all corresponding data point. Following the switch from SRM-A diet to the experimental diets (comparison of 0 month and 1 month data) triglyceride concentrations increased in the APOE*3-Leiden mice on LFC diet ($P<0.005$) but decreased in non-transgenic and APOE*3-Leiden mice on HFC and HFC/0.5% cholate diets ($P<0.005$). Symbols: ○, SRM-A diet; ●, LFC diet; □, HFC diet; ■, HFC/0.5% cholate diet. Note again the difference in scale between Fig 2A and B. Abbreviations: SRM-A diet: standard rat/mouse chow; LFC: low fat and cholesterol diet; HFC: high fat plus cholesterol diet; HFC/0.5%:cholate, high fat and high cholesterol diet, supplemented with 0.5% cholate.

the major source of energy and are based on formulations developed by Nishina et al.(10, 11) to limit the side effects (liver damage and gall stone formation) seen with traditional atherogenic mouse diets of chow, supplemented with fat, cholesterol and bile salts. The effects of the experimental diets in the present study was investigated for a period of up to 3 months (diets LFC and HFC/0.5% cholate) or 6 months (diet HFC). Compared to a standard mouse diet all three semi-synthetic diets increased serum cholesterol concentrations in transgenic mice as well as control animals while serum triglyceride concentrations were decreased rather than increased on the HFC and HFC/0.5% cholate diets. On all three semi-synthetic diets and at all time points tested, serum lipids were many fold (2-13x) higher in the APOE*3-Leiden mice than in the corresponding control groups, differences being the most prominent on the HFC/0.5% cholate diet (compare Fig 1A and 1B for cholesterol and Fig 2A and 2B for triglycerides). All differences in serum lipids between APO *E3 Leiden and non-transgenic control mice were highly statistically significant. The distribution of lipids among the different lipoprotein classes was analysed by Superose 6B column chromatography on pools of serum from animals in the same experimental group. Lipoprotein profiles of animals after 2 months of feeding these experimental diets are shown in Fig. 3. Control animals on the LFC and HFC diet carry most of their serum cholesterol in the HDL fraction. On the same diets, transgenic mice showed a marked increase in cholesterol associated with VLDL/LDL sized fractions which accounted for more than 50% of total plasma cholesterol in these animals. The shifts in the profiles were more pronounced in animals on the HFC diet than on the LFC diet. On the HFC/0.5% cholate diet, the differences between the control

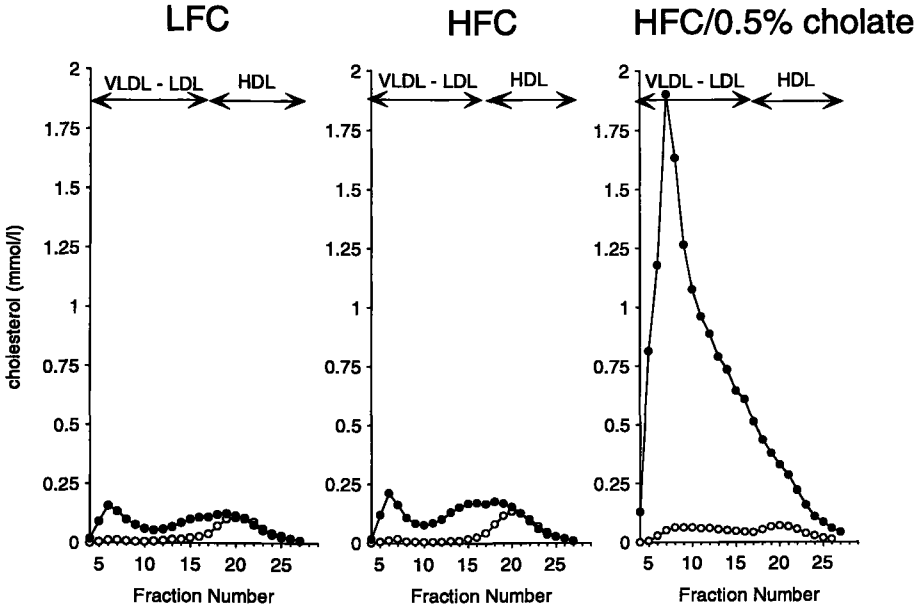


*Figure. 3 Serum lipoprotein cholesterol distribution in APOE*3-Leiden mice and non-transgenic controls fed one of 3 experimental diets for a period of 2 months.* Pooled serum samples(200 µl, 6 animals per group) were fractionated by Superose 6B FPLC column chromatography as described in Methods and fractions were analysed for cholesterol content. VLDL to LDL sized particles are found in fractions 4-16, HDL-sized particles in fraction 17-27. Symbols: ●, APOE*3-Leiden mice; ○, non-transgenic controls. Abbreviations; LFC, low fat and cholesterol diet; HFC, high fat and cholesterol diet; HFC/0.5% cholate, high fat and cholesterol plus 0.5% cholate diet.

and transgenic animals were even more outspoken. In control animals this diet raised cholesterol associated with the VLDL/LDL sized fraction several fold but in the transgenic animals increases were massive. Although FPLC analysis of hyperlipidaemic serum does not result in a complete separation of HDL from VLDL/LDL, the profiles in Fig 3 (at least for the LFC and HFC diets) indicate that HDL concentrations in transgenic animals on these diets are similar or higher when compared to control animals on the same diet. Similar lipoprotein distribution profiles as shown in Fig 3 were found in the corresponding 1, 3 and 6 months groups (data not shown).

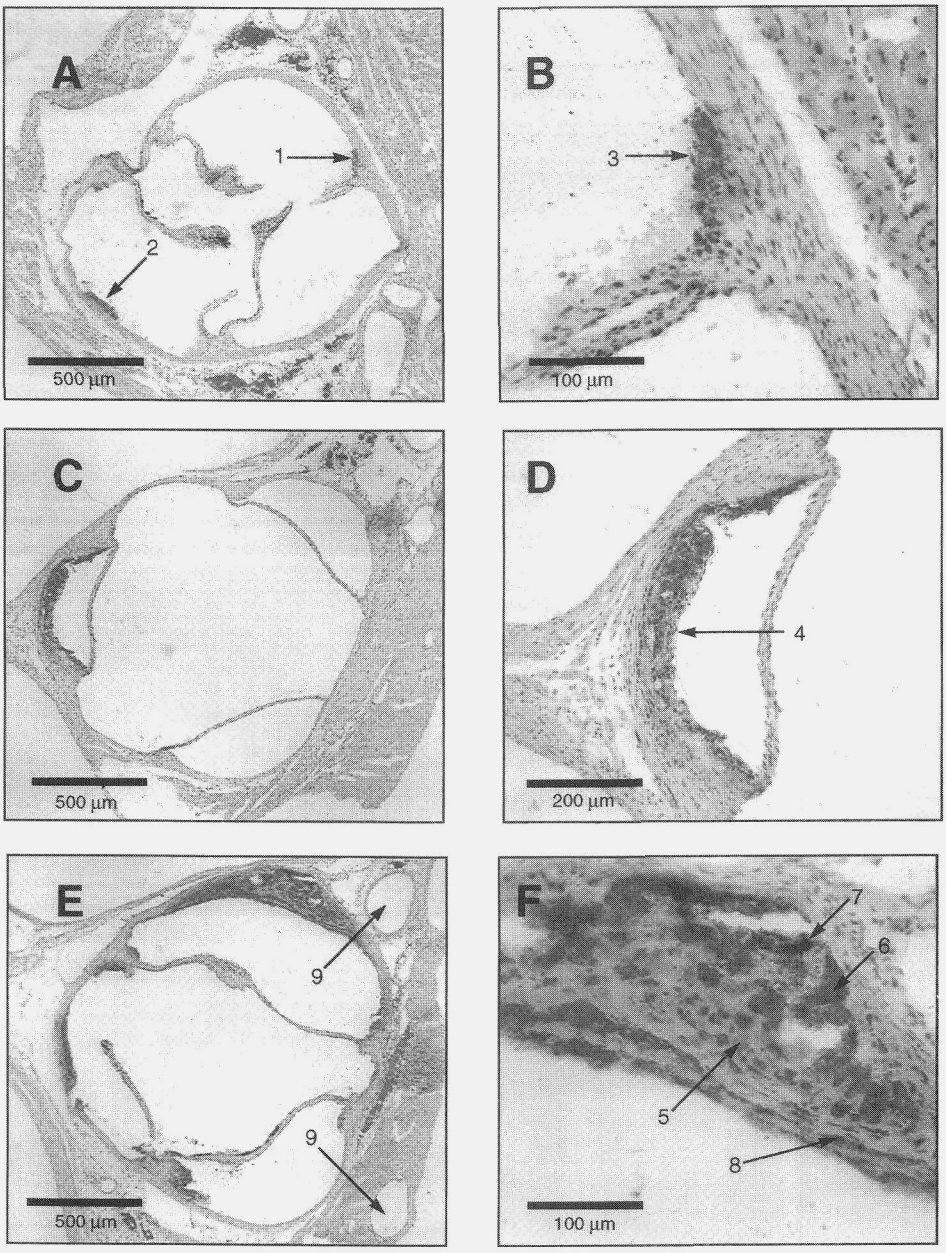
Effect of different diets on the development of atherosclerosis in the aortic sinus.

On all diets tested, APOE*3-Leiden transgenic mice showed enhanced aortic atherosclerosis compared with non-transgenic controls. Dramatic differences were seen in lesion development between transgenic animals fed the HFC/0.5% cholate, HFC and LFC diets. Atherosclerosis in control animals was minimal except in those fed the HFC/0.5% cholate diet for 2 and 3 months. Photomicrographs of various stages of lesion development

in cross sections of the aortic root are shown in Fig 4 A to F. Using the nomenclature used by Qiao et al (13), early lesions in all groups were mainly of the type I (oil-red O-positive plaques, related to aortic valves, that is on valve attachments, valve cusps and valve residuals) although type II lesions (oil-red O- positive lesions on the free aortic wall) were also seen. Early type I lesions consisted of small, sometimes intensely oil-red O stained plaques (arrows 1 and 3 in Fig 4 A and B, APOE*3-Leiden mice on HFC diet for 2 months). These early lesions are rather superficial and contain a single or double layer of lipid-laden foam cells. Early type II lesions are very similar in appearance. An example of an early type II lesion is also seen in Fig 4 A (arrow 2). The early type I and II plaques develop into more extensive lesions where the entire arterial wall within the cusp is covered (Fig 4 C and D, APOE*3-Leiden mice on HFC diet for 3 months). Those lesions are raised and rich in lipid-laden foam cells underlying an apparently intact layer of endothelial cells (arrow 4 in Fig 4 D). Interestingly, extensive lesion development can sometimes be seen in a single cusp while the other cusps in the same section are totally unaffected (see Fig 4C). In transgenic mice, exposed to the atherogenic HFC/0.5% cholate diet for 3 months even more complex lesions are seen (Fig 4 E and F). These lesions often have a core that stains intensively with oil-red O and a cap of several layers of spindle-shaped cells (Fig 4 F). Extracellular lipid dominates in the core (arrow 6 in Fig 4 F) but lipid-laden foam cells are also present, both in the core as well as among the spindle-shaped cells in the cap. The core of some of these more complex lesions contain areas that stain purple with haematoxylin, suggesting the presence of calcification (Qiao et al, (13), arrow 7 in Fig 4 F). Many of the complex lesions are very friable as plaques easily shear (e.g. the white area to the left of arrow 7 in Fig 4 F)and even detach from the aortic wall during sectioning. In none of the animals, even after 3 month on the HFC/0.5% cholate diet, were lesions seen in the proximal coronary arteries (arrow 9 in Fig 4 E) but lesions were present in discrete areas in the ascending aorta, aortic arch and descending aorta. Fig 5 A and B show the presence of these lesions in the inner curvature of the aortic arch and branch points with the carotid arteries. Most of these lesions are rather superficial and represent early fatty streaks, although raised lesions eg at the branch point of carotid arteries were also seen. No lesions were present in non-transgenic littermates exposed to the same dietary regimen (Fig 5 C) as compared with APOE*3-Leiden transgenics (Fig 5 D).

Quantification of lesion area.

Atherosclerotic lesions were quantified in cross sections of the aortic roots of all animals using video image analysis as described in the Methods section. Initially, all sections from the animals on the HFC/0.5% cholate diet were imaged and analysed. As shown in Fig. 6, APOE*3-Leiden mice on this diet developed lesions reasonably uniformly over the whole of this part of the aorta. Based on these results, it was decided to analyse all the other animals in the study using sections 1 to 10 only. The results of this analysis are shown in Fig 7. Massive difference were seen in lesion areas between animals on LFC, HFC and HFC/0.5%


A
1
2
500 µm
B
3
100 µm
C
500 µm
D
4
200 µm
E
9
9
500 µm
F
7
6
5
8
100 µm

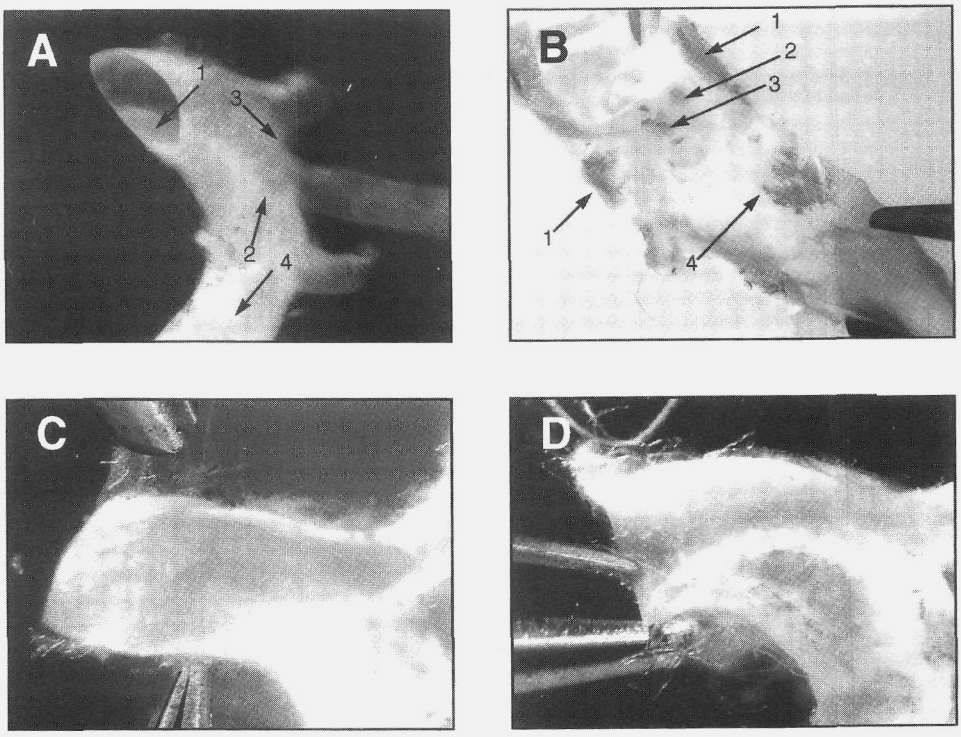


*Figure 5. Atherosclerosis in the aortic arch of APOE*3-Leiden mice.* A) photomicrograph of the oil-red O stained aortic arch of an APOE*3-Leiden mouse, fed the atherogenic HFC/0.5% cholate diet for 3 month. B) photomicrograph of the same aorta as shown in Fig 5A, after opening the aorta longitudinally along the inner curvature of the arch. The same lesions in Fig 5A and B carry the same numbers. C) and D) en face view of the aortic arch of a control mouse and an APOE*3-Leiden mouse respectively after feeding the the high fat and cholesterol plus 0.5% cholate (HFC/0.5% cholate) diet for 3 month.

*Figure 4. Photomicrographs of cross sections of the aortic root of APOE*3-Leiden transgenic mice.* All slides were stained with oil-red O and hematoxilin. A) early lesions in the aortic root of an APOE*3-Leiden mouse, fed the HFC diet for 2 month. Note the early type I lesion in the valve attachment area (arrow 1) and a flat type II lesion on the wall of the aorta in the valve cusp (arrow 2). B) high magnification of the valve attachment lesion in Fig 4A). Note the presence of lipid-laden foam cells (arrow 3). C) raised valve cusp lesion in the aortic root of an APOE*3-Leiden mouse, fed a HFC diet for 3 month. D) higher magnification of the lesion shown in Fig 4 C). Note the presence of abundant lipid-laden foam cells, under an apparently intact endothelium (arrow 4). E) severe lesion in the aortic root of an APOE*3-Leiden mouse, fed the atherogenic HFC/0.5% cholate diet for 3 months. F) higher magnification of the lesion shown in Fig 4E. Note the presence of many lipid-laden foam cells (arrow 5) as well as large amounts of extracellular lipid in the core of the lesion (arrow 6). Also note the dark blue stained deposits (arrow 7), indicative for calcification. (arrow 7) and oil red O-positive spindle shaped cells (arrow 8). Note also that the proximal coronary arteries do not show any sign of lesion development (arrow 9). Abbreviations; LFC, low fat and cholesterol diet; HFC, high fat and cholesterol diet; HFC/0.5%cholate, high fat and cholesterol plus 0.5% cholate diet.

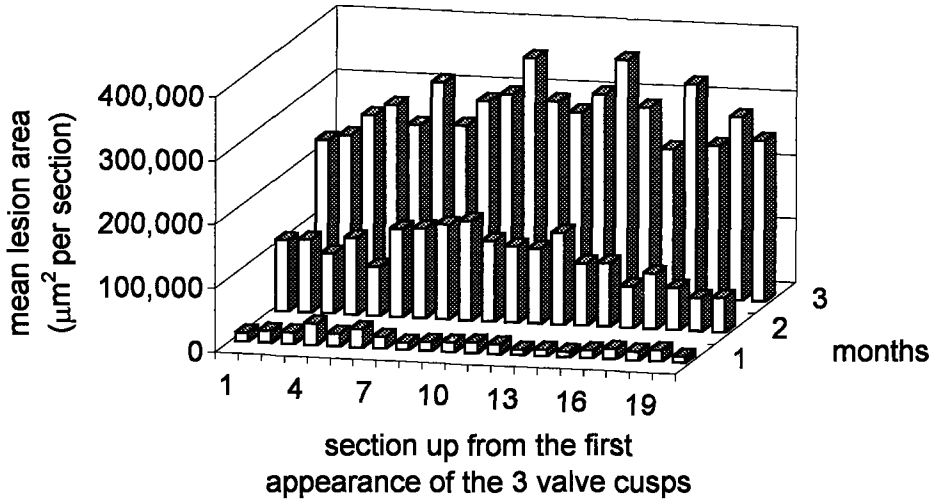


*Figure 6. Mean lesion area in the aortic root (y-axis) as a function of section number (x axis) and months exposure of APOE*3-Leiden mice to the high fat and cholesterol plus cholic acid (HFC/0.5% cholate) diet. (z axis).* Section 1 is the first cross section of aorta where all three valve leaflets were seen. Section number increases in the direction of the aortic arch and covers a travel distance of 400 μm. Data are the mean of 6 animals. For further details see the Methods section.

cholate diet (note the difference in scale of the y-axis in Fig 7 A, B and C). On control LFC diet, atherosclerotic lesions were small and variable. Only at 2 months were differences between non-transgenic and transgenic animals significantly different. On the HFC diet a clear time-dependent increase in lesion area was seen in the transgenic mice and differences with control animals were large (5-10x) and statistically significant (except for the 3 month time point where inter-animal variation in the transgenic group was particularly large). On the HFC/0.5% cholate diet, a time-dependent increase in lesion area was again observed, especially in transgenic mice, the average levels being about 10 times higher than in the corresponding groups on the HFC diet. Inter-animal variability in all groups was appreciable.In order to determine the relationship between plasma cholesterol levels and lesion area in APOE*3-Leiden mice, cholesterol exposures were calculated for each animal as described in the Methods section and plotted against the mean area of plaque measured. As discussed in that section, data required a $\log_{10}$ transformation and were analysed by ANOVA. The results of this analysis are given in Fig 8. Impressive correlations were found in each of the 1, 2, and 3 month subgroups (r=0.82 to 0.85) and pairwise testing indicated that the data of the 2, 3 and 6 month groups could be combined while those of the 1 month group showed a somewhat different relationship. Assuming a linear log/log relationship,the data could be best described by two closely parallel regression lines, one for the 1 month group (y=1.32+2.34x, r=0.82) and one for the combined 2,3 and 6 months groups (y=0.72+2.34x, r=0.84). (The inability to fit the one month data on the same regression

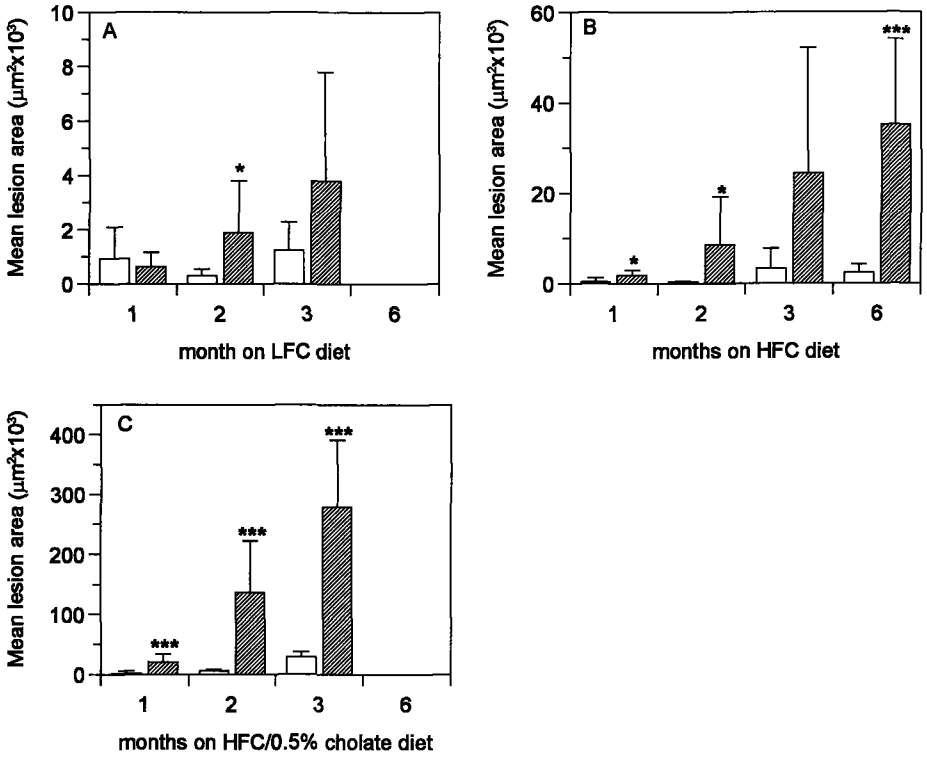


*Figure. 7. Mean lesion area per section ± SD as a function of the number of months on the experimental diets of APOE*3-Leiden trangenic mice and their non-transgenic controls.* The open bars and hatched bars represent non-transgenic- and APOE*3-Leiden transgenic animals respectively. A) bargraph for animals on diet LFC. B) bargraph for animals on diet HFC. C) bargraph for animals on diet HFC/0.5% cholate. Symbols: *) ,**) and ***), statistically significant difference between non-transgenic and APOE*3-Leiden transgenic mice with $P<0.05$; $P<0.01$ and $P<0.001$ respectively. Abbreviations; LFC, low fat and cholesterol diet; HFC, high fat and cholesterol diet; HFC/0.5% cholate, high fat and cholesterol plus 0.5% cholate diet.

line as the combined 2, 3 and 6 months groups may be due to an underestimation of the cholesterol exposure in the former group as serum cholesterol may not increase linearly during the first month, as assumed in the AUC calculation, but faster). These interesting results indicate that in APOE*3-Leiden transgenic mice plasma cholesterol concentrations and the duration of hyperlipidemia are major determinants of plaque formation.

DISCUSSION

Previously, we have shown that transgenic mice, carrying the dysfunctional human APOE*3-Leiden gene, develop hyperlipidemia and premature atherosclerosis (8,9). In the present study we performed a quantitative analysis of the effects of duration of exposure to

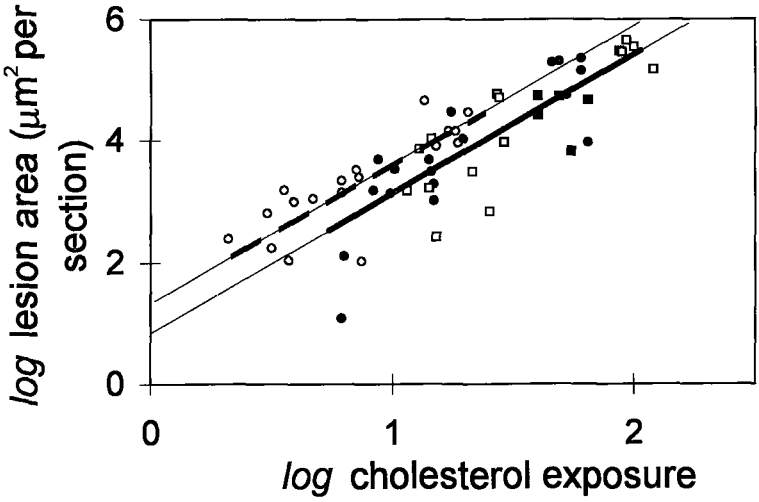


*Figure. 8. X/Y scatter plot of log transformed data of cholesterol exposure (x-axis) and lesion area of APOE*3-Leiden transgenic mice (y-axis), exposed to different diets.* Animals sacrificed after 1, 2 ,3 and 6 month are indicated with a ○, ●, □ and ■, respectively. Hatched line: best fit linear regression line for the 1 month dietary treatment group ($y=1.32+2.34x$, $r=0.82$). Solid line: best fit linear regression line for the combined 2, 3 and 6 months dietary treatment groups ($y=0.72+2.34x$, $r=0.84$).

different diets on the development of atherosclerotic lesions in these animals. Compared with non-transgenic littermates on the same regimen, APOE*3-Leiden mice show a 5 to10 times increased lesion area when fed a HFC or HFC/0.5% cholate diet. On both these diets, a clear phenotype is also seen in the hypercholesterolemic response with serum cholesterol concentrations being increased 5 to 10 fold those of their non-transgenic littermates and mainly confined to cholesterol associated with the VLDL/LDL sized lipoprotein fractions. A clear phenotype was also seen in VLDL/LDL cholesterol concentrations in mice on the LFC diet but the hyperlipidemia was mild compared with the HFC and HFC/0.5% cholate diet and this LFC dietary regimen did not result in a consistent significantly increased lesion area over the 3 month period.

Morphological analysis of the lesions showed a pattern, seen in many other transgenic mice models with increased susceptibility for atherosclerosis. Early aortic lesions were seen in the valve cusps and valve attachments and these lesions progress into raised complex lesions with an extracellular lipid core covered by a cap after longer exposure to atherogenic diets. On the HFC/0.5% cholate diet, distinct fatty streak like lesions were also seen in the inner curvature of the aortic arch and at branch points of major arteries in the APOE*3-Leiden transgenics but not in their non-transgenic littermates.

Comparison of the phenotype in this transgenic mouse model with that of related models

is of interest. Several groups, including ours, have generated mice in which the *Apoe* gene has been silenced by gene targeting and these *Apoe* knockout mice were shown to develop severe hyperlipidemia and atherosclerosis, even when fed a normal chow diet (15-18). Heterozygous *Apoe* knockout mice, when fed an atherogenic diet, were also shown to have hyperlipidemia and an increased susceptibility for atherosclerosis, demonstrating that the subnormal expression of the *Apoe* gene also leads to a (mild) phenotype (17). Based on inhouse experience with all three models, APOE*3-Leiden mice are more susceptible for atherosclerosis than the heterozygous *Apoe* knockout mouse. For comparison, we previously showed (17) that female heterozygous *Apoe* knockout mice on the HFC/0.5% cholate diet for 3 month developed lesions with a surface area of 61,000 μm^2 per cross section as compared to 270,000 μm^2 in APOE*3-Leiden mice, found in the present study. However, APOE*3-Leiden mice were less susceptible than the homozygous *Apoe* knockout mouse, which develops lesion even on a normal chow diet. In contrast to *Apoe* knockout mice, APOE*3-Leiden mice have the ability to synthesise functional endogenous apo E and although this apo E may not be very effective as a ligand on triglyceride-rich lipoproteins for uptake via lipoprotein receptors (presumably due to competition with the dysfunctional APOE*3-Leiden, 6,7) other functions may not be affected. For instance, lipid-laden macrophages synthesise large quantities of apo E (19) and this process may be associated indirectly or directly with cholesterol efflux from these cells (19-22). This process presumably is still functional in APOE*3-Leiden mice but not in *Apoe* knockout mice. In this context, two other murine models, the bone marrow transplanted *Apoe* knockout mice (23) and mice carrying an apo E transgene under control of a promotor that stimulates expression in cells of the arterial wall (24), are of major interest. In the first model, restoration of apo E synthesis in monocyte-derived macrophages by bone marrow transplantation decreases the susceptibility of these mice for atherosclerosis. However, this effect may not be solely be due to stimulated cholesterol efflux from arterial macrophages as this intervention also decreases serum lipids, presumably due to apo E synthesis by liver fixed macrophages (Kupffer cells) and consequently stimulated hepatic uptake of VLDL and chylomicron remnants. In the second model a tissue specific overexpression of apo E in arterial tissue was induced by introduction of an apo E transgene under the control of a H2 Ld promotor. These transgenic mice showed a decreased susceptibility for diet-induced atherosclerosis as compared to non-transgenic controls but no differences in serum lipids, supporting the concept of a direct protective effect of locally synthesised apo E on arterial lesion development. We therefore postulate that the less severe atherogenic phenotype of APOE*3-Leiden mice as compared to homozygous *Apoe* knockout mice is at least partly due to their unaltered ability to synthesise functional apo E in extrahepatic cells including vascular macrophages.

After our initial report on the APOE*3-Leiden mouse (8), Fazio et al (25) described another transgenic mouse model, overexpressing a human dysfuctional human APOE gene (APO*E Arg112,Cys142). These mice also developed hyperlipidemia and an increased susceptibility for diet-induced atherosclerosis. Whether the phenotype of those mice is similar

or differs from that found in the APO*E 3 Leiden remains to be seen.

The data, collected in the present study allowed us to address the question of whether the level of hyperlipidemia and the period of exposure of the vascular tree to the hyperlipidemia are indeed important determinants for lesion area. Previously, Nishina et al (11) have shown in C57BL/6J mice on various lipid-rich diets that the lesion area is positively correlated with the ratio of VLDL+LDL to HDL cholesterol and negatively with HDL cholesterol concentrations. However, no correlation was found between lesion area and plasma cholesterol and/or triglycerides. Studies in WHHL rabbits (14) have also failed to show a clear relationship between serum cholesterol and the severity of aortic atherosclerosis. In contrast, in the present study a strong positive correlation was observed between the vascular exposure to plasma cholesterol in APOE*3-Leiden transgenics and lesion area in the aortic root. As differences in serum cholesterol between animals on different diets is mainly localized in the VLDL to LDL fraction, it is tempting to speculate that this fraction is responsible for the observed correlation. It should be noted that the lesion areas found in the present study in APOE*3-Leiden transgenics span a far greater range (0-450,000 μm^2) than in Nishina's studies (0-4000 μm^2) underlining the significance of the present finding. As HDL cholesterol concentrations were not specifically measured in the present study and are difficult to determine from Superose 6B profiles with dominant VLDL/LDL contributions, a possible negative relation between lesion area and exposure to HDL cholesterol could not be assessed.

Transgenic mice with a susceptibility for diet induced hyperlipidemia and atherosclerosis are potentially useful small animal models for the testing of hypolipidemic and anti-atherosclerotic agents. In the light of the excellent correlation between cholesterol exposure and lesion area, we postulate that the APOE*3-Leiden mice may be particular useful in that respect. Studies along this line are presently in progress.

ACKNOWLEDGEMENTS

Miss H. Belinda van 't Hof is thanked for expert technical assistance and Mr Amit Bhattacharyya is greatfully acknowledged for support in the statistical analysis. Dr Nigel Toseland is thanked for advise and reading the manuscript.

This research was supported by the Netherlands Heart Foundation and the Netherlands Organisation for Scientific Research (projects #900-504-092 and 900-539-117).

REFERENCES

1. Havel RJ., Chow YS., Windler E, Kotite L, and Guo LSS. Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathophysiology of familial dysbetalipoproteinemia. *Proc. Natl. Acad. Sci USA* 1980; 77: 4349-4353.
2. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988; 240: 622-630.

3. Mahley RW., Innerarity TL, Rall SC, Weisgraber KH, and Taylor JM. Apolipoprotein E: genetic variants provide insight into its structure and function. *Curr. Opin. Lipid.* 1990; 1:87-95.
4. Havekes L., de Wit E, Gevers Leuven J, Klasen E, Utermann G, Weber W, and Beisiegel U. Apolipoprotein E3 Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. *Hum. Genet.* 1986; 73: 157-163.
5. van den Maagdenberg AM., de Knijff P, Stalenhoef AFM, Gevers Leuven J, Havekes LM, and Frants RR. Apolipoprotein E3 Leiden allele results from a partial gene duplication in exon 4. *Biochem. Biophys. Res. Commun.* 1989; 165: 851-857.
6. Wardell MR., Weisgraber KH, Havekes LM, and Rall SC. Apolipoprotein E3 Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121 to 127. *J. Biol. Chem.* 1989; 264:21205-21210.
7. Fazio SY., Horie KH, Weisgraber KH, Havekes LM, and Rall SC. Preferential association of apolipoprotein E Leiden with very low density lipoproteins of human plasma. *J. Lipid Res.* 1993; 34: 447-453.
8. Van den Maagdenberg, AMJM., Hofker MH, Krimpenfort PJA, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM and Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 1993; 268: 10540-10545
9. Van Vlijmen BJM., van den Maagdenburg AMJM, Gijbels MJJ, van den Boom H, HogenEsch H, Frants RR, Hofker MH, and Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice *J. Clin. Invest.* 1994; 93: 1403-1410.
10. Nishina PM., Verstuyft J, and Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J.Lipid Res.* 1990; 31, 859-869.
11. Nishina PM., Lowe S, Verstuyft J, Naggert JK, Kuypers FA, Paigen B. Effects of dietary fats from animal and plant sources on diet-induced fatty streak lesions in C57Bl/6J mice. J. Lipid Res. 1993; 34: 1413-1422.
12. Paigen BA., Morrow A, Holmes PA, Mitchell D, and Williams RA. Quantitative assessment of atherosclerosis in mice. *Atherosclerosis* 1987; 68: 231-240.
13 Qiao J-H, Xie P-Z, Fishbein MC, Kreuzer J, Drake TA, Demer LL, and Lusis AJ. Pathology of atheromatous lesions in inbred and genetically engineered mice. *Arteriosclerosis and Thrombosis* 1994; 14, 1480-1497.
14. Shiomi M., Ito T, Shiraishi M, and Watanabe Y. Inheritability of atherosclerosis and the role of lipoproteins as risk factors in the development of atherosclerosis in WHHL rabbits: risk factors related to coronary atherosclerosis are different from those related to aortic atherosclerosis. *Atherosclerosis* 1992; 96, 43-52.
15. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyf JG, Rubin EM, and Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice, created by homologous recombination. *Cell* 1992; 71: 343-353.
16. Zhang S, Reddick RL, Piedrahita JA, and Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992; 258: 468-471.
17. Van Ree JH., van den Broek WJAA, Dahlmans VEH, Groot PHE, Vidgeon-Hart M, Frants RR, Wieringa E, Havekes LM, and Hofker MH. Diet-induced hypercholesteroemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis* 1994; 111: 25-37.
18. Nakashima Y., Plump AS, Raines EWR, Breslow JL, and Ross R. Apo E-deficient mice develop lesions in all phases of atherosclerosis throughout the arterial tree *Arteriosclerosis and Thrombosis* 1994; 14: 133-140.
19. Basu SK., Goldstein JL, and Brown MS. Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science* 1983; 219: 871-873.
20. Rosenfeld ME., Butler S, Ord VA, Lipton BA, Dyer CA, Curtiss LK, Palinski W and Witztum JL. Abundant expression of apolipoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arteriosclerosis and Thrombosis* 1993; 13: 1382-1389
21. Schmitz G, Robenek H, Krause R, Schurek A, and Niemann R. Ca ++ antagonists and ACAT inhibitors promote cholesterol efflux from macrophages by different mechanisms. I. Characterization of cellular lipid metabolism. *Arteriosclerosis* 1988; 8: 46-56.
22. Kruth HS, Skarlatos SI, Gaynor PM and Gamble W. Production of cholesterol-enriched nascent high density lipoproteins by human monocyte-derived macrophages is a mechanism that contributes to

macrophage cholesterol efflux. *J. Biol. Chem.* 1994; 269: 24511-24518.
23. Linton RF., Atkinson JB, and Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science* 1995; 267: 1034-1037.
24. Shimano H., Ohsuga JO, Shimada M, Namba Y, Gotoda T, Harada K, Katsuki M, Yazaki Y, and Yamada N. Inhibition of diet-induced atheroma formation in transgenic mice expressing apolipoprotein E in the arterial wall. *J. Clin. Invest.* 1995; 95: 469-476.
25. Fazio S, Sanan DA, Lee Y-L, Ji Z-S, Mahley RW and Rall SC. Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E (arg112, cys 142). *Arteriosclerosis and Thrombosis* 1994; 14: 1873-1879.

CHAPTER 4

MODULATION OF VERY LOW DENSITY LIPOPROTEIN PRODUCTION AND CLEARANCE CONTRIBUTES TO AGE- AND GENDER-DEPENDENT HYPERLIPOPROTEINEMIA IN APOLIPOPROTEIN E3-LEIDEN TRANSGENIC MICE.

Bart J.M. van Vlijmen[1], H. Belinda van 't Hof[1], Marc J.T.M. Mol[2], Hans van der Boom[1], André van der Zee[3], Rune R. Frants[3], Marten H. Hofker[3], and Louis M. Havekes[1]

[1]TNO Prevention and Health, Gaubius Laboratory, Leiden; [2]Department of Medicine, Division of General Internal Medicine, University Hospital Nijmegen, Nijmegen; [3]Department of Human Genetics, Leiden University, Leiden, The Netherlands.

Reproduced from
The Journal of Clinical Investigation, 1996, 97:1184-1192
by copyright permission of
The American Society for Clinical Investigation

ABSTRACT

Apolipoprotein E3-Leiden (APOE*3-Leiden) transgenic mice have been studied to identify factors modulating chylomicron and VLDL remnant lipoprotein metabolism. Transient elevated levels of VLDL/LDL sized lipoproteins occurred in these mice with maximal levels during the period of rapid growth (optimum at 45 days of age). After about 100 days of age, serum cholesterol and triglyceride levels stabilized to slightly elevated levels as compared to control mice. The expression of the APOE*3-Leiden transgene was not age-dependent. In young mice the in vivo hepatic production of VLDL-triglycerides was 50% increased as compared to older mice. This is sustained by in vivo VLDL-apoB turnover studies showing increased (75%) VLDL-apoB secretion rates in young mice, whereas the VLDL-apoB clearance rate appeared not to be age-dependent.

On a high fat/cholesterol (HFC) diet, females displayed significantly higher cholesterol levels than males (10 versus 7.0 mmol/l, respectively). Serum levels of VLDL/LDL sized lipoproteins increased upon adminstration of estrogens, whereas administration of testosterone gave the opposite result. As compared to male mice, in female mice the hepatic VLDL-triglyceride production rate was significantly elevated. Injection of estrogen in males also resulted in increased VLDL-triglyceride production, although not statistically significant. In vivo VLDL-apoB turnover experiments showed that the VLDL secretion rate tended to be higher in females. Although, the fractional catabolic rate of VLDL-apoB is not different between males and females, administration of estrogens in males resulted in a decreased clearance rate of VLDL, whereas administration of testosterone in females resulted in an increased clearance rate of VLDL. The latter presumably due to an inhibiting effect of testosterone on the expression of the APOE*3-Leiden transgene.

We conclude that hyperlipidemia in APOE*3-Leiden transgenic mice is strongly affected by age via its effect on hepatic VLDL production rate, whereas gender influences hyperlipidemia by modulating both hepatic VLDL production and clearance rate.

INTRODUCTION

In Western society people spend most of their life-span under non-fasting conditions. As a result, there is a continuous release into the circulation of newly synthesized intestinal and hepatic lipoproteins i.e. chylomicrons and VLDL lipoproteins. Normally, the remnants of these lipoproteins are rapidly removed from the circulation via receptor mediated uptake in the liver. Apolipoprotein E (apoE) is a structural component of these remnant lipoproteins and serves as a ligand in the receptor mediated uptake by the liver (1,2). In patients suffering from familial dysbetalipoproteinemia (FD), a defective apoE leads to impaired hepatic uptake of remnant lipoproteins (1). The consequent accumulation of these lipoproteins leads to a predisposition of these patients to coronary and/or peripheral atherosclerosis (1,3).

Several *in vivo* and *in vitro* studies have demonstrated the unequivocal role of apoE in

remnant lipoprotein metabolism (4,5). However, in homozygous carriers of the mutant APOE*2(Arg158→Cys) allele, the presence of binding defective apoE2 does not explicitly result in the accumulation of remnant lipoproteins. Obviously, other environmental or genetic factors interfere with remnant metabolism (1).

In humans, heterozygosity for the rare mutant APOE*3-Leiden allele is also associated with an accumulation of remnant lipoproteins due to an impaired remnant clearance, despite the presence of normal apoE (6). Thus, in carriers of the apoE3-Leiden mutation, FD is inherited in a dominant fashion although also in this case, additional environmental and genetic factors do modulate the severity of the disease.

Because of heterogeneity in environmental and genetic background, these additional factors can hardly be studied in man. Therefore, to elucidate subtle factors that may modulate the metabolism of remnant lipoproteins, a suitable experimental animal model is required. Experimental animals, like inbred mice and rats, efficiently remove remnant lipoproteins, even under severe dietary stress conditions (7), and are thus less suitable for these studies. Recently, we reported that transgenic mice expressing the human APOE*3-Leiden mutation also demonstrate a hyperlipidemic phenotype (8). Furthermore, in these mice severe hyperlipidemia was observed after feeding high fat/cholesterol containing diets which positively correlated with the level of expression of the transgene (9). In addition, on high fat/cholesterol containing diets the high expressing lines developed atherosclerotic lesions of which the severity was positively correlated with serum levels of cholesterol-rich remnant lipoproteins. Thus, also in these transgenic mice apoE3-Leiden behaves like a dominant trait in the accumulation of remnant lipoproteins.

We reasoned that APOE*3-Leiden mice display a defect in plasma clearance of remnant lipoproteins which allows us to study the effect of more subtle factors involved in remnant metabolism under highly standardized conditions. The present study shows that in APOE*3-Leiden transgenic mice the plasma level of VLDL remnants is strongly influenced by age and gender related factors. Age appeared to affect the hepatic VLDL production rate, whereas gender modulates both the VLDL production and clearance rate.

METHODS

Animals

Transgenic mice of line #2, heterozygous for the expression of the human APOE*3-Leiden and human APOC1 gene were used (8). Transgenic and non-transgenic littermates were obtained by breeding with C57BL/6J mice (The Broekman Institute bv, Someren, The Netherlands). Mice of the F5 and F6 generation, were included in the trials. Transgenic mice were identified by sandwich ELISA for the presence of human apoE in the serum (method see below). Immediately after weaning (at age 25 days) mice were equally divided into two groups, consisting of female and male transgenic or non-transgenic mice. One group of mice was fed a regular mouse diet (SRM-A: Hope Farms, Woerden, The Netherlands), the other group of mice was fed a semi-synthetic high fat/cholesterol (HFC) diet. This HFC diet is a basic semi-synthetic diet, which was composed essentially according to Nishina et al. (7), supplemented with cocoa butter (15%, by weight) and cholesterol (0.25%, by

weight) and was purchased from Hope Farms. Mice were housed under standard conditions in conventional cages and given free access to food and water. None of the transgenic or non-transgenic mice were lost during the study.

Lipid and lipoprotein analysis

After a 12 hour fasting period, mice were weighted and approximately 50 μl of blood was obtained from each individual mouse through tail-bleeding. Blood samples were taken at different time points as indicated in the Figures for both diets. Total serum cholesterol and triglyceride levels (without measuring free glycerol) were measured enzymatically using commercially available kits: #236691 (Boehringer Mannheim, Germany) and #337-B (Sigma, Mo.).

For size fractionation of lipoproteins, some 200 μl of pooled serum (from at least 12 mice), was injected onto a 25 ml Superose 6B column (Pharmacia, Uppsala, Sweden) connected to a HPLC pump system and eluted at a constant flow rate of 0.5 ml/min with phosphate-buffered saline (pH 7.4). The effluent was collected in 0.5 ml fractions. Cholesterol and triglyceride concentrations in lipoprotein fractions were measured enzymatically, using kits #236691 and #701904 (Boehringer Mannheim, Germany), respectively.

Human ApoE3-Leiden protein measurements

Human apoE concentrations were measured by sandwich ELISA. Affinity purified polyclonal goat anti-human apoE antibodies were used for coating. Affinity purified polyclonal rabbit anti-human apoE antibodies were used as second antibodies. Thereafter, the plates were incubated with swine anti-rabbit IgG antibodies conjugated to horse radish peroxidase (HRP). Finally, HRP detection was performed using HRP substrate tetramethylbenzidine. Pooled plasma from healthy human subjects with known apoE level, was used as a standard.

Human ApoE and mouse Ldlr mRNA measurements in the liver

Total RNA was isolated from liver using the RNAZOL procedure (Cinna/Biotecx, Houston, TX). RNA samples (10 μg per lane) were separated by electrophoresis through a denaturing agarose gel (1.2% w/v) containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond N+, Amersham) according to the manufacturer's recommendations. For analysis of the mouse *Ldlr* expression, polyA$^+$ RNA was isolated using the PolyATtract mRNA isolation kit (Promega, Madison, WI). Blots were subsequently hybridized with a ^{32}P-labelled probes of human APOE cDNA (10), mouse *Ldlr* cDNA (mLDLRc90) (11) and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA at 55°C in a solution containing 50% formamide. The intensity of the hybridization signal was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amounts of APOE*3-Leiden and LDL receptor mRNA were related to the level of GAPDH mRNA.

Hormone treatment

HFC fed, male and female transgenic mice, fourteen weeks of age were injected subcutaneously along the back every 14 days with testosterone decanoate or estradiol decanoate dissolved in arachis oil. Blood sampling and serum analysis were performed before and after 28 days of hormone treatment as described above. Estradiol decanoate and testosterone decanoate were a kind gift from Drs H. Verheul and F. Deckers, Organon International bv., Oss, The Netherlands.

In vivo hepatic triglyceride production

After a 12 hour fasting period mice were injected intravenously with Triton WR1339 (500 mg/kg body weight) (12) using 15% (wt/vol) Triton solution in 0.9% NaCl. At 0, 45 and 90 minutes after injection blood samples were drawn and analyzed for triglycerides as described above. Production of hepatic triglyceride was calculated from the slope of the curve and expressed as mmol/hr/kg body weight. For calculation of mouse total serum volume we used the equation: serum volume = 0.33 x body weight (13).

Isolation and labeling of VLDL

Blood was collected from 6-8 fasted transgenic or 15-20 fasted non-transgenic mice. Sera were pooled and ultracentrifuged to obtain the VLDL fraction ($d < 1.006$ g/ml). VLDL cholesterol and triglyceride were determined enzymatically as described above, and VLDL protein was determined using the method of Lowry (14). To determine apoB content of the VLDL, some 25 μg of VLDL protein was subjected to a 4-20% gradient SDS-PAGE. After staining with Coomassie Brilliant Blue R and destaining in 30% methanol/10% acetic acid the gels were scanned using a HP ScanJet Plus, Hewlett Packard, Santa Clara, CA. and the amount of apoB(100+48) relative to total protein was calculated. The percentage of total apoB ranged from 20 to 40% of total VLDL protein. VLDL was radiolabeled with ^{125}I by the iodine monochloride method (15). The fraction of ^{125}I-label present in apoB was determined by isopropanol precipitation (16,17) and ranged from 30 to 65%.

In vivo removal of ^{125}I-labeled VLDL-apoB

Fasted mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 10 μg of ^{125}I-labeled VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t=1, 3, 5, 10, 30, 60 and 90 minutes after injection. The serum content of ^{125}I-labeled apoB was measured by isopropanol precipitation followed by counting ^{125}I-label. A bi-exponential model was used to estimate the area under the ^{125}I-apoB decay curve and subsequent calculation of VLDL-apoB kinetics. VLDL secretion rate was calculated from VLDL fractional catabolic rate and serum apoB pool. Since more than 85% of serum apoB and triglycerides were associated with the $d < 1.006$ lipoprotein fraction, we determined the serum apoB pool by measuring serum triglyceride levels 48 hours prior to injection and the apoB to triglyceride ratio in the isolated $d < 1.006$ lipoproteins (VLDL) of mice from the same group (autologous). Serum triglycerides on 48 hours prior to injection were approximately 20% higher as compared to serum triglyceride levels at the end of the turnover experiment. However, this decreasing effect was similar for all mice injected.

RESULTS

Effect of age, gender and diet on serum lipid and lipoprotein levels.

The increase in body weight with age for male and female transgenic and non-transgenic mice is shown in Figure 1. No difference in growth rate was observed between transgenic and control mice and between both diets administered. At an early age, the gain in body weight was more rapid than at later age and more pronounced for male than for female mice.

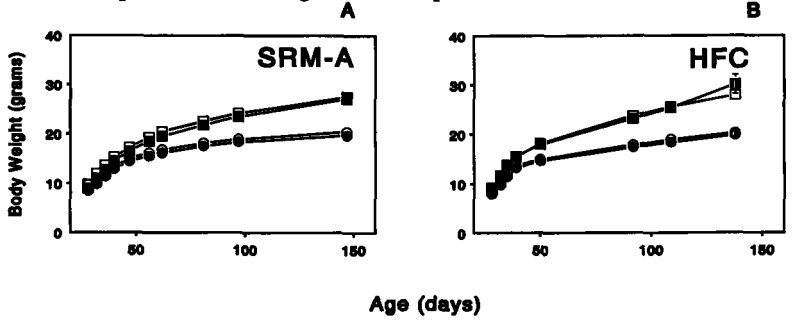


Figure 1. Age and sex dependent body weight. Body weight changes due to the increase in age was followed over time in male (squares) and female (circles) mice fed a SRM-A diet (panel A) or a high fat/cholesterol (HFC) diet (panel B). All groups contain at least 13 mice, which were weaned at day 25 $\pm$ 1. Body weight was determined after an overnight fast. Values are expressed as mean $\pm$ S.E.(error bar not visible when S.E. < symbol size). Black symbols, APOE*3-Leiden transgenic mice; open symbols, control mice.

Figure 2 (panels A-C) shows the effect of age on serum cholesterol (panel A), triglyceride (panel B) and the apoE3-Leiden protein levels (panel C) for SRM-A fed mice. At weaning, transgenic and non-transgenic animals showed comparable levels of serum cholesterol and triglycerides. Transgenic animals showed a rapid significant increase ($P<0.01$) in both serum cholesterol and triglycerides with a maximum around 45 days of age. This post-weaning increase was more pronounced for male than for female transgenic mice. Serum apoE3-Leiden levels also displayed a significant increase ($P<0.01$) in levels directly after weaning. However sex differences were not observed during this period. At an early age non-transgenic animals also showed an increase for both serum cholesterol and triglyceride levels, however this increase was less pronounced as compared to transgenic animals.

The increases at an early age in transgenics in levels of cholesterol, triglycerides and apoE3-Leiden appeared to be transient. After 150 days of age, serum cholesterol and triglycerides had decreased to almost basal levels of about 2.5 and 2.0 mmol/l, respectively.

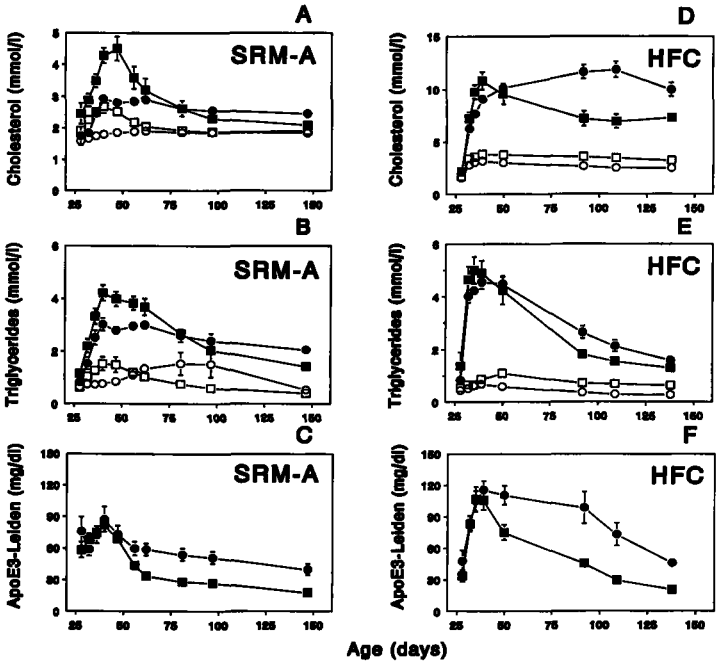


Figure 2. Age and sex dependent serum cholesterol, triglycerides and apoE3-Leiden levels. Mice were fed a SRM-A diet (panel A-C) or a HFC diet (panel D-F). Serum cholesterol (panel A and D), triglycerides (panel B and E) and apoE3-Leiden levels (panel C and F) were followed in time in male (squares) and female (circles) APOE*3-Leiden and control mice. All groups contain at least 13 mice, which were weaned at day 25 $\pm$ 1. Blood samples were drawn by tail bleeding after an overnight fast. Vertical bars represent standard error of the means. Statistical analysis was performed using the repeated measuring ANOVA to determine the influence of age and gender on cholesterol, triglycerides and apoE3-Leiden levels. Black symbols, APOE3-Leiden mice, open symbols, control mice.

At this age females had higher levels of cholesterol and triglycerides than males. In addition, females showed significantly higher apoE3-Leiden levels than males (40 vs 18 mg/dl).

Figure 2 (panel D-F) shows the serum cholesterol, triglyceride and apoE3-Leiden levels after feeding a cholesterol-rich HFC diet. The time course profiles for serum triglycerides (panel E) resembled those observed after regular SRM-A feeding (panel B), whereas after feeding HFC the respective levels of apoE3-Leiden (panel F) were about twice as high as during SRM-A feeding (panel C). At an early age, serum cholesterol levels (panel D) were 2 to 3-fold higher as compared to SRM-A feeding. In contrast to SRM-A feeding, HFC feeding led to prolonged elevated levels of cholesterol, although for males a slight decrease in serum cholesterol levels to about 7.0 mmol/l was observed at a later age.

Non-transgenic animals at an early age also showed increased levels of cholesterol and triglycerides after a feeding HFC diet, but to a much smaller extent as compared to transgenic animals. In contrast to transgenic mice, non-transgenic male mice at a later age had slightly higher lipid levels as compared to non-transgenic females.

Figure 2 suggests that the effect of HFC feeding on lipid levels at an early age is a result of both age, as observed during SRM-A feeding, and an additional dietary component. To study the effect of HFC feeding on serum lipid levels solely, mice of 200 days of age and fed a SRM-A diet were switched to the HFC diet. As illustrated in Figure 3A, transgenic animals displayed an increase in serum cholesterol levels but less pronounced than at an early age (as shown in Figure 2), and again the females were more responsive than the males (upto

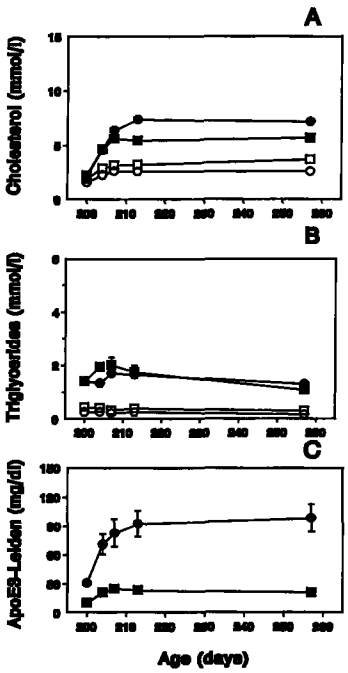


Figure 3. The effect of HFC feeding at a later age. Serum cholesterol (panel A), triglycerides (panel B) and apoE3-Leiden (panel C) were followed in time for male (squares) and female (circles) mice which switched from SRM-A diet to HFC at 200 days of age. All groups contains at least 13 mice. Blood samples were drawn by tail bleeding after an overnight fast. Vertical bars represent standard error of the means. Statistical analysis was performed using the repeated measuring ANOVA to determine the influence of age and gender on cholesterol, triglycerides and apoE3-Leiden levels. Black symbols, APOE3-Leiden mice; open symbols, control mice.

7.5 and 5.5 mmol/l, respectively). Remarkably, serum triglycerides (panel B) showed only a small and transient increase, whereas ApoE3-Leiden levels (panel C) strongly increased from 7 to 26 and from 30 to 90 for males and females, respectively. In non-transgenics, serum cholesterol levels were much less severely elevated after feeding HFC diet at a later age, whereas serum triglyceride levels were not affected at all.

The results described above, clearly indicate that serum lipids in transgenic mice are affected by age, nutrition and gender. To investigate in which lipoprotein classes these changes in serum lipids mainly occur, we analyzed lipoprotein profiles by FPLC gelpermeation chromatography using a Superose 6B column and calculated the distribution of cholesterol among VLDL/LDL and HDL-sized lipoproteins, respectively. Feeding both a SRM-A or HFC diet, the major changes in serum cholesterol were confined to the VLDL/LDL-sized fractions. In addition, the higher serum cholesterol levels in females as compared to males during the diets at a later age are also confined to this lipoprotein fraction (results not shown).

We further investigated the effect of age and gender on hyperlipoproteinemia in the APOE*3-Leiden mice, separately. For investigating the effect of age we used only young (45 days of age) and older (>100 days of age) SRM-A fed male mice, since the effect of age on serum lipid levels was more pronounced in male than in female mice. For studying the effect of gender we used older male and female mice (>100 days) fed a HFC diet, since the effect of gender on hyperlipoproteinemia was more pronounced in older than in young mice.

Effect of age on VLDL metabolism in vivo.

Our previous study has shown that the level of hyperlipidemia corresponds to the expression level of the APOE*3-Leiden gene (8,9). To study whether the transient accumulation of VLDL/LDL sized lipoproteins at an early age on either diet is due to transient higher expression levels of the transgene, we determined APOE*3-Leiden mRNA in the liver by Northern blotting. Livers of mice with maximum hyperlipidemia (45 days of

*Table I. Age and Diet Dependent APOE*3-Leiden mRNA Expression in Mouse Liver.*

Sex	Age	SRM-A	HFC
	d	%	
Males	45	100.0 ± 19.0	118.2 ± 12.6
	>100	80.9 ± 5.6	117.2 ± 24.7

APOE*3-Leiden RNA in liver was quantified for young (45 days of age) and old male mice (>100 days of age). After an overnight fast, mice were anesthesized, liver was excised and APOE*3-Leiden RNA was quantified as described in methods section. Mice were fed SRM-A diet or HFC diet. APOE*3-Leiden RNA concentrations are relative to internal standard GAPDH and expressed as a percentage of male (45 days of age) SRM-A fed group. Values are the mean ± S.D. of 3 to 5 mice. Values are not statistically different ($P<0.05$; using nonparametric Mann-Whitney test)

age) were compared to older mice (> 100 days of age). From the results presented in Table I, it is obvious that no significant differences in E3-Leiden mRNA levels in the liver were observed between young and old male mice when fed a SRM-A diet. Feeding HFC diet resulted in slightly but not significantly elevated levels of E3-Leiden mRNA as compared to SRM-A feeding. These results indicate that the age-dependent serum lipid and apoE3-Leiden protein levels were not related to changes in expression of the transgene.

To investigate whether the effect of age on serum levels of VLDL/LDL-lipoproteins was related to changes in endogenous VLDL production by the liver we determined the *in vivo* hepatic VLDL-triglyceride production rate. Therefore, young male mice of 45 days of age and old male mice (> 100 days of age), both transgenic and non-transgenic, were injected with Triton WR1339 and the increase in serum triglycerides after injection was determined. As presented in Table II, VLDL-production rates were significantly affected by age, young male mice displaying about 50% higher VLDL-triglyceride production rate than older mice irrespective of the presence of the APOE*3-Leiden transgene.

Table II. Age Dependent In Vivo Hepatic VLDL-Triglyceride Production

	VLDL-triglyceride production rate	
Age	non-transgenics	transgenics
d	*mmol/hr/kg body wt*	
45	0.180 ± 0.021*	0.150 ± 0.011*
>100	0.112 ± 0.017	0.102 ± 0.018

After an overnight fast SRM-A fed male mice were injected with Triton WR1339 (see Methods section). The serum triglycerides were determined just prior to injection (0 min.) and at 45 and 90 min. following Triton injection. Production of hepatic triglyceride rate was calculated from the slope of the curve and is expressed as mmol/hr/kg body weight. Values are expressed as mean ± S.D. (n=4). *$P<0.05$, indicating the difference between mice of 45 days of age and mice of >100 days of age, using nonparametric Mann-Whitney test.

To investigate whether the effect of age on serum levels of VLDL/LDL-lipoproteins was also due to changes in VLDL clearance rate, *in vivo* VLDL turnover studies were performed. Young male transgenic and non-transgenic mice of 45 days of age and old male transgenic mice (>100 days of age), were injected with 10 μg of autologous ^{125}I-labeled VLDL, and the ^{125}I-apoB disappearance from the circulation was determined. As illustrated in Figure 4A and presented in Table III, VLDL fractional catabolic rate (VLDL-FCR) was clearly affected by transgene expression. Young transgenic mice displayed a significant lower VLDL-FCR as compared to young non-transgenic mice. In contrast, no effect of transgene expression on VLDL secretion rate (VLDL-SR) was observed, indicating that the higher serum lipid levels in transgenic mice as compared to non-transgenic mice were due to an impaired VLDL clearance rate, as expected.

An age-related change in VLDL-FCR was not observed as young and old transgenic mice

Table III. Age Dependent VLDL-apoB Fractional Catabolic Rates and Secretion Rates

Mice	Age	FCR	SR
	d	*pools/hr*	*μg apoB/hr/g body wt*
non-transgenics	45	3.94 ± 1.22	3.67 ± 1.81
transgenics	45	1.45 ± 0.34*	4.24 ± 0.95
transgenics	>100	2.00 ± 1.23	2.45 ± 1.05‡

FCR, fractional catabolic rate; SR, secretion rate. After an overnight fast SRM-A fed male mice were injected with 10 μg of autologous ^{125}I-labeled VLDL protein. ^{125}I-apoB disappearance from the circulation was determined and FCR and SR were calculated (see methods section). Values are expressed as mean ± S.D. (n=7). *P<0.05, indicating the difference between transgenic and non-transgenic mice of age 45 days, ‡P<0.05, indicating the difference between transgenic mice of 45 days of age and >100 days of age, using nonparametric Mann-Whitney test.

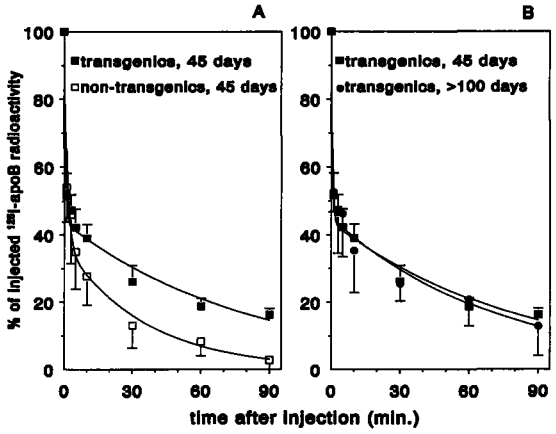


Figure 4. The effect of age on VLDL-apoB removal. SRM-A fed male mice were injected with autologous ^{125}I-labeled VLDL. 25 μl of blood was drawn at each time point and ^{125}I-apoB radioactivity of the serum sample was measured. Values are the mean ± S.D. of seven mice. Curves were calculated from the mean data using a bi-exponential curve fit model.

displayed comparable VLDL-FCR (Figure 4B and Table III), In contrast, a strong and significant effect of age on VLDL-SR is observed (Table III, 4.24 versus 2.45 μg apoB/hr/g body weight in young and old transgenic mice, respectively). This decreasing VLDL-SR with age corresponds with the observed lower hepatic VLDL-triglyceride production rate as determined by the Triton method (Table II).

Effect of sex hormones on serum lipid levels and on VLDL metabolism in vivo

Maximal gender related differences in lipid levels in APOE*3-Leiden mice were observed at a later age (>100 days) after feeding HFC. Thus, to investigate these gender related differences, older mice on an HFC diet were treated with either testosterone or estradiol. Mice were injected subcutaneously either a physiological or a pharmacological dose

*Table IV. Serum Lipid Concentrations after 4 Weeks of Hormone Treatment of APOE*3-Leiden Transgenic Mice*

Sex	Treatment	TC	TG
		mmol/liter	
Males	Placebo	6.20 ± 1.20	2.37 ± 0.39
	Estradiol decanoate (20 μg/mouse)	5.47 ± 0.66	3.06 ± 0.60*
	Estradiol decanoate (100 μg/mouse)	6.71 ± 0.32	4.57 ± 0.65*
	Testosterone decanoate (250 μg/mouse)	5.45 ± 0.32	2.49 ± 0.65
	Testosterone decanoate (1250 μg/mouse)	5.18 ± 0.75	2.66 ± 0.44
Females	Placebo	9.46 ± 1.50	3.36 ± 0.79
	Estradiol decanoate (20 μg/mouse)	10.70 ± 1.72	4.62 ± 0.64*
	Estradiol decanoate (100 μg/mouse)	10.69 ± 1.58	5.09 ± 0.73*
	Testosteron decanoate (250 μg/mouse)	8.10 ± 1.01	2.47 ± 0.55
	Testosteron decanoate (1250 μg/mouse)	6.64 ± 1.15*	2.26 ± 0.33*

TC, total cholesterol; TG, triglyceride. Total cholesterol and triglyceride values are the mean serum levels±SD of five APOE*3-Leiden transgenic mice per group. *$P < 0.05$, indicating the difference between hormone and placebo treated groups of mice of the same sex, using nonparametric Mann-Whitney tests.

of either hormone. As presented in Table IV, in male mice, treatment with estradiol resulted in a significant increase in serum triglyceride levels, whereas treatment with testosterone decreased serum cholesterol levels, although not significantly. Also for female mice, treatment with estradiol resulted in significantly increased serum triglyceride levels, whereas treatment with a pharmacological dose of testosterone significantly decreased both serum cholesterol and triglyceride levels.

To investigate in which lipoprotein classes these changes in serum lipid levels mainly occur, we generated lipoprotein profiles by FPLC gelpermeation chromatography using Superose 6B. In male mice (Figure 5, panel A) treatment with estradiol resulted in a slight dose dependent decrease of HDL cholesterol, whereas an increase of cholesterol in the VLDL-sized lipoprotein fractions is observed. Thus, leading to an unaffected total serum cholesterol level as presented in Table IV. The decrease in serum cholesterol and triglycerides levels observed after testosterone treatment of female mice was confined to VLDL/LDL sized-lipoprotein fractions (Figure 5, panel B).

To investigate whether changes in lipid levels after hormone treatment were initiated by changes in transgene expression, we also determined APOE*3-Leiden mRNA levels in the liver of both HFC fed male and female mice, treated with either testosterone or estradiol. As presented in Table V, for both males and females, estradiol administration had no significant effect on the expression of the APOE*3-Leiden gene. In contrast, administration of testosterone resulted in a significant lowering of APOE*3-Leiden mRNA levels for both male and female mice. In a parallel experiment untreated male mice (placebo) displayed

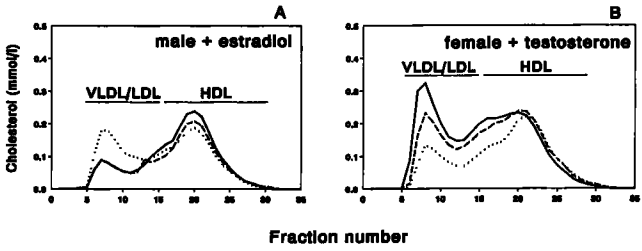


Figure 5. Distribution of serum cholesterol among lipoprotein fractions after hormone treatment. Lipoprotein fractions were separated by permeation chromatography using a 25-ml Superose 6B column as described in Methods. Lipoprotein profiles are shown for male (panel A) and female transgenic mice (panel B) which were treated with estradiol decanoate or testosterone decanoate, respectively. Each run is performed with a pool of at least 5 mice of the same group. Fraction numbers 5-15 and 16-30 correspond to VLDL/LDL and HDL, respectively. Solid, dashed and dotted lines represent mice treated with placebo, physiological dose and pharmacological dose of hormone, respectively (see Table IV).

significantly lower expression of the APOE*3-Leiden transgene than untreated female mice (69.0 ± 14.5 versus 100 ± 13.2%). Since hepatic LDL receptors are known to be regulated by estradiol administration, at least in rats (18-20) and humans (21) we also determined LDL receptor mRNA levels in estradiol treated female mice.In female mice hepatic *Ldlr* mRNA levels were not affected by estradiol administration (100 ± 25.3 versus 102 ± 25.9 % for placebo and estradiol treated animals, respectively). We investigated whether the changes in serum levels of VLDL/LDL-sized lipoproteins upon sex hormone administration were related to a concurrent change in hepatic VLDL triglyceride production rate.As presented in Table VI, placebo treated female mice displayed a significantly higher VLDL-triglyceride

*Table V. Hepatic APOE*3-Leiden mRNA Levels after 4 Weeks of Hormone Treatment*

Treatment	APOE*3-Leiden mRNA	
	Males	Females
	% of control	
Placebo	100.0 ± 22.7	100.0 ± 9.6
Estradiol decanoate (20 μg/mouse)	100.5 ± 19.6	98.8 ± 12.5
Estradiol decanoate (100 μg/mouse)	74.8 ± 26.9	105.4 ± 3.4
Testosterone decanoate (250 μg/mouse)	74.1 ± 19.4*	94.7 ± 13.2
Testosterone decanoate (1250 μg/mouse)	65.3 ± 15.5*	83.4 ± 8.9*

mRNA levels were measured in livers of mice treated, as described, for 4 weeks. mRNA concentrations were relative to internal standard GAPDH and expressed as a percentage of placebo group (control) of same sex. Values are the mean ± S.D. of five mice. *P < 0.05, indicating the difference between hormone and placebo treated groups of mice of the same sex, using nonparametric Mann-Whitney tests.

*Table VI. In Vivo Hepatic VLDL-Triglyceride Production after 4 Weeks of Hormone Treatment of APOE*3-Leiden Transgenic Mice*

Treatment	VLDL-triglyceride production rate	
	Males	Females
	% of placebo treated males	
Placebo	100.0 ± 33.0	135.6 ± 33.0*
Estradiol decanoate (100 μg/mouse)	119.6 ± 38.6	N.D.
Testosteron decanoate (1250 μg/mouse)	N.D.	135.1 ± 32.5

After an overnight fast hormone treated APOE*3-Leiden transgenic mice were injected with Triton WR1339 (see methods section). The serum triglycerides were determined just prior to injection (0 min.) and at 45 and 90 min. following Triton injection. Hepatic VLDL triglyceride production rate was calculated from the slope of the curve and is expressed as mmol/hr/kg body weight. Values are the mean ± S.D. Since two independent experiment are combined in order to increase the number of animals per groups (n=14-16) values are expressed as a percentage of the hepatic VLDL triglyceride production in placebo treated male mice in the same experiment. *$P<0.05$, indicating the difference between male and female mice, using non-parametric Mann-Whitney test. N.D.; not determined

production rate as compared to placebo treated male mice. VLDL-triglyceride production rate in female mice did not change upon testosterone treatment, whereas a 20% increase in VLDL-triglyceride production rate was observed in males after estrogen treatment.

To evaluate whether sex hormones were also influencing the plasma clearance rate of VLDL, we performed *in vivo* VLDL turnover studies. Placebo treated male and female mice displayed comparable VLDL-FCR's (Table VII). As illustrated in Figure 6A and presented in Table VII, VLDL-FCR in males decreased significantly following estrogen treatment, whereas a significant increase in VLDL-FCR was observed in females after testosterone treatment (Figure 6B; Table VII). Opposite effects were observed for VLDL-SR: VLDL-SR in male mice increased following estrogen treatment, whereas a slight decrease in VLDL-SR

*Table VII. VLDL-apoB Fractional Catabolic Rates and Secretion Rates after 4 Weeks of Hormone Treatment of APOE*3-Leiden Transgenic Mice*

Sex	Treatment	FCR	SR
		pools/hr	*μg apoB/hr/g body wt*
Males	Placebo	1.06 ± 0.27	1.65 ± 0.34
	Estradiol decanoate (100 μg/mouse)	0.68 ± 0.17*	1.94 ± 0.48
Females	Placebo	1.11 ± 0.20	2.13 ± 0.79
	Testosteron decanoate (1250 μg/mouse)	1.46 ± 0.42*	1.94 ± 0.41

FCR, fractional catabolic rate; SR, secretion rate. After an overnight fast HFC fed mice were injected 10 μg of autologous ^{125}I-labeled VLDL protein. ^{125}I-apoB disappearance from circulation was determined and FCR and SR were calculated (see methods section). Values are the mean ± S.D. of 4-6 mice per group. *$P<0.05$, indicating the difference between hormone and placebo treated groups of mice of the same sex, using nonparametric Mann-Whitney tests.

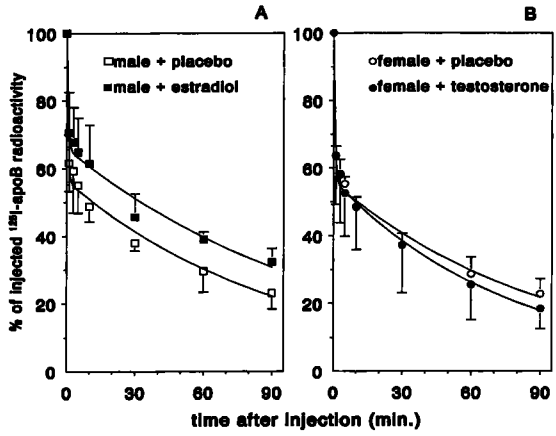


Figure 6. The effect of 4 weeks of hormone treatment on VLDL-apoB removal. HFC fed transgenic mice (>100 days of age) were injected with autologous ^{125}I-labeled VLDL. Some 25 μl of blood was drawn at each time point and ^{125}I-apoB radioactivity of the serum sample was measured. Values are the mean $\pm$ S.D. of 4-6 mice. Curves were calculated from mean data using a bi-exponential curve fit model.

is observed in female mice after testosterone treatment. In addition, placebo treated female mice displayed a higher VLDL-SR as compared to placebo treated male mice. Although these effects on VLDL-SR were not significant, these changes in VLDL-SR correspond with changes upon hormone treatment in serum lipid levels (Table IV) and in hepatic VLDL-triglyceride production rate (Table VI)

DISCUSSION

In the present study we showed that hyperlipidemia in APOE*3-Leiden mice was severely affected by age (Figures 2 and 3). At early age in the APOE*3-Leiden mice an enhanced production of hepatic VLDL was observed, whereas the plasma clearance of VLDL was not age-dependent (Tables II and III). Apparently, since APOE*3-Leiden mice are unable to efficiently remove VLDL remnants from the circulation (Figure 4A and Table III), the extra supply of newly synthesized hepatic VLDL, as occurring during rapid growth, is primarily responsible for the enhanced accumulation of these lipoproteins in the plasma at young age. Increased VLDL production rate has also been reported to occur in young rapidly growing rats, due to a stimulation of the hepatic triglyceride synthesis by growth hormone (22,23). In adult humans VLDL synthesis is also known to be stimulated upon body weight gain. Similar to APOE*3-Leiden mice, in humans carrying mutant APOE alleles, like APOE*3-Leiden, APOE*2(Lys146→Gln) and the APOE*2(Arg158→Cys), high body weight and body weight gain are strong aggravating factors in the accumulation of VLDL remnants (Familial Dysbetalipoproteinemia, FD) (1,6,24).

APOE*3-Leiden transgenic mice displayed a marked effect of gender on the expression of hyperlipidemia (Figures 2 and 3). Estrogens increase serum lipid levels, whereas testosterone showed the opposite effect (Table IV, Figure 5). Previously, we reported that in APOE*3-Leiden mice the level of serum lipids is positively correlated with the level of

the expression of the transgene in the liver (9). In the present study we found that expression of the APOE*3-Leiden gene results in a reduced clearance rate of VLDL, whereas the production of VLDL was not affected by the presence of the transgene (Table III). Thus, the observation that testosterone administration results in a reduced expression of the APOE*3-leiden transgene in the liver (Table V) can explain why testosterone administration resulted to lower serum VLDL/LDL lipoprotein levels, i.e. testosterone increases the clearance rate of VLDL, without an effect of testosterone on VLDL production rate (Table VI and VII). The reason for the effect of testosterone on the expression of the transgene is at present subject to speculation. Transgene expression was not affected by estrogen administration (Table V), and thus, cannot explain the observed reducing effect of estrogen on VLDL clearance (Table VII). We present evidence that the increased serum lipid levels observed in females, and after estrogen administration in males (Table IV) can, at least partly, be explained by a relative high rate of VLDL production (Table VI and VII).

The respective effects of both steroid hormones on the serum lipids and lipoprotein profile, hepatic VLDL triglyceride production and VLDL-apoB secretion are in line with the observed differences in lipid levels between untreated male and female APOE*3-Leiden mice. However, although both testosterone and estrogen treatment displayed considerable but opposing effects on VLDL clearance rate, differences in VLDL clearance were not found between untreated male and female mice. Similarly, although estrogens decreased VLDL clearance rate in males, the only significant difference between untreated males and females was increased VLDL production rate in females. These observations suggest discrepancies in the overall observed gender effect. However, in untreated males and females endogenous sex hormones form only part of the whole set of factors determining the final phenotype. The use of castrated males and ovariatectomized females instead of the respective sex hormone injected animals might have avoided these apparent discrepancies. In this study, addition of (supra) physiological doses of exogenous sex hormones was carried out only in order to evaluate the direction of the effect rather than that it aimed to extrapolate the effect to the basal in vivo situation in a quantitative sense.

Clearly, estrogens play an important role in mediating the gender differences observed in APOE*3-Leiden mice. The estrogen-mediated stimulation of hepatic VLDL production is well documented for humans (25) and rats (26) and suggests estrogen to be a risk factor for FD. Paradoxally, in humans FD is more prevalent in men than in women (1) and estrogen-replacement therapy is frequently used in reducing the expression of FD (27). Obviously, in man estrogens play a protective rather than an aggravating role in the accumulation of VLDL remnant lipoproteins. An explanation for this beneficial effect of estrogens in humans is commonly assumed to be the fact that estrogens stimulate the expression of hepatic LDL receptors (18-21). The fact that an increase in hepatic LDL receptor mRNA upon estrogen treatment does not occur in hamsters and mice (28-30) including the present APOE*3-Leiden transgenic mice, could explain the opposing effects of estrogen in man and mice regarding the accumulation of remnants.

It could be possible that in mice hepatic LDL receptor activity is less susceptible to regulation than it is in humans. This is also supported by the observations that feeding C57BL/6J mice a high fat/cholesterol diet results in downregulation of HMG-CoA reductase activity whereas LDL receptor expression is relatively unaffected (31,32). We hypothesize that an increased hepatic VLDL production increases the hepatic demand for cholesterol, but in mice this is not followed by upregulation of hepatic LDL receptors like in humans. Consequently, in APOE*3-Leiden mice, which are unable to efficiently metabolize hepatic lipoproteins, the increase in VLDL production upon the administration of estrogen will not be compensated by a simultaneous upregulation of LDL receptors and, thus, will lead to an extra accumulation of these lipoproteins in the plasma.

For the APOE*3-Leiden mice used in the present study, the APOC1 gene was included to be sure to have included an element that mediates liver-specific expression of the APOE gene. Recently, we have generated transgenic mice expressing the APOE*3-Leiden gene without the human APOC1 gene but containing the hepatic control region mediating liver expression. These mice have comparable expression of the APOE*3-Leiden transgene on the level of mRNA and on the level of serum apoE protein. In addition, these mice display similar diet-induced hyperlipoproteinemia and also show a similar effect of age and gender on the hyperlipidemic phenotype (results not shown). Hence, a role for the human APOC1 gene in inducing the age and sex dependent hyperlipidemic phenotype can be excluded.

Transgenic mice expressing the defective APOE(Arg112,Cys142) variant, also display diet-induced elevated levels of plasma cholesterol and triglycerides due to the accumulation of remnant lipoproteins (33,34). However, in these mice a similar gender and age related effect on plasma lipid levels has not been reported. On the contrary, in heterozygous apoE deficient mice elevated plasma lipid levels in females as compared to males have also been observed, after being fed severe cholesterol-rich diets (35). Such a gender effect has not been reported (35-39) in homozygous apoE deficient mice, either due to the extreme phenotype of these mice under normal conditions or the absence of apoE itself.

In this study, we have shown that age and gender related changes in serum lipid levels of APOE*3-Leiden mice at least in part were related to mild changes in hepatic VLDL production. These results are consistent with our previous study (9) showing that the serum lipid levels in APOE*3-Leiden mice are also highly responsive to sucrose feeding which is known to increase hepatic lipogenesis and thereby the production and secrétion of VLDL triglyceride by the liver (7,40,41). We conclude that introducing a defective apoE, like in the APOE*3-Leiden transgenic mice, leads to a high responsiveness of the serum lipid levels to relative mild changes in chylomicron -and VLDL production rate. Consequently, this animal model can easily be used for studying the effect on plasma lipid levels of (subtle) environmental factors, like nutrition and drugs that are supposed to modulate VLDL production in the liver or lipid absorption and chylomicron production in the intestine. In addition, our results strongly sustain the hypothesis that in carriers of mutant apoE alleles the production rate of both liver and intestinal lipoproteins are important determinants of the

clinical expression of familial dysbetalipoproteinemia.

ACKNOWLEDGEMENTS

We are grateful to Mrs Marga M. van Eck for technical assistance and we would like to thank Dr. Ko Willems van Dijk for his helpful discussions.

This research was supported by the Netherlands Heart Foundation and the Netherlands Organisation for Scientific Research (projects #900-504-092 and #900-539-117). Dr. M.Mol is a recipient of a post-doc fellowship from the Royal Netherlands Academy of Arts and Sciences

REFERENCES

1. Mahley, R.W. and S.C. Rall. 1989. Type III hyperlipoproteinemia (dysbetalipoproteinemia): The role of apolipoprotein E in normal and abnormal metabolism. In: *The metabolic basis of inherited disease*, edited by Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. New York: McGraw-Hill, p. 1195-1213.
2. Mahley, R.W., T.L. Innerarity, S.C. Rall Jr., K.H. Weisgraber and J.M. Taylor. 1990. Apolipoprotein E: genetic variants provide insight into its structure and function. *Curr. Opin. Lipid.*1:87-95.
3. Havel, R.J., Y.-S. Chao, E. Windler, L. Kotite, and L.S.S. Guo. 1980. Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia. *Proc. Natl. Acad. Sci. USA.* 77:4349-4353.
4. Sherill, B.C., T.L. Innerarity, and R.W. Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. *J. Biol. Chem.* 255:1804-1807.
5. Mahley, R.W. 1988. Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science.* 240:622-630.
6. de Knijff, P., A.M.J.M. van den Maagdenberg, A.F.H. Stalenhoef, J.A. Gevers Leuven, P.N.M. Demacker, L.P. Kuyt, R.R. Frants, and L.M. Havekes, L.M. 1991. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *J. Clin. Invest.* 88:643-655.
7. Nishina, P.M., J. Verstuyft, and B. Paigen. 1990. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* 31:859-869.
8. van den Maagdenberg, A.M.J.M., M.H. Hofker, P.J.A. Krimpenfort, I.H. de Bruijn, B.J.M. van Vlijmen, H. van der Boom, L.M. Havekes, and R.R. Frants. 1993. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 268:10540-10545.
9. van Vlijmen, B.J.M, A.M.J.M. van den Maagdenberg, M.J. Gijbels, H. van der Boom, H. HogenEsch, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1994. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* 93:1403-1410.
10. Zannis, V.I., J. McPherson, G. Goldberger, S.K. Krathanasis, and J.L. Breslow. 1984. Synthesis, intracellular processing, and signal peptide of human apolipoprotein E. *J. Biol. Chem.* 259:5495-5499.
11. Hoffer, M.J.V., M.M. van Eck, F. Pretrij, A. van der Zee, E. de Wit, D. Meijer, G. Grosveld, L.M. Havekes, M.H. Hofker and R.R. Frants. 1993. The mouse low density receptor gene: cDNA sequence and exon-intron structure. *Biochem. Biophys. Res. Commun.* 191:880-886.
12. Otway, S., and D.S. Robinson. 1967. The use of a non-ionic detergent (Triton WR1339) to determine rates of triglyceride entry into the circulation of the rat under different physiological conditions. *J. Biol. Chem.* 244:4406-4412.
13 .Bernstein, S.E. 1975. Physiological characteristics. *In* Biology of the Laboratory Mouse. 2nd ed. E.L. Green, editor. Dover Publications, Inc., New York. 337-350.
14. Lowry, O.H. N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 249:5133-5162.
15. Bilheimer, D.W., S.Eisenberg, and R.I. Levy. 1972. The metabolism of very low density lipoprotein

proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* 260:212-221.
16. Kita, T., M.S. Brown, D.W. Bilheimer, and J.L. Goldstein. 1982. The metabolism of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. *Proc. Natl. Acad. Sci. USA.* 79:5693-5697.
17. Holmquist, L., K. Carlson, and L.A. Carlson. 1978. Comparison between the use of isopropanol and tetramethylurea for the solubilization and quantitation of human serum very low density apolipoproteins. *Anal. Biochem.* 88:457-460.
18. Kovanen, P.T., M.S. Brown, and J.L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17α-ethinyl estradiol. *J. Biol. Chem.* 254:11367-11373.
19. Chao, Y-S., E.E. Windler, G.C. Chen, and R.J, Havel. 1979. Hepatic catabolism of rat and human lipoproteins in rats treated with 17α-ethinyl estradiol. *J. Biol. Chem.* 254:11360-11366.
20. Windler, E.E.T., P.T. Kovanen, Y-S. Chao, M.S. Brown, R.J. Havel, and J.L. Goldstein. 1980. The estradiol-stimulated lipoprotein receptor of rat liver. A binding site that mediates the uptake of rat lipoproteins containing apolipoproteins B and E. *J. Biol. Chem.* 255:10464-10471.
21. Angelin, B., H. Olivecrona, E. Reihner, M. Rudling, D. Stahlberg, M. Eriksson, S. Ewerth, P. Hendrikson, and K. Einarsson. 1992. Hepatic cholesterol metabolism in estrogen-treated men. *Gastroenterology* 103:1657-1663.
22. Elam, M.B., C.P. Simkevich, S.S. Solomon, H.G. Wilcox, and M. Heimberg. 1988. Stimulation of in vitro triglyceride synthesis in the rat hepatocyte by growth hormone treatment in vivo. *Endocrinology.* 122:1397-1402.
23. Elam, M.B., H.G. Wilcox, S.S. Solomon, and M. Heimberg. 1992. In vivo growth hormone treatment stimulates secretion of very low density lipoprotein by the isolated perfused rat liver. *Endocrinology.* 131:2717-2722.
24. Knijff, P., A.M.J.M. van den Maagdenberg, D.I. Boomsma, A.F.H. Stalenhoef, A.H.M. Smelt, J.J.P. Kastelein, A.D. Marais, R.R. Frants, and L.M. Havekes. 1994. Variable expression of familial dysbetalipoproteinemia in apolipoprotein E*2(Lys146→Gln) allele carriers. *J. Clin. Invest.* 94:1252-1262.
25. Walsh, B.W., I. Schiff, B. Rosner, L. Greenberg, V. Ravnikar, and F.M. Sacks. 1991. Effects of postmenopausal estrogen replacement on concentrations and metabolism of plasma lipoproteins. *N. Engl. J. Med.* 325:1196-1204.
26. Weinstein, I, F.C. Turner, C. Soler-Argilaga, and M. Heimberg. 1978. Effects of ethynyl estradiol on serum lipoprotein lipids in male and female rats. *Biochim. et Biophys.* 530:394-401.
27. Stuyt, P.M.J., P.N.M. Demacker, and A. van 't Laar. 1986. A study of the hypolipidemic effect of estrogen in Type III hyperlipoproteinemia. *Horm. Metab. Res.* 18:607-610.
28. Srivastava, R.A.K., D. Baumann, and G. Schonfeld. 1993. In vivo regulation of low density receptors by estrogen differs at the post-transcriptional level in rat and mouse. *Eur. J. Biochem.* 216:527-538.
29. Himber, J., B. Missano, and H. Kuhl. 1994. Lack of effect on the low density lipoprotein receptor in hamsters treated with 17α-ethinyl estradiol. *Biochim. et Biophys. Acta.* 1211:359-363.
30. Tang, J., R.A.K. Srivastava, E.S. Krul, D. Baumann, B.A. Pfleger, R.T. Kitchens, and G. Schonfeld. 1991. In vivo regulation of apolipoprotein A-I gene expression by estradiol and testosterone occurs by different mechanisms in inbred strains of mice. *J. Lipid. Res.* 32:1571-1585.
31. Dueland, S., J. Drisko, L. Graf, D. Machleder, A.J. Lusis, and R.A. Davis. 1993. Effect of dietary cholesterol on 7 alpha-hydroxylase and hepatic LDL receptors in inbred mice. *J. Lipid. Res.* 34:923-931.
32. Rudling, M. 1992. Hepatic mRNA levels for the LDL receptor and HMG-CoA reductase show coordinate regulation in vivo. *J. Lipid. Res.* 33:493-501.
33. Fazio, S., Y.L. Lee, Z.S. Ji, and S.C. Rall Jr. 1993. Type III Hyperlipoproteinemic phenotype in transgenic mice expressing dysfunctional apolipoprotein E. *J. Clin. Invest.* 92:1497-1503.
34. Fazio, S., D.A. Sanan, Y-L. Lee, Z-S. Ji, R.W. Mahley and S.C. Rall Jr. 1994. Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E(Arg 112,Cys142). *Arteriosclerosis and Thrombosis* 14:1873-1879.
35. van Ree, J.H., J.A.A. van den Broek, V.E.H. Dahlmans, P.H.E. Groot, M. Vidgeon-Hart, R.R. Frants, B. Wierenga, L.M. Havekes, and M.H. Hofker. 1994. Diet-Induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.* 111:25-37.
36. Zhang, S.H., R.L. Reddick, J.A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258:468-471.
37. Plump. A.S., J.D. Smith, T. Hayek, K. Aalto Setälä, A. Walsh, J.G. Verstuyft, E.M. Rubin, and J.L.

Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71:343-353.

38. Reddick, R.L., S.H. Zhang, and N. Maeda. 1993. Atherosclerosis in mice lacking apoe E. Evaluation of lesional development and progression. *Arteriosclerosis and Thrombosis* 14: 141-147.
39. Nakashima, Y., A.S. Plump, E.W. Raines, J.L. Breslow, and R. Ross. 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arteriosclerosis and Thrombosis* 14:133-140.
40. Grundy, S.M. and M.A. Denke. 1990. Dietary influences on serum lipids and lipoproteins. *J. Lipid Res.* 31:1149-1172.
41. Witztum, J.L. and G. Schonfeld. 1978. Carbohydrate diet induced changes in very low density lipoprotein composition and structure. *Diabetes.* 27:1215-1229.

CHAPTER 5

ENHANCED HYPERLIPIDEMIA IN APOLIPOPROTEIN E3-LEIDEN TRANSGENIC MICE WITH ONLY ONE FUNCTIONAL ENDOGENOUS APOLIPOPROTEIN E GENE

Bart J.M. van Vlijmen[1], Janine H. van Ree[2], H. Belinda van 't Hof[1], Patrick J.J. van Gorp[2], Hans van der Boom[1], Marten H. Hofker[2], and Louis M. Havekes[1,3]

[1]TNO-Prevention and Health, Gaubius Laboratory, Leiden, [2]MGC-Department of Human Genetics, Leiden University, Leiden, and [3]Departments of Cardiology and Internal Medicine, Academic Hospital Leiden, Leiden, The Netherlands

ABSTRACT

Apolipoprotein (APO) E3-Leiden transgenic mice develop hyperlipidemia and atherosclerosis, depending on the expression level of the transgene. High-expressing transgenic line #2 mice accumulate VLDL particles even on a chow diet, while the low-expressing line #195 is normolipidemic, even after high/fat cholesterol (HFC) feeding. In the present study the protective role of the endogenous mouse apolipoprotein E (*Apoe*) gene in remnant metabolism of both the hyperlipidemic and normolipidemic APOE*3-Leiden transgenic mice was investigated. Therefore, *Apoe* knockout (*Apoe-/-*) mice were bred with line #195 and line #2 mice to obtain mice with reduced expression of the endogenous *Apoe* gene. Loss of one of the two Apoe alleles did not affect plasma lipid levels in wild type mice, both on a normal and HFC diet. In contrast, loss of one apoE allele did affect lipid and lipoprotein levels in transgenic mice. Removal of one apoE allele in line #195 mice (#195·*Apoe*+/-) yielded normolipidemic mice on a chow diet. However, after feeding a HFC diet, #195·*Apoe*+/- mice had significantly higher ($P<0.05$) serum lipid levels as compared to #195·*Apoe*+/+ mice (5.0 ± 0.6 versus 4.0 ± 0.24 mmol/l and 0.4 ± 0.2 versus 0.2 ± 0.1 mmol/l for cholesterol and triglycerides, respectively), and there was a dramatic change in lipoprotein profile, characterized by accumulation of VLDL. The loss of one apoE allele in hyperlipidemic line #2 mice (#2·*Apoe*+/-) did not affect plasma lipid levels on chow diet. However, on HFC diet #2·*Apoe*+/- mice displayed a more pronounced increase of lipid levels than #2·*Apoe*+/+ mice (15.8 ± 4.9 versus 9.9 ± 1.4 mmol/l and 5.8 ± 2.5 versus 2.7 ± 0.7 mmol/l for cholesterol and triglycerides, respectively). *In vivo* turnover studies revealed that the VLDL-fractional catabolic rate followed the order #195 > #195·*Apoe*+/- > #2 = #2·*Apoe*+/- which inversely relates to the level of hyperlipidemia. We also show that the ratio of apoE3-Leiden to mouse apoE on VLDL varies from low to high in order of line #195, #195·*Apoe*+/-, #2 and #2·*Apoe*+/-. These results indicate that the VLDL clearance and therefore the hyperlipidemic phenotype in APOE*3-Leiden mice is determined by the ratio between apoE3-leiden and endogenous mouse apoE protein.

INTRODUCTION

Apolipoprotein (apo) E is a major structural component of various plasma lipoproteins, including chylomicrons, very low density lipoprotein (VLDL), and their remnants (1). ApoE serves as the ligand in the receptor-mediated uptake of these lipoproteins by the liver through interaction with the LDL receptor or with the LDL receptor-related protein (LRP) (1-3). *In vitro* studies have shown that enrichment of VLDL with apoE stimulates the uptake of these lipoproteins (4,5). Injection of exogenous apoE into normal and Watanabe heritable hyperlipidemic (WHHL) rabbits resulted in a transient decrease in plasma cholesterol concentrations, by increasing the clearance of lipoproteins from the circulation (6,7). In apoE-transgenic mice, stable overexpression of rat apoE also resulted in a sustained reduction

of plasma lipid levels because of an increased clearance of VLDL. These mice also showed resistance to diet-induced hypercholesterolemia (8-10) indicating that (extra) apoE can protect against (diet-induced) hyperlipidemia, by accelerating the clearance of VLDL/LDL lipoproteins.

Humans carrying the receptor-defective apoE variant APOE*3-Leiden, have impaired metabolism of chylomicron and VLDL remnant particles, despite the presence of one normal apoE allele (11). Recently, we described transgenic mice expressing the human APOE*3-Leiden gene (12). APOE*3-Leiden mice develop diet-induced hyperlipidemia and atherosclerosis despite the presence of two endogenous mouse apoE alleles (12-15). High-expressing transgenic line #2 accumulate VLDL particles even on a chow diet, while the low-expressing line #195 is normolipidemic, even after high/fat cholesterol feeding (13). Possibly, in normolipidemic low expressing APOE*3-Leiden mice the amount of normal apoE is sufficiently high to protect these mice against (diet-induced) hyperlipidemia.

In the present study the protective role of the endogenous mouse apolipoprotein E (*Apoe*) gene in remnant metabolism of both normolipidemic and hyperlipidemic APOE*3-Leiden mice was investigated. Therefore, low and high expressing APOE*3-Leiden mice were crossbred with *Apoe* knockout mice to reduce the expression level of the endogenous *Apoe* gene. The present study shows that the lack of one of the two mouse *Apoe* alleles did not affect plasma lipid levels under normal feeding conditions. However, upon feeding a mild atherogenic diet, the lack of one of the two *Apoe* alleles exacerbated the hyperlipidemia in high expressing APOE*3-Leiden mice, while low expressing APOE*3-Leiden mice also develop a hyperlipidemic phenotype after feeding this diet. The data in this study indicate that the quantitative ratio between apoE3-Leiden and endogenous apoE protein, determines the VLDL clearance rate and, consequently, the level of hyperlipidemia.

MATERIALS AND METHODS

Animals

Transgenic mice of lines #2 and #195, expressing human APOE*3- Leiden and human APOC1 genes were previously obtained from microinjection in (C57BL/6J x CBA/J) F1 fertilized eggs (12). Mice of F7 and F8 generation of backcrosses to C57BL/6J have been used as mating partner for F4 generation of apoE-deficient mice, with a hybrid 129 Sv and C57BL/6 background (16), and as mating partner for C57BL/6J mice. The resulting female offspring was used for this study.

Presence of the transgene was measured by an ELISA specific for human apoE, as described previously (14). The endogenous *Apoe* genotype was identified through tail tip analysis, as described earlier (16). Animals were housed under standard conditions with free access to water and food.

Diets

Before starting the dietary treatment, mice were fed regular mouse chow (standard rat mouse diet [SRM-A]). The semi-synthetic mild high fat/cholesterol diet (HFC) is composed essentially according to Nishina et al. (17) and contains 40.5% sucrose, 15% cocoa butter, 0.25% cholesterol, 10% cornstarch, 1% corn oil and 6% cellulose (% by weight). Both diets were obtained from Hope Farms, Woerden, The Netherlands.

Lipid and lipoprotein analyses

After a 4-h fasting period (from 8.30 a.m. to 12.30 p.m.) about 200 μl of whole blood was obtained from each individual mouse through tail bleeding. Levels of total serum cholesterol and triglyceride (without measuring free glycerol) were measured enzymatically using commercially available kits (#236691 from Boehringer Mannheim and #337-B from Sigma, respectively).

For fast protein liquid chromatography (FPLC) size fractionation of lipoproteins, 100 μl of pooled serum from 12 fasted mice per group was applied onto a 25 ml Superose 6B prep grade column (Pharmacia, Uppsala, Sweden) and processed as described previously (14).

Serum apoE measurements

Human ApoE3-Leiden and mouse apoE protein measurements were performed by sandwich ELISA as described before (14).

For determination of serum mouse apoE concentrations, some 1 μl of serum was subjected to a 4-20% gradient SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) followed by incubation with polyclonal rabbit antisera against mouse apoE. Donkey anti-rabbit ^{125}I-IgG (Amersham, Litle Chalfont, UK) was used as a secondary antibody, and detection was performed by scanning the blots with a PhosphorImager. Mouse serum apoE level is expressed as percentage of mouse apoE level present in pooled serum isolated from normal mice fed a the standard chow diet.

Isolation, characterization and labeling of VLDL

Blood was collected from 6-10 mice. Sera were pooled and ultracentrifuged to obtain the VLDL fraction ($d < 1.006$ g/ml). VLDL protein was determined using the method of Lowry (18). To determine apolipoprotein composition of the VLDL, some 4.5 μg of VLDL protein was subjected to a 4-20% gradient SDS-PAGE. Proteins were either stained with Coomassie Brilliant Blue R or transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) followed by incubation with polyclonal goat anti-human apoE and polyclonal rabbit anti-mouse apoE (kind gift of Dr. K. Weisgraber, Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, CA). The primary antibodies were detected by rabbit anti-goat and goat anti-rabbit secondary antibodies, respectively (Nordic Immunology, Tilburg, The Netherlands), followed by the immunoperoxidase procedure, using 4-chloro-1-naphthol as substrate. Bands were then quantified by densitometric scanning.

VLDL was radiolabeled with ^{125}I by the iodine monochloride method (19). The fraction of ^{125}I-label present in apoB was determined by isopropanol precipitation (20,21) and ranged from 30 to 65% of total label.

In vivo removal of ^{125}I-labeled VLDL-apoB

Fasted mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 10 μg of ^{125}I-labeled VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t=2, 5, 10, 30, 60 and 90 minutes after injection. The serum content of ^{125}I-labeled apoB was measured by isopropanol precipitation followed by counting ^{125}I-label. A bi-exponential model was used to estimate the area under the ^{125}I-apoB decay curve and subsequent calculation of VLDL-apoB kinetics. VLDL secretion rate was calculated from VLDL fractional catabolic rate and serum apoB pool. Since more than 85% of serum apoB and triglycerides were associated with the $d < 1.006$ lipoprotein fraction, we determined the serum apoB pool by measuring serum triglyceride levels 48 hours prior to injection and the apoB to triglyceride ratio in the isolated $d < 1.006$ lipoproteins (VLDL) of mice from the same group (autologous)

RESULTS

Serum lipid and apoE levels

Serum lipid and apoE levels in APOE*3-Leiden mice with either two or one functional *Apoe* gene were measured at age 8-10 weeks, and are presented in Table I. On a normal chow diet, non-transgenic mice showed a significant reduction of serum mouse apoE levels upon reduction of the endogenous *Apoe* gene expression level. Although not significant, also in APOE*3-Leiden transgenic mice, reduced mouse apoE levels were observed upon reduction of the expression of the endogenous *Apoe* gene. The reduction of endogenous *Apoe* gene expression had no effect on the serum lipid in chow fed control and transgenic mice. In addition, in transgenic mice, apoE3-leiden levels were not affected.

The animals were then challenged with the HFC diet (containing 15% fat and 0.25% cholesterol) for 8 weeks. Serum cholesterol and triglyceride levels of line #2 mice (9.9 mM and 2.7 mM, respectively) were markedly increased compared to control mice (2.8 mM and 0.2 mM, respectively). Reduction of endogenous *Apoe* expression in #2·*Apoe*+/- resulted

*Table I. Serum Lipid and ApoE Levels in APOE*3-Leiden Mice with One or Two Functional Apoe Genes*

Mouse line	Diet	Cholesterol	Triglycerides	mouse apoE	human apoE
		mmol/l	*mmol/l*	*R.U.*	*mg/dl*
control	SRM-A	1.9 ± 0.3	0.4 ± 0.2	1.0 ± 0.6	-
Apoe+/-	SRM-A	1.8 ± 0.2	0.3 ± 0.1a	0.5 ± 0.3a	-
#195	SRM-A	2.4 ± 0.2[a]	0.7 ± 0.2[a]	1.0 ± 0.7	1.1 ± 0.1
#195·*Apoe*+/-	SRM-A	2.4 ± 0.3[a]	0.7 ± 0.2[a]	0.7 ± 0.5	1.1 ± 0.1
#2	SRM-A	2.8 ± 0.6[a]	1.7 ± 0.6[a]	0.8 ± 0.5	14.9 ± 1.9
#2·*Apoe*+/-	SRM-A	2.7 ± 0.5[a]	1.5 ± 0.6[a]	0.5 ± 0.4	14.2 ± 3.1
control	HFC	2.8 ± 0.3	0.2 ± 0.1	0.9 ± 0.4	-
Apoe+/-	HFC	3.0 ± 0.6	0.1 ± 0.1	0.4 ± 0.3[a]	-
#195	HFC	4.0 ± 0.2[a]	0.2 ± 0.1[a]	1.1 ± 0.4	1.0 ± 0.1
#195·*Apoe*+/-	HFC	5.0 ± 0.6[a,b]	0.4 ± 0.2[a]	0.6 ± 0.3	1.1 ± 0.2
#2	HFC	9.9 ± 1.4[a]	2.7 ± 0.7[a]	1.1 ± 0.4	24.7 ± 3.8
#2·*Apoe*+/-	HFC	15.8 ± 4.9[a,b]	5.8 ± 2.5[a,b]	0.8 ± 0.3	26.1 ± 6.5

SRM-A, standard rat/mouse-A diet; HFC, high fat/cholesterol diet; R.U., relative units. Serum mouse apoE levels are expressed relative to serum mouse apoE levels in control mice fed a SRM-A diet. [a]$P<0.05$, indicating significantly different from control mice fed the same diet. [b]$P<0.05$, indicating significantly different from mice with the same transgene and diet but with two *Apoe* genes.

in even more pronounced hypercholesterolemia (15.8 mM) and hypertriglyceridemia (5.8 mM). In the low-expressing #195 mice a significant influence of endogenous *Apoe* genotype on serum lipid levels was also observed, whereas in control mice loss of one *Apoe* allele did not affect the serum lipid levels on the HFC diet. This indicates that lack of one functional *Apoe* gene affects the hyperlipidemia caused by APOE*3-Leiden expression. For both control and transgenic mice decreased serum mouse apoE levels were observed in mice with the *Apoe*+/- genotype. Upon HFC feeding, ApoE3-Leiden concentrations doubled in #2 and #2·*Apoe*+/- mice, whereas these levels remained unchanged in the low expressing mice.

Lipoprotein profiles

The distribution of cholesterol among the various lipoproteins was determined by size fractionation of serum lipoproteins, using Superose 6B gel permeation chromatography. In Figure 1, lipoprotein profiles are displayed for control, #195 and #2 mice with either two or one functional endogenous *Apoe* allele(s) on a chow diet (A) or after 8 weeks of HFC diet (B). On a chow diet, the presence of only one *Apoe* allele apparently had no effect on the

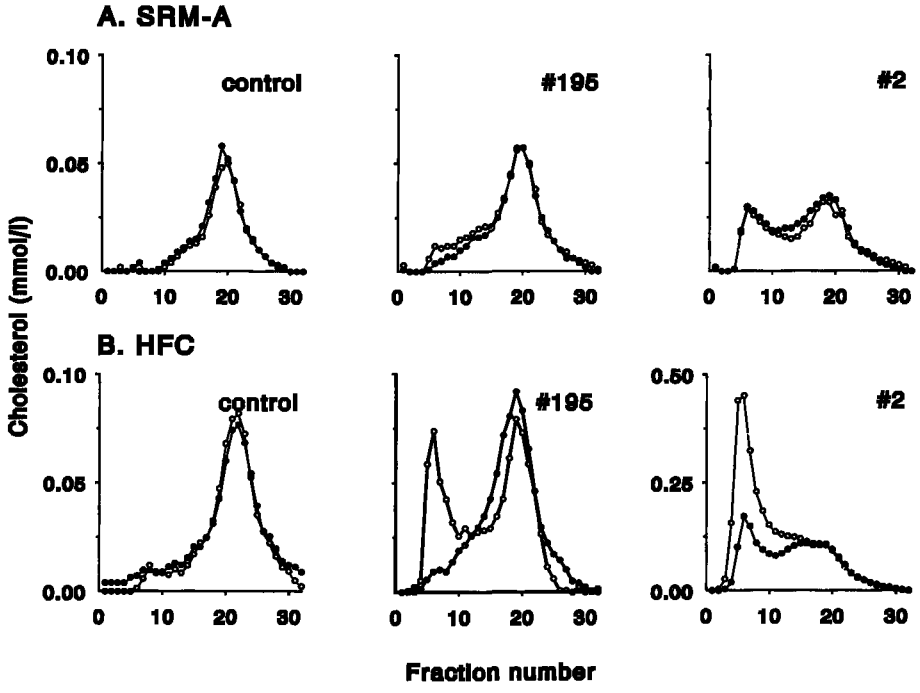


Figure 1. FPLC profiles of 100 µl of pooled mouse serum. The distribution of serum cholesterol among lipoprotein fractions of nontransgenic mice and transgenic mice of line #195 and #2 with either two (closed symbols) or one (open symbol) mouse *Apoe* gene(s) was determined as described in Materials and Methods. Mice were fed a chow diet (panel A) or the HFC diet (panel B). Fractions 13-19, VLDL; fractions 20-24, IDL/LDL; fractions 25-35, HDL.

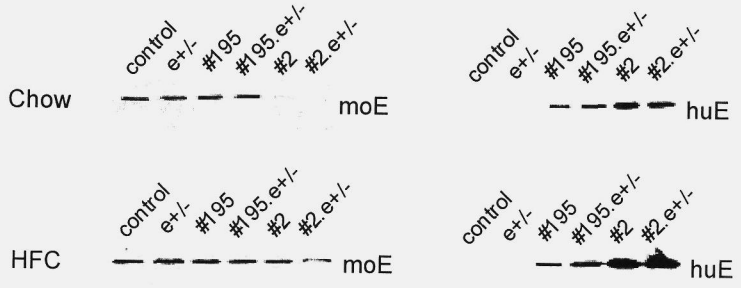


Figure 2. Western blot analysis of mouse VLDL from mice fed a chow diet (upper panel) or the HFC diet (lower panel). Lipoproteins of d < 1.006 g/ml were isolated by ultracentrifugation and subjected to SDS/PAGE (4-20% gradient gels) and transferred to a nitrocellulose membrane (equal amount of VLDL protein was loaded per lane). The first antibody was either polyclonal rabbit anti-mouse apoE or goat anti-human apoE.

distribution of cholesterol (Figure 1A). While line #195 and control mice carry most of the cholesterol in the HDL-sized fraction, line #2 mice exhibit a fifty-fifty distribution of cholesterol among VLDL/LDL- and HDL-sized particles.

Wild type mice carrying only one endogenous *Apoe* allele responded to the change of diet from chow to HFC diet similar to mice carrying two *Apoe* alleles, as is evident from the lipoprotein profile (Figure 1B). The low-expressing transgenic line #195 with two endogenous *Apoe* alleles appears to be normal and have a lipoprotein profile similar to that of control mice on both diets. However, for its counterpart with only one *Apoe* allele (#195·*Apoe*+/-) the reduction of the *Apoe* gene expression strongly increased the VLDL-cholesterol level to about 30% of total serum cholesterol. In line #2 mice, the relative amount of VLDL-cholesterol rose from approximately 30% to 50% of total serum cholesterol upon reducing the number of endogenous *Apoe* genes from two to one, provided the lipoprotein metabolism is stressed with HFC diet.

Composition of the d < 1.006 lipoproteins

The effect of reducing of the expression of the endogenous *Apoe* gene on the apolipoprotein composition of the accumulating VLDL lipoproteins was investigated. Therefore, d < 1.006 lipoproteins (VLDL) were isolated by ultracentrifugation from mice fed the chow diet or the HFC diet, subjected to SDS/PAGE followed by western blotting. Blots were incubated with either anti-mouse apoE or anti-human apoE (Figure 2). The intensity of the bands was determined by densitometric scanning (data not shown). The amount of

endogenous mouse apoE on the VLDL particles appears to be slightly reduced in the transgenic heterozygous apoE-deficient mouse lines. This is most evident in the #2·*Apoe*+/- line on the chow diet, in which mouse apoE is below the detection limit. The endogenous apoE level on VLDL is similar in control and line #195 mice, whereas this level is reduced in the high-expressing line #2. The quantity of apoE3-Leiden on VLDL from mice fed either chow or HFC is higher for #2 and #2·*Apoe*+/- mice than for #195 and #195·*Apoe*+/- mice. In addition, more human apoE is found in line #195·*Apoe*+/- than in line #195, although the endogenous *Apoe* genotype apparently does not affect the apoE3-Leiden level on VLDL of line #2 and #2·*Apoe*+/- mice. Taken these results together, the ratio of apoE3-Leiden to mouse apoE on VLDL varies from low to high in order of line #195, #195·*Apoe*+/-, #2 and #2*Apoe*+/-, parallel to the severity of the hyperlipidemia.

In vivo VLDL turnover

To evaluate whether enhanced hyperlipidemic phenotype in *Apoe*+/- mice were related to decreased VLDL clearance rate or VLDL production rate, we performed *in vivo* VLDL turnover studies. However, for line #2 mice no effect of *Apoe* genotype on VLDL-apoB FCR was observed. As illustrated in Figure 3 and presented in Table II, VLDL-apoB FCR was significantly reduced in mice of line #2 when compared to control mice, as was

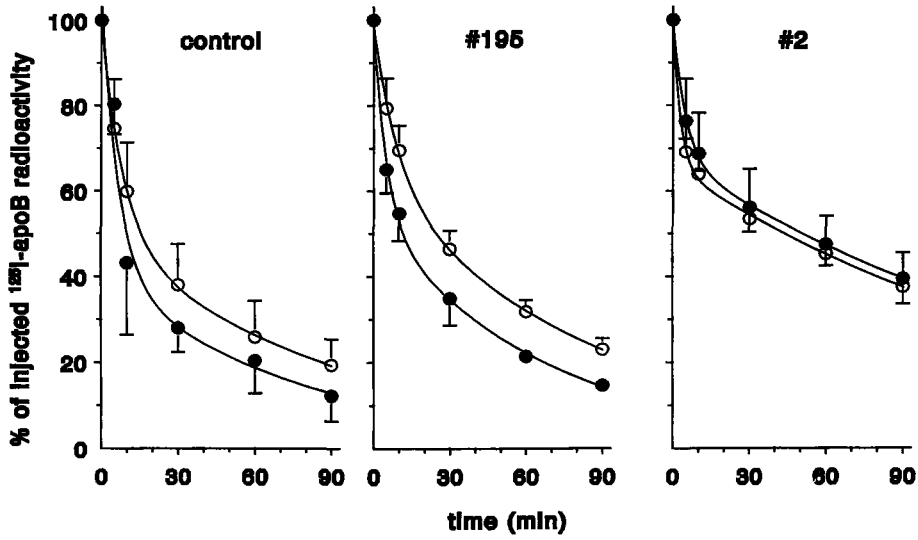


Figure 3. VLDL-apoB removal. HFC fed female control (left panel), line 195 (middle panel) or line 2 (right panel) mice with either two (closed symbols) or one functional *Apoe* genes (open symbols) were injected with autologous ^{125}I-labeled VLDL. 25 μl of blood was drawn at each time point and ^{125}I-apoB radioactivity of the serum sample was measured. Values are the mean $\pm$ S.D. of seven mice. Curves were calculated from the mean data using a bi-exponential curve fit model.

*Table II. VLDL-apoB Fractional Catabolic Rates and Secretion Rates in APOE*3-Leiden with One or Two Endogenous Mouse ApoE Alleles*

line	VLDL-apoB FCR	VLDL-apoB pool	VLDL-apoB SR
	pools/hr	*μg/g mouse*	*μg/hr/g mouse*
control	16.9 ± 7.1	0.65 ± 0.24	11.5 ± 7.4
Apoe+/-	11.1 ± 2.4	0.67 ± 0.99	6.3 ± 7.5
#195	15.4 ± 1.4	0.42 ± 0.04	6.4 ± 0.6
#195·*Apoe+/-*	10.0 ± 1.2[a,b]	1.28 ± 0.54[a,b]	12.7 ± 4.7
#2	5.3 ± 1.5[a]	3.01 ± 1.10[a]	15.0 ± 3.8
#2·*Apoe+/-*	5.6 ± 1.3[a]	5.34 ± 3.18[a]	30.7 ± 22.2

After 6 hour fasting period female mice were injected 10 μg of autologous labeled ^{125}I-labeled VLDL protein. ^{125}I-apoB disappearance from circulation was determined and FCR and SR were calculated (see Methods). Values are the mean±SD of 4-8 mice per group. [a]$P<0.05$, indicating significant difference when compared to non-transgenic mice, using nonparametric Mann-Whitney tests. [b]$P<0.05$, indicating significant difference when compared to mice with the same transgene, using nonparametric Mann-Whitney tests.

expected. Line #195 mice with only one functional *Apoe* gene had significant lower VLDL-apoB FCR as compared to its counterpart with two functional *Apoe* genes. Since, VLDL-apoB SRs for all groups were not significantly affected, the reduction of VLDL-apoB FCRs seems to be the sole factor contributing to the observed increased VLDL-apoB pool in the hyperlipidemic mice.

DISCUSSION

Mice that carry the human APOE*3-Leiden gene develop diet-induced hyperlipidemia and atherosclerosis, depending on the degree of expression of the transgene (12-15). While in the high- expressing transgenic line #2 VLDL particles accumulate even on a chow diet, the low-expressing line #195 is normolipidemic, even after high/fat cholesterol feeding (13). In the present study the protective role of the endogenous mouse apolipoprotein E (*Apoe*) gene in remnant metabolism of both the hyperlipidemic and normolipidemic APOE*3-Leiden transgenic mice was studied. Therefore, APOE*3-Leiden mice with different expression levels of the transgene were crossbred with *Apoe* knockout mice to reduce the expression level of the endogenous *Apoe* gene. Our results show that on a normal chow diet for both the low and high expressing line plasma lipid levels were not dependent on the expression level of endogenous *Apoe*. However, one functional *Apoe* gene was clearly not enough to protect remnant metabolism of both high and low expressing APOE*3-Leiden mice when fed a mild hypercholesterolemic diet. High expressing mice displayed an enhancement of the

hyperlipidemia upon loss of one *Apoe* allele on this diets. In low expressing mice with one *Apoe* allele this diet resulted in a moderate increase in absolute plasma lipid levels, and a dramatic change of lipoprotein profile i.e. the accumulation of remnant lipoproteins. This accumulation was at least in part caused by a decrease in VLDL clearance rate. We also demonstrated that an increased transgene expression or decreased endogenous *Apoe* expression decreased, or the combination of both, led to a decreased amount of mouse apoE relative to apoE3-Leiden on the VLDL particles. Therefore, we postulate that the rate of VLDL clearance, and consequently the level of hyperlipidemia, largely depends on the ratio of apoE3-Leiden over normal endogenous apoE. This is in line with *in vitro* observations showing that the amount of apoE present on remnant particles is an important determinant of lipoprotein receptor binding affinity (4,5).

Our findings correlate well with the observations in hyperlipidemic humans carrying the APOE*3-Leiden allele. The ratio of mutant over normal apoE on the accumulating VLDL in the plasma of these hyperlipidemic humans is about 7:1 (11), which suggested that also in these hyperlipidemic humans the relative amount of apoE on the VLDL particle is too low prevent accumulation of remnant lipoproteins. A possible explanation for the observed increased ratio of mutant over normal apoE on the accumulating VLDL particle can be preferential associating of apoE3-Leiden with VLDL particles as was shown *in vitro* by Fazio et al. (22). The importance of the ratio of defective over normal apoE is also illustrated by hyperlipidemic humans and mice expressing APOE*3(Arg142→Cys) (23-25), showing that the accumulating particles displayed an increased mutant over wild type apoE ratio.

Finding ways to increase the amount of functional apoE per VLDL particle has clinical implications. *In vivo* studies revealed that introduction of exogenous apoE in apoE-deficient mice by adenoviral transfection (26,27) or bone marrow transplantation (28) could almost completely normalize the severe hypercholesterolemic phenotype in these mice. In these mice, a low plasma level of normal apoE (12.5% of control) was sufficient to achieve the normalization of plasma lipoprotein levels. However, in this study, we showed that one apoE allele was not enough to protect low expressing APOE*3-Leiden mice against diet-induced hyperlipidemia. In line with this, van Ree (16) showed that heterozygous apoE-deficient mice developed hypercholesterolemia when challenged with a severe high/fat cholesterol diet. Thus, under dietary stress conditions the amount of (functional) apoE can be the limiting factor. It was shown that protection against diet-induced-induced hyperlipidemia requires high levels of extra apoE (9). Since, in the Western societies, people live under non-fasting conditions with continuous release of chylomicron and VLDL in the circulation during most of their life-span. Thus, under these metabolic stressful conditions apoE may become limiting as well, with as a consequence causing hyperlipidemia. Further studies on the role of apoE in the protection against hyperlipidemia in relation to diet, will provide valuable information for strategies to lower plasma lipid levels and, consequently, risk for atherosclerosis in humans.

ACKNOWLEDGEMENTS

This work was supported in part by the Medical Genetics Center South-West Netherlands (MGC), the Netherlands Heart Foundation and the Netherlands Organisation for Scientific Research (projects #901-04-092 and #900-539-117). Financial contribution of the specific RTD Programme of the European commission (BIOMED-2, #BMH4-CT96-0898) is gratefully acknowledged. Dr. Marten Hofker is an Established Investigator of the Netherlands Heart Foundation.

REFERENCES

1. Mahley, R.W. 1988. Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science.* 240:622-630.
2. Ishibashi, S., J. Herz, N. Maeda, J. L. Goldstein, and M. S. Brown. 1994. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc. Acad. Sci. U. S. A.* 91:4431-4435.
3. Willnow, T. E., Z. Sheng, S. Ishibashi, and J. Herz. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor anatagonist. *Science.* 264: 1471-1474.
4. Windler, E., Y. Chao, and R.J. Havel. 1980. Regulation of hepatic uptake of triglyceride-rich lipoproteins in the rat. *J.Biol.Chem.* 255:8303-8307
5. Eisenberg, S. G. Friedman, and T. Vogel. 1988. Enhanced metabolism of normolipidemic human plasma very low density lipoprotein in cultured cells by exogenous apolipoprotein E-3 *Arteriosclerosis* 8:480-487.
6. Yamada, N., H. Shimano, H. Mokuno, S. Ishibashi, T. Gotohda, M. Kawakami, Y. Watanabe, Y. Akanuma, T. Murase, T., F. Takaku. 1989. Increased clearance of plasma cholesterol after injection of apolipoprotein E into Watanabe heritable hyperlipidemic rabbits. *Proc. Natl. Acad. Sci. USA* 86:665-669.
7. Mahley, R.W., K.H. Weisgraber, M.M. Hussain, B. Greenman, M. Fisher, T. Vogel, M. and Gorecki. 1989. Intravenous infusion of apolipoprotein E accelerates clearance of plasma lipoproteins in rabbits. *J. Clin. Invest.* 83:2125-2130.
8. Shimano, H., N. Yamada, M. Katsuki, K. Yamamoto, T. Gotoda, K. Harada, M. Shimada, and Y. Yazaki. 1992a. Plasma lipoprotein metabolism in transgenic mice overexpressing apolipoprotein E. Accelerated clearance of lipoproteins containing apolipoprotein B. *J. Clin. Invest.* 90:2084-2091.
9. Shimano, H., N. Yamada, M. Katsuki, M. Shimada, T. Gotoda, K. Harada, T. Murase, C. Fukazawa, F. Takaku, and Y. Yazaki. 1992b. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet- induced hypercholesterolemia. *Proc. Natl. Acad. Sci. USA* 89: 1750-1754.
10. Shimano, H., Y. Namba, J. Ohsuga, M. Kawamura, K. Yamamoto, M. Shimada, T. Gotoda, K. Harada, Y. Yazaki, and N. Yamada. 1994. Secretion-recapture process of apolipoprotein E in hepatic uptake of chylomicron remnants in transgenic mice. *J. Clin. Invest.* 93:2215-2223
11. de Knijff, P., A.M.J.M. van den Maagdenberg, A.F.H. Stalenhoef, J.A. Gevers Leuven, P.N.M. Demacker, L.P. Kuyt, R.R. Frants, and L.M. Havekes, L.M. 1991. Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *J. Clin. Invest.* 88:643-655.
12. van den Maagdenberg, A.M.J.M., M.H. Hofker, P.J.A. Krimpenfort, I.H. de Bruijn, B.J.M. van Vlijmen, H. van der Boom, L.M. Havekes, and R.R. Frants. 1993. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 268:10540-10545.
13. van Vlijmen, B.J.M, A.M.J.M. van den Maagdenberg, M.J. Gijbels, H. van der Boom, H. HogenEsch, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1994. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* 93:1403-1410.
14. van Vlijmen, B.J.M., H.B. van 't Hof, M.J.T.M. Mol, H. van der Boom, A. van der Zee, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1996. Modulation of very low density lipoprotein production and clearance contributes to age- and gender dependent hyperlipoproteinemia in apolipoprotein E3-Leiden

transgenic mice. *J. Clin. Invest.* 97:1184-1192.

15. Groot, P.H.E., B.J.M. van Vlijmen, G.M. Benson, M.H. Hofker, R. Schiffelers, M. Vidgeon-Heart, and L.M. Havekes. 1996. Quantitative assessment of aortic atherosclerosis in APO E3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Ath. Tromb. Vasc. Biol.* 16:926-933.
16. van Ree, J. H., J. A. A. van den Broek, V. E. H. Dahlmans, P. H. E. Groot, M. Vidgeon-Hart, R. R. Frants, B. Wierenga, L. M. Havekes, and M. H. Hofker. 1994. Diet-Induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.* 111:25-37.
17. Nishina, P.M., J. Verstuyft, and B. Paigen. 1990. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* 31:859-869.
18. Lowry, O.H. N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 249:5133-5162.
19. Bilheimer, D.W., S.Eisenberg, and R.I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* 260:212-221.
20. Holmquist, L., K. Carlson, and L.A. Carlson. 1978. Comparison between the use of isopropanol and tetramethylurea for the solubilization and quantitation of human serum very low density apolipoproteins. *Anal. Biochem.* 88:457-460.
21. Kita, T., M.S, Brown, D.W. Bilheimer, and J.L. Goldstein. 1982. The metabolism of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. *Proc. Natl. Acad. Sci. USA.* 79:5693-5697.
22. Fazio, S., Y. Horie, K.H. Weisgraber, L.M. Havekes, and S.C. Rall Jr. 1993. Preferential association of apolipoprotein E Leiden with very low density lipoproteins of human plasma. *J. Lipid. Res.* 34:447-453.
23. Horie, Y., S. Fazio, J.R. Westerlund, K.H. Weisgraber, and S.C. Rall Jr. 1992. The functional characteristics of a human apolipoprotein E variant (cysteine at residue 142) may explain its association with dominant expression of type-III hyperlipoproteinemia. *J. Biol. Chem.* 267:1962-1968.
24. Fazio, S., Y. L. Lee, Z. S. Ji, and S. C. Rall Jr. 1993. Type III Hyperlipoproteinemic phenotype in transgenic mice expressing dysfunctional apolipoprotein E. *J. Clin. Invest.* 92:1497-1503.
25. *Fazio, S., D. A. Sanan, Y-L. Lee, Z-S. Ji, R. W. Mahley and S. C. Rall Jr. 1994. Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E(Arg 112,Cys142). Arteriosclerosis and Thrombosis* 14:1873-1879.
26. Kashyap, V. S., S. Santamarina-Fojo, D. R. Brown, C. L. Parrott, D. Applebaum-Bowden, S. Meyn, G. Talley, B. Paigen-B, N. Maeda, and H. B. Brewer Jr. 1995. Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. *J. Clin. Invest.* 96:1612-1620
27. Stevenson, S. C., J. Marshall-Neff, B. Teng, C. B. Lee, S. Roy, and A. P. McClelland. 1995. Phenotypic correction of hypercholesterolemia in apoE-deficient mice by adenovirus-mediated in vivo gene transfer. *Arterioscler. Thromb. Throb. Biol.* 15:479-484
28. Linton, M. F., J. B. Atkinson, and S. Fazio. 1995. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science.* 267:1034-1037

CHAPTER 6

APOLIPOPROTEIN E3-LEIDEN TRANSGENIC MICE AS A TESTMODEL FOR HYPOLIPIDAEMIC DRUGS

Bart J.M van Vlijmen[1], Nigel J. Pearce[2], Martin Bergö[3], Bart Staels[4], John W. Yates[2], Andy D. Gribble[2], Brian C. Bond[2], Marten H. Hofker[5], Louis M. Havekes[1,6] and Pieter H.E. Groot[2].

[1]TNO-PG, Gaubius Laboratory, Leiden, The Netherlands, [2]SmithKline Beecham Pharmaceuticals, Welwyn, Herts, United Kingdom, [3]Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, Sweden, [4]Département d'Atherosclérose, Institut Pasteur, Lille, France, [5]Department of Human Genetics, Leiden University, Leiden, and [6]Departments of Cardiology and Internal Medicine, Academic Hospital Leiden, Leiden, The Netherlands.

Submitted

ABSTRACT

Apolipoprotein (APO) E*3-Leiden mice with impaired chylomicron and VLDL remnant metabolism display hyperlipidaemia and atherosclerosis. In the present study, these mice were used for testing the hypolipidaemic effect of two marketed agents, Lovastatin and Gemfibrozil, as well as a novel, compound SB 204990, a potent inhibitor of cholesterol and fatty acid synthesis at the level of ATP-citrate lyase. APOE*3-Leiden mice were fed a saturated fat and cholesterol-rich diet supplemented with either 0.05 or 0.1% w/w of Lovastatin, 0.1 or 0.2% w/w of Gemfibrozil or 0.1 or 0.2% w/w of SB 204990. Lovastatin showed dose-related decrease in plasma cholesterol levels (up to -20%) due to a lowering LDL and HDL-cholesterol (-20 and -18%, respectively), while plasma triglyceride levels were unaffected. Gemfibrozil had no effect on plasma total cholesterol levels but gave significant dose-dependent decreases in plasma (VLDL) triglyceride levels (up to 53%). SB 204990 resulted in a dose-dependent reduction of plasma cholesterol (up to 29%) by lowering VLDL, LDL and HDL-cholesterol (-50, -20 and -20%, respectively). In addition, a strong dose dependent reduction of plasma (VLDL) triglycerides up to 43% was observed with this compound. Although the effects of Gemfibrozil and SB 204990 were not simply explained by changes in a single determinant of VLDL metabolism - no effects of these drugs were seen on post-heparin plasma lipoprotein lipase activity, *in vivo* rate of VLDL synthesis or hepatic apoC-III mRNA levels - APOE*3-Leiden mice were found to give robust hypolipidaemic responses on these test compounds. The responsiveness to hypolipidaemic therapy combined with a clear relationship between aortic lesion size and plasma cholesterol exposure (Groot et al. (1996) Ath. Thromb. Vasc. Biol. 16, 926-933), makes this mouse an attractive model for the testing of anti-atherosclerotic properties of hypolipidaemic drugs.

INTRODUCTION

For the development of novel hypolipidaemic and anti-atherosclerotic drugs, small animal models in which both the hypolipidaemic properties of candidate drugs and their potential to affect lesion formation could be tested, would be of great benefit. Normal rats and mice are not very suitable as plasma lipids in these animals are very low, even on high fat/cholesterol diets making rodents very resistant against the development of atherosclerosis (1). Although hamsters become hyperlipidaemic on high fat/cholesterol diets and have a lipoprotein profile more similar to that seen in human also this species does not easily develop atherosclerotic lesions (2).

With the recent progress in techniques for gene insertion and gene silencing in murine germ- and embryonic cells this picture has changed. These developments have resulted in the production of a number of murine strains with defects in plasma lipid and lipoprotein metabolism. Some of these strains were shown to develop spontaneous or diet-induced hyperlipidaemia and advanced atherosclerotic lesions in the aorta and other main arteries.

Recently, we described the generation of transgenic mice expressing the human apolipoprotein (APO) E*3-Leiden gene (3). These mice have an impaired clearance of chylomicron and VLDL remnant lipoproteins from the blood circulation by the liver (3-5). As a consequence, these mice have raised plasma cholesterol and triglyceride levels due to increases in VLDL-LDL lipoproteins. In addition, in these mice the plasma cholesterol and triglyceride levels are highly responsive to small changes in chylomicron and VLDL metabolism (4,5). In a subsequent study we have shown that APOE*3-Leiden mice are susceptible for diet-induced atherosclerosis and we demonstrated that the size of the aortic lesions correlates very well with the exposure of the vasculature to raised concentrations of plasma cholesterol (4,6). Their more human-like lipoprotein profile, the extreme sensitivity of plasma lipid levels to changes in lipoprotein metabolism and the clear relation between aortic lesion size and cholesterol exposure suggested to us that APOE*3-Leiden mice may serve as a suitable animal model for the testing of lipid lowering and anti-atherosclerotic effects of hypolipidaemic drugs.

In the present study we have evaluated the responsiveness of plasma lipids in these mice for hypolipidaemic drugs using two marketed agents, Lovastatin and Gemfibrozil, as well as a novel experimental compound SB 204990, an inhibitor of de novo cholesterol- and fatty acid synthesis at the level of hepatic ATP-citrate lyase (7).

METHODS

Animals

All studies were performed with male APOE*3-Leiden transgenic mice (line #2) (3). Transgenic mice were obtained by mating male transgene carriers with C57BL/6J females (The Broekman Institute bv, Someren, The Netherlands). Mice of the F7 generation were used for all studies. Transgenic mice were identified by sandwich ELISA for the presence of human apoE in the serum as described previously (5). At the time of the study animals were 3-4 months of age.

Study design

The study had a parallel group design and included 6 experimental groups (9 animals per group and 2 dose levels of each of the drugs) and a control group of 18 mice. Animals were distributed over these groups, stratifying for a balanced age distribution. They were housed (3 per cage) in shoebox cages with hoppers that allowed feeding of a powdered diet and weighing of food consumption. Animals had free access to food and water. Two weeks prior to the start of drug administration, mice were switched from a chow diet to a powdered semi-synthetic sucrose-rich diet, containing cocoa butter (15% w/w) and cholesterol (0.25% w/w). The diet was composed essentially according to Nishina et al. (8), and described by us earlier (5) (Western or HFC diet, Hope Farms, Woerden, The Netherlands). During the subsequent testing period of two weeks, mice were fed the same powdered diet supplemented with 0.05 or 0.1% w/w of Lovastatin (Zocor®, a gift of Merck, Sharp and Dohme, Rahway, NJ), 0.1 or 0.2% w/w of Gemfibrozil (Lopid®, a gift of Warner-Lambert, Pharmaceutical Co., Ann Arbor, MI) or 0.1 or 0.2% w/w of the SB204990 (the 5-ring lactone of + (3R*,5S*) 3-carboxy-11-(2,4-dichlorophenyl)-3,5- dihydroxyundecanoic acid, (7)).

At the beginning and end of the experimental period mice were fasted from 7 to 1 pm, weighed and approximately 50 μl of blood was obtained in an EDTA coated vial through tail-bleeding under light isofluothane anaesthesia. Plasma samples were stored on ice until lipid and lipoprotein analysis.

Following the post-drug blood sampling, animals were sacrificed, livers were excised, frozen into liquid nitrogen and stored at -70 degrees until RNA isolation.
For determination of post-heparin plasma lipoprotein lipase (LPL) activity (methods see below), indicated parts of the experiment were repeated under identical conditions using mice with comparable genetic background, sex and age. *In vivo* hepatic VLDL triglyceride production rate (methods see below) was determined in mice that were used for determining post-heparin plasma LPL activity. Therefore, these mice were fed the experimental diets for one additional week. 3 wk plasma lipid data were not different from the 2 wk data (not shown).

Lipid and lipoprotein analysis

Total plasma cholesterol and triglyceride levels (without measuring free glycerol) were measured enzymatically using commercially available kits: #997-64909 (Wako Chemicals GmbH, Neuss, Germany) and #14149 (Merck, Darmstadt, Germany).
For size fractionation of lipoproteins, some 30 μl of pooled serum (from 3 mice), containing 2.5 mM EDTA, was injected onto a Superose 6B column (SMART system, Pharmacia, Uppsala, Sweden) eluted at a constant flow rate of 30 μl/min with 150 mM NaCl, 1 mM EDTA, pH 8.0. The effluent was collected in 30 μl fractions. Cholesterol and triglyceride concentrations in lipoprotein fractions were measured enzymatically, as described above.

Post-heparin plasma lipoprotein lipase activity

For measurements of post-heparin plasma lipoprotein lipase activity fasted mice received an intravenous injection of heparin (Leo Pharmaceutical products bv, Weesp, The Netherlands, dose: 100 U/kg body weight) at 1 pm, following a 6 hour fasting period. After ten minutes 200 μl blood was drawn from the tail vein and stored on ice. Plasma was frozen in liquid nitrogen and shipped on dry ice to Umeå for LPL activity measurements. Plasma LPL activity was assayed as described by Bengtsson Olivecrona et al. (9). In brief, postheparin plasma samples were incubated with a goat anti-hepatic lipase IgG antibody for 2 hours at 4 degrees (10). 20 μl of the samples were incubated with substrate (Intralipid into which ^{3}H-labelled triolein had been incorporated by sonication) in the presence of 10 μl of heat-inactivated rat serum (as source of apo CII) and 6% (w/v) BSA in a total volume of 200 μl. The assay temperature was 25 °C. Enzyme activity is expressed in mU, corresponds to 1 nmol of fatty acid released per minute.

In vivo hepatic triglyceride production

After a six hour fasting period mice were injected intravenously via a tail vein with Triton WR1339 (500 mg/kg body weight) (11) using 15% (wt/vol) Triton solution in 0.9% by wt NaCl. At 0, 15, 30 and 45 minutes after injection blood samples were drawn from the tail and analyzed for triglycerides as described above. Production rate of hepatic triglyceride was calculated from the slope of the curve and expressed as mmol/hr/kg body weight. Plasma volume was assumed to be 3.3% of the body weight (12).

Hepatic apoC-III mRNA measurements in the liver

Mouse hepatic apoC-III mRNA were determined as described previously (13). In brief, total cellular RNA was prepared from the liver using the guanidinium thiocyanate/phenol-chloroform method (14). Northern blots of total cellular RNA were subsequently hybridized with a ^{32}P-labelled probes of rat apoC-III and rat ribosomal 36B4 cDNA (15). The intensity of the hybridization signal was quantified and the level of apoC-III mRNA was related to the level of 36B4 mRNA.

Statistical analysis

For analysis of treatment effects on serum cholesterol and triglyceride levels, analysis of variance was performed. The pre-study cholesterol levels, pre-study triglyceride level and the pre-study body weight were both found to be important factors, therefore included as covariates in the analysis of the

cholesterol and triglyceride levels. A logarithmic scale was used in the analysis of the lipid data. The variability of these measurements tended to increase with the level of response. Using a logarithmic scale standardises the variability producing better analysis. Geometric means for each treatment and the ratio of treatment relative to the control mean for plasma lipid levels are presented, along with their respective 95% confidence intervals. A 95% confidence interval for the ratio not containing one indicates a statistically significant difference at 5% level.

Analysis of VLDL production rate, postheparin plasma LPL activity and hepatic apoC-III mRNA levels were performed by analysis of variance including terms for treatment and cage. Means for each treatment and the difference of treatment means relative to the control mean are presented, along with their 95% confidence intervals. A 95% confidence interval of a difference not containing zero indicates a statistically significant difference at the 5% level.

RESULTS

The effect of Lovastatin, Gemfibrozil and SB204990 on plasma lipids and lipoproteins.

Feeding a Western-type diet markedly increased plasma cholesterol concentrations in APOE*3-Leiden mice as compared to animals fed a normal rodent chow (5.60 versus 2.64 mM) while triglycerides were unaltered (1.58 versus 1.77 mM, n=18). Two weeks after the switch to the Western-type diet, animals were put on diet supplemented with Lovastatin,

*Table I. The Effect of Lovastatin, Gemfibrozil and SB 204990 on Plasma Lipids of APOE*3-Leiden Mice.*

Compound (dose)	n	Cholesterol			Triglycerides		
		Geometric Mean	Ratio	95% C.I.	Geometric Mean	Ratio	95% C.I.
		mmol/l			*mmol/l*		
Control	18	5.60			1.58		
Lovastatin (0.05 %w/w)	9	5.20	0.93	(0.84, 1.03)	1.37	0.86	(0.73, 1.02)
Lovastatin (0.1 %w/w)	9	**4.50**	**0.80**	**(0.73, 0.88)[a]**	1.85	1.17	(0.99, 1.38)
Gemfibrozil (0.1 %w/w)	9	5.87	1.00	(0.92, 1.10)	**1.03**	**0.65**	**(0.55, 0.76)[a]**
Gemfibrozil (0.2 %w/w)	9	5.30	0.95	(0.86, 1.04)	**0.74**	**0.47**	**(0.40, 0.55)[a]**
SB 204990 (0.1 %w/w)	9	**4.36**	**0.78**	**(0.71, 0.86)[a]**	**1.11**	**0.70**	**(0.59, 0.83)[a]**
SB 204990 (0.2 %w/w)	9	**3.97**	**0.71**	**(0.65, 0.78)[a]**	**0.91**	**0.57**	**(0.49, 0.68)[a]**

n, number of animals; C.I., confidence interval. Male APOE*3-Leiden mice were fed for two weeks a Western diet supplemented with Lovastatin, Gemfibrozil, or SB 204990 at indicated doses. After a six hours fasting period, mice were bled and plasma cholesterol and triglycerides were determined. Geometric means for plasma cholesterol and triglyceride levels for each treatment and the ratio of treatment means to the control means are presented along with their 95% confidence intervals. [a]95% confidence interval for the ratio not containing one, indicating a statistically difference at 5% level (indicated in bold)

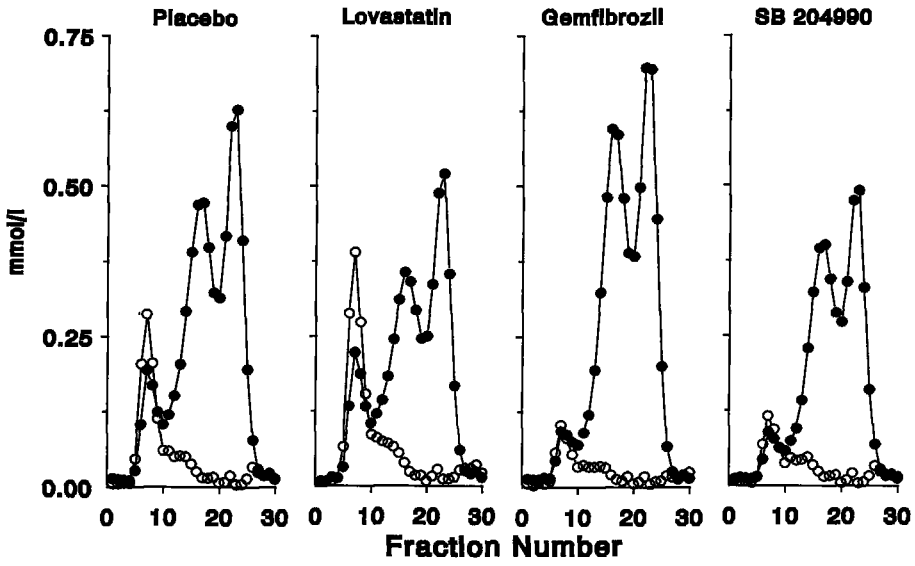


*Figure 1. The effect of Lovastatin, Gemfibrozil and SB 204990 on lipoprotein profile of APOE*3-Leiden mice fed a Western diet.* Pool plasma of three mice was applied to a Superose 6B column as described in the Methods section. Fractions were analyzed for cholesterol (●) and triglycerides (○). Lipoprotein profiles are shown for untreated Western fed APOE*3-Leiden mice and Western fed APOE*3-Leiden mice treated with Lovastatin (0.1 %w/w), Gemfibrozil (0.2 %w/w) and SB 204990 (0.2 %w/w). The profiles shown are the mean profiles of three seperate pools (six for the control group). Fractions 4-10, 11-20 and 21-27 correspond with VLDL, IDL/LDL and HDL, respectively.

Gemfibrozil or SB 204990 and the effects on plasma lipids were tested after a two week treatment period. The effects of hypolipidaemic drug therapy on plasma lipids are given in Table I. Compared with the control (no drug) group, Lovastatin showed a dose-related decrease in plasma cholesterol but the difference only reached statistical significance in the 0.1% w/w dose group (-20%). SB 204990 also gave a dose-related hypocholesterolaemic response, both dosing levels reaching statistical significance (up to 30% decrease compared with the no drug group). No effect on plasma cholesterol was seen in the animals treated with Gemfibozil. Interesting effects of the hypolipidaemic drugs were also seen on plasma triglyceride concentrations. Both Gemfibrozil and SB 204990 decreased plasma triglycerides in a dose-related manner (up to -53%) while no clear effects were seen in the groups treated with Lovastatin. Although in all drug treatment groups statistically significant effects on body weight development were seen (weight increments compared with the control group were 0.4-1.2 gram at the end of the 14 day dosing period) these differences were small in comparison with body weights (around 27 gram). No significant differences were seen in

food intake between any of the treatment groups and controls (average food intake around 12 gram/day per cage of 3 mice).

To investigate which lipoprotein classes are affected by the drug treatment, plasmas of mice were analyzed by high-performance gel filtration chromatography and results of these analyses are shown in Figure 1. Profiles in this figure represent means for 9 (18 for the control group) animals, analyzed in 3 (6 for the control group) plasma pools, each composed of 3 animals. Statistical analysis of the differences in lipoprotein lipids in comparison with the control (no drug) group showed that Lovastatin treatment significantly decreased LDL and HDL cholesterol at 0.1% w/w dosing level (by -22% and -18% respectively). SB 204990 significantly decreased VLDL cholesterol and triglycerides at the 0.1% and 0.2% w/w dosing levels (by up to -50% and -55% respectively) and decreased LDL and HDL cholesterol at the highest (0.2% w/w) dose (both by 20%). Gemfibrozil at both dosing levels significantly decreased VLDL cholesterol and triglycerides (by up to -55% and -60% respectively) but increased rather than decreased LDL and HDL cholesterol, although not statistically significant (+10%). Although not entirely identical to what would be predicted from documented effects of Lovastatin and Gemfibrozil therapy in human, clear parallels can be seen in lipoprotein changes in APOE*3-Leiden mice and men (Lovastatin's main effect on plasma (LDL) cholesterol and not on triglycerides; Gemfibrozil's main effect on plasma (VLDL) triglycerides with a small but non-significant rise in HDL cholesterol but also LDL cholesterol). Responses of the mice to SB 204990, an inhibitor of hepatic cholesterol and fatty acid synthesis, were also as anticipated, with clear dose-related decreases in plasma (VLDL and LDL) cholesterol and plasma (VLDL) triglycerides.

Effect of hypolipidaemic drugs on post-heparin plasma LPL activity, hepatic VLDL triglyceride production rate and hepatic apoC-III expression.

As Gemfibrozil and SB 204990 were found to induce major changes in plasma VLDL concentrations, the underlying mechanism of these effects was investigated in more detail. The major determinants of the plasma concentration of VLDL are the rate of VLDL triglyceride degradation, mediated by endothelial lipoprotein lipase, and the rate of secretion of VLDL by the liver. To investigate whether the hypotriglyceridaemic properties of Gemfibrozil and SB 204990 was due to increased VLDL triglyceride catabolism, groups of mice, exposed to the same dietary and drug regimen as described for the plasma lipid studies, were injected intravenously with heparin and post-heparin plasma LPL activity was determined as described in Methods. Results of these experiments are given in Table II. Neither Gemfibrozil nor SB 204990 administration resulted in a clear statistically significant change in post-heparin LPL activity, suggesting that the hypotriglyceridaemic properties of either drug are not well explained at the level of lipoprotein lipase activity.

To investigate whether the hypolipidaemic response to Gemfibrozil and SB 2044990 were related to changes in VLDL synthesis, hepatic VLDL triglyceride production rates were determined *in vivo* in animals exposed to a dietary and drug treatment regimen as described

for the plasma lipid studies. For hepatic VLDL triglyceride synthesis measurements animals received i.v. Triton WR 1339 as described in Methods and from the increase in plasma triglyceride levels over a period of up to 45 minutes, production rate were calculated. The results of those experiments are given in Table II. In APOE*3-Leiden control (no drug) mice on a Western-type diet the mean hepatic VLDL-triglyceride production rates was 0.125 mmol/hr/kg body weight. However, no clear effects of Gemfibrozil or SB 204990 on VLDL-triglyceride production rate were found (Table II), suggesting again that the hypotriglyceridaemic properties of either drugs are not well explained at the level of hepatic VLDL synthesis.

As neither lipoprotein lipase activity nor VLDL production rate could give a satisfactory explanation for the observed hypotriglyceridaemic responses, differences in the composition of VLDL particles between the controls and the drug treated animals were considered. VLDL apolipoprotein C-III abundancy is known to affect the rate of lipolysis by lipoprotein lipase (16,17). As the hepatic apoC-III gene mRNA was found to be decreased in rats after fenofibrate treatment (13), this option was investigated using the livers of animals used for the study shown in Table I and Fig 1. The results of these measurements are given in Table III. Once again, differences between mice in the control (no drug) and high dose Gemfibrozil or SB 204990 were small and not statistically significant.

*Table II. The Effect of Gemfibrozil and SB204990 on Post-Heparin Plasma LPL Activity and In Vivo Hepatic VLDL-Triglyceride Production Rate in APOE*3-Leiden Mice*

		Post-Heparin Plasma LPL Activity			Hepatic VLDL-Triglyceride Production Rate		
Compound (dose)	n	Mean	Difference	95% C.I.	Mean	Difference	95% C.I.
		mU/ml			*mmol/hr/kg body weight*		
Control	18	1141			0.125		
Gemfibrozil (0.1 %w/w)	8	1056	-85	(-353, 183)	0.112	-0.014	(-0.064, 0.037)
Gemfibrozil (0.2 %w/w)	10	1278	137	(-131, 405)	0.150	0.025	(-0.026, 0.075)
SB 204990 (0.1 %w/w)	8	1112	-29	(-297, 239)	0.126	0.001	(-0.050, 0.050)
SB 204990 (0.2 %w/w)	10	949	-192	(-460, 76)	0.114	-0.011	(-0.062, 0.040)

n, number of animals; C.I., confidence interval. Male APOE*3-Leiden mice were fed a Western diet were treated for two weeks a Western diet supplemented with Gemfibrozil or SB 204990 at indicated doses. After a six hour fasting period, mice received an intravenous injection of heparin and postheparin plasma LPL activity was determined (see methods). After one additional week of feeding the experimental diets, following a six hour fasting period mice were intravenously injected with Triton WR1339 and hepatic VLDL triglyceride production rate was determined (see Methods). Means for each treatment and the difference of treatment means relative to control means, with 95% confidence intervals are presented. A 95% confidence interval of a difference not containing zero indicates a statistically significant difference at the 5% level.

*Table III. The Effect of Gemfibrozil and SB204990 on Hepatic Mouse ApoCIII mRNA Levels of APOE*3-Leiden Mice.*

Compound (dose)	n	mRNA apoC-III/36B4		
		Mean	Difference	95% C.I.
		Arbitrary Units		
Control	8	2.13		
Gemfibrozil (0.2 %w/w)	8	2.15	-0.02	(-0.56, 0.60)
SB 204990 (0.2 %w/w)	8	1.73	-0.40	(-0.98, 0.18)

n, number of animals; C.I., confidence interval. Male APOE*3-Leiden mice were fed for two weeks a Western diet supplemented with Gemfibrozil or SB 204990, at indicated doses. After a six hour fasting period mice were bled for determination of plasma cholesterol and triglyceride levels (see Table I). Thereafter, mice were killed and liver was excised. Total liver RNA was isolated and 10 μg was used for norther blot analysis followed by hybridization with a ^{32}P-labeled probe of mouse apoCIII (see Methods). RNA levels are relative to internal standard 36B4. Means for each treatment and the difference of treatment means relative to control means, with 95% confidence intervals are presented. A 95% confidence interval of a difference not containing zero indicates a statistically significant difference at the 5% level.

Thus, APOE*3-Leiden transgenic mice exhibit significant hypolipidaemic responses in absence of a detectable effect on plasma LPL activity, hepatic VLDL production, or hepatic apoC-III mRNA expression.

DISCUSSION

For the development of hypolipidaemic and anti-atherosclerotic drugs, a small animal model in which both efficacy endpoints could be tested would be of great advantage. The APOE*3-Leiden mouse overexpresses a human dysfunctional apo E variant and develops hyperlipidaemia and atherosclerosis when fed lipid/cholesterol-enriched diets (3-6). In the present study, APOE*3-Leiden transgenic mice were evaluated as a testmodel for hypolipidaemic drugs by treating these mice with three types of hypolipidaemic compounds, Lovastatin, Gemfibrozil and SB 204990.

Lovastatin does not affect plasma cholesterol levels in normal mice and rats, due to a strong compensating upregulation of the 3-hydroxy-3-methylglutaryl-Coenzyme A reductase gene expression and the relative minor role of LDL receptors in the removal from plasma of apo B containing lipoproteins (18,19). Interestingly, this drug expressed a mild hypolipidaemic response in APOE*3-Leiden mice fed a Western-type diet. In the APOE*3-Leiden mice removal from plasma of remnants of triglyceride-rich lipoproteins is hampered due to the enrichment of dysfunctional apoE3-Leiden on these particle (3-5). Consequently, the importance of the LDL receptor in the removal of IDL and LDL may be increased and it is speculated that an increase in LDL receptor activity, induced by Lovastatin, may have greater impact on plasma IDL and LDL concentrations in apo E3-Leiden mice than in normal

animals.

The hypotriglyceridemic effect of Gemfibrozil in APOE*3-Leiden mice is in line with the observed hypotriglyceridemic response in normal mice (20) and rats (21). The possible mechanism by which Gemfibrozil, and fibrates in general, lower plasma triglycerides include upregulation of lipoprotein lipase activity (22,23), reducing VLDL synthesis (24,25) and down-regulation of hepatic apoC-III gene expression (13). In the present study, a clear hypotriglyceridemic response of this drug in APOE*3-Leiden mice was observed. However, this response could not well be explained by either one of these effects. Whether a combination of small changes in these parameters combined with others e.g. VLDL size and composition, are responsible for the hypotryglyceridaemic response remains to be seen. If so, plasma VLDL concentrations in APOE*3-Leiden mice may be very sensitive to small changes in triglyceride-rich lipoprotein metabolism, a conclusion supported by our earlier work with this model (4,5).

This sensitivity of plasma VLDL concentrations in APOE*3-Leiden mice is also evident when mice were treated with SB 204990. SB 204990 inhibits de novo cholesterol and fatty acid synthesis in rat hepatocytes and Hep G2 cells and decreases plasma cholesterol, triglycerides and the rate of VLDL synthesis in rats by up to 50% when mixed in the diet at levels of 0.05%-0.25% w/w (7). APOE*3-Leiden mice exhibit a clear hypotriglyceridaemic response to SB 204990 treatment but surprisingly, this response was not clearly explained by a reduced hepatic VLDL production rate. Post-heparin lipoprotein lipase activity was also unaltered as was the level of mRNA for apoC-III. Again, whether a combination of small changes in these parameters are responsible for the hypotriglyceridaemic response remains to be seen. Notwithstanding the lack of hard data to underpin the underlying mechanisms, APOE*3-Leiden mice seem to be susceptible for treatment with statins and fibrates as well as SB 204990, the lacton precursor of a potent inhibitor of ATP-citrate lyase. This responsiveness to hypolipidaemic therapy combined with the demonstration elsewhere (6) of a clear relationship between aortic lesion size and plasma cholesterol exposure, makes this mouse an attractive model for the testing of anti-atherosclerotic properties of hypolipidaemic drugs.

ACKNOWLEDGEMENTS

This research was supported by the Netherlands Heart Foundation and the Netherlands Organisation for Scientific Research (projects #900-504-092 and #900-539-117). Financial contribution of the specific RTD Programme of the European commission (BIOMED-2, #BMH4-CT96-0898) is gratefully acknowledged. Dr. Marten Hofker is an Established Investigator of the Netherlands Heart Foundation.

REFERENCES

1. Sukling, K.E., and Jackson, B. (1993) Prog. Lipid. Res. 32, 1-24
2. Sima, A., Bulla, A. and Simionescu, N. (1990) J. Submicrosc. Cytol. Pathol. 22, 1-16.
3. van den Maagdenberg, A.M.J.M., Hofker, M.H., Krimpenfort, P.J.A., de Bruijn, I.H., van Vlijmen, B.J.M., van der Boom, H., Havekes, L.M. and Frants, R.R. (1993) J. Biol. Chem. 268, 10540-10545.
4. van Vlijmen, B.J.M, van den Maagdenberg, A.M.J.M., Gijbels, M.J., van der Boom, H., HogenEsch, H., Frants, R.R., Hofker, M.H. and Havekes. L.M. (1994) J. Clin. Invest. 93, 1403-1410.
5. van Vlijmen, B.J.M., van 't Hof, H.B., Mol, M.J.T.M., van der Boom, H., van der Zee, A., Frants, R.R., Hofker, M.H. and Havekes, L.M. (1996) J. Clin. Invest. 97, 1184-1192.
6. Groot, P.H.E., van Vlijmen, B.J.M., Benson, G.M., Hofker, M.H., Schiffelers, R., Vidgeon-Heart, M. and Havekes, L.M. (1996) Ath. Tromb. Vasc. Biol. 16, 926-933.
7. Groot, P.H.E., Pearce, N.J., Yates, J.W., Gribble, A.D., Shaw, A.N., Tew, D.G., Camilleri, P., Wiggins, D. and Gibbons, G.F. (1994) Atherosclerosis. 109, 322
8. Nishina, P.M., Verstuyft, J. and Paigen, B. (1990) J. Lipid Res. 31, 859-869.
9. Bengtsson-Olivecrona, G. and Olivecrona, T. (1991) in Lipoprotein analysis (Converse, C. and Skinner, E.R. eds) pp 169-185, Oxford University Press, Oxford, England.
10. Peterson, J., Olivecrona, T., and Bengtsson-Olivecrona, G. (1985) Biochim. Biophys. Acta. 837, 262-270
11. Otway, S., and Robinson, D.S. (1967) J. Biol. Chem. 244, 4406-4412.
12. Bernstein, S.E. (1975) in Biology of the Laboratory Mouse. 2nd ed. (Green, L., ed.) pp 337-350, Dover Publications, Inc., New York.
13. Staels B., Vu-Dac, N., Kosykh, V.A., Saladin, R., Fruchart, J.C. and Dallongeville, J. (1995) J. Clin. Invest. 95, 705-712.
14. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
15. Laborda, J. (1991) Nucl. Acid. Res. 19, 3998.
16. Wang, C.S., McConathy, Kloer, H.U., and Alaupovic, P. (1985) J. Clin. Invest 75, 384-390.
17. Quarfordt, S.H., Michalopoulos, G., Schirmer, B. (1982) J. Biol. Chem. 257, 14642-14647.
18. Dueland, S., Drisko, J., Graf, L., Machleder, D., Lusis, A.J. and Davis, R.A. (1993) J. Lipid. Res. 34, 923-931.
19. Rudling, M. (1992) J. Lipid. Res. 33, 493-501.
20. Olivier, P., Plancke, M.O., Marzin, D., Clavey, V., Sauzieres, J. and Fruchart, J.C. (1988) Atherosclerosis. 70, 107-114.
21. Krause, B.R. and Newton, R.S. (1985) J. Lipid. Res. 26, 940-949.
22. Nikkilä, E.A., Huttunen, J.K. and Enholm, C. (1977) Metab. Clin. Exp. 26, 179-186.
23. Goldberg, A.P., Applebaum-Bowden, D.M., Bierman, E.L., Hazard, W.R., Haas, L.B., Sherrard, D.J., Brunzell, J.D., Huttunen, J.K., Enholm, C. and Nikkilä, E.A. (1979) N. Eng. J. Med. 301, 1073-1076.
24. Kosykh, V.A., Prodez, E.A., Novikov, D.K., Victorov, A.V., Dolbin, A.G., Repin, V.S. and Smirnov, V.N. (1987) Atherosclerosis. 68, 67-76.
25. Laker, M.E., and Mayes, P.A. (1979) Biochem. Pharmacol. 28, 2813-2827.

CHAPTER 7

THE EFFECTS OF DIETARY FISH OIL ON SERUM LIPIDS AND VLDL-KINETICS IN HYPERLIPIDEMIC APOLIPOPROTEIN E*3-LEIDEN TRANSGENIC MICE.

Bart J.M. van Vlijmen[1], Ronald P. Mensink[2], H. Belinda van 't Hof[1], Rene F.G. Offermans[2], Marten H. Hofker[3], and Louis M. Havekes[1,4]

[1]TNO Prevention and Health, Gaubius Laboratory, Leiden, [2]Department of Human Biology, Maastricht University, Maastricht, [3]MGC-Department of Human Genetics, Leiden University, Leiden, and [4]Departments of Cardiology and Internal Medicine, Academic Hospital Leiden, Leiden, The Netherlands.

Submitted

ABSTRACT

Studying the effect of dietary fish oil on VLDL metabolism in man, is subject to both large intra- and interindividual variability. In the present study we used hyperlipidemic apolipoprotein (APO) E*3-Leiden mice, with impaired chylomicron and VLDL remnant metabolism, to study the effects of dietary fish oil on serum lipids and VLDL kinetics under highly standardized conditions. Therefore, APOE*3-Leiden mice, were fed a fat and cholesterol containing diet supplemented with either 0, 3 or 6% w/w of fish oil. Fish oil fed mice showed a significant dose dependent decrease in serum cholesterol (up to -43%) and triglyceride levels (up to -60%), mainly due to a reduction of VLDL lipoproteins (-80%). LDL and HDL-cholesterol levels were not affected by fish oil feeding. VLDL-apoB kinetic studies showed that fish oil feeding results in a significant 2-fold increase in VLDL-apoB FCR, whereas hepatic VLDL-apoB production was unaffected. In addition, VLDL-triglyceride turnover studies revealed that fish oil significantly decreased hepatic VLDL-triglyceride production rate (-60%), whereas significant increase in VLDL-triglyceride FCR was observed (+70%). The increase in VLDL-triglyceride FCR was secondary to the decrease in VLDL-triglyceride production rate, and did not relate to increased lipolytic activity. We conclude that APOE*3-Leiden mice are highly responsive to dietary fish oil. The observed strong reduction in serum VLDL is primarily due to the decreasing effect of fish oil on hepatic VLDL triglyceride production rate and an increasing effect on VLDL-apoB FCR.

INTRODUCTION

Dietary fish oils, rich in long-chain n-3 fatty acids, lower plasma triglyceride and very low density lipoprotein (VLDL) levels in both normal and hyperlipidemic subjects (for review ref. 1). VLDL-triglyceride kinetic studies have been performed in humans showing that the hypotriglyceridemic effect of fish oil appears to be caused primarily by an inhibition of VLDL-triglyceride synthesis (2-5). However, effects on VLDL production and/or VLDL-triglyceride removal rate via increased lipoprotein lipolysis cannot be ruled out (2, 6). The increasing effect of fish oil supplementation on low density lipoprotein (LDL) cholesterol levels have been variable and somewhat confusing. In some studies, LDL levels decreased upon fish oil administration; in others they increased (for review see ref. 1). It has been suggested that n-3 fish oils increase plasma levels of LDL by reducing the number LDL receptors in liver and peripheral tissues (7-10), or by increasing the conversion of VLDL into LDL (11).

To date, most studies involving the metabolic consequences of dietary fish oil feeding were performed in humans. However, human studies are hampered by a highly variable response to dietary fish oil (1). Examining the metabolic effects of fish oil in an animal model may circumvent these problems, because studies can be carried out under highly

standardized conditions i.e. a homogenous genetic and environmental background.

Recently, we described the generation of transgenic mice expressing the human APOE*3-Leiden gene (12). These mice have an impaired clearance of chylomicron and VLDL remnant lipoproteins from the blood circulation by the liver. As a consequence, these mice have elevated plasma cholesterol and triglyceride levels due to increases in levels of VLDL/LDL-sized lipoproteins. In addition, in these mice the plasma cholesterol and triglyceride levels are highly responsive to small changes in chylomicron and VLDL production and clearance (13, 14). The "human-like" lipoprotein profile and the extreme sensitivity of plasma lipid levels to changes in VLDL metabolism suggested to us that APOE*3-Leiden mice may serve as an suitable animal model for studying in detail the metabolic consequences of fish oil feeding under highly standardized conditions.

The present study shows that the plasma lipid levels in APOE*3-Leiden transgenic mice are highly responsive to dietary fish oil. A strong reduction in the serum cholesterol and triglycerides was observed due to a reduction in serum levels of VLDL-sized lipoproteins. We found that dietary fish oil lowers serum VLDL levels primarily by a decreasing effect on hepatic VLDL triglyceride production rate, and secondly by an increasing effect on VLDL-apoB clearance rate.

METHODS

Animals

Female APOE*3-Leiden transgenic mice (line #2) were used (12). Transgenic mice were obtained by breeding with C57BL/6J mice (The Broekman Institute bv, Someren, The Netherlands). Mice of the F10 generation were included in the trial. Transgenic mice were identified by sandwich ELISA for the presence of human apoE in the serum as described previously (14). At the time of the study animals were 3-4 months of age.

Study design

The study had a parallel group design and included 2 experimental groups (10 animals per group, 2 dose levels) and 1 control group (12 animals). Animals were distributed over these groups, stratifying for a balanced age distribution. They were housed in shoebox cages (5-6 per cage) with hoppers that allowed feeding of a powdered diet. Three weeks prior to the experimental feeding, mice were fed a powdered semi-synthetic sucrose-rich diet, containing cocoa butter (15% w/w) and cholesterol (0.25% w/w). The diet was composed essentially according to Nishina et al. (15), and described by us earlier (14) (western-type or high fat/cholestrol (HFC) diet, Hope Farms, Woerden, The Netherlands). During the following four weeks mice were fed the same powdered diet to which 3% w/w or 6% w/w of fish oil (kindly provided by Hoffmann LaRoche GmbH, Basel, Switzerland) was added at the expense of cacao butter (for composition of experimental diets see table I). Experimental diets were stored at -20°C and mice were given a fresh aliquot daily at 5 pm. Animals had free access to food and water.

At time 0, after two weeks and at the end of the experimental period (4 weeks) mice were fasted from 8 am to 1 pm, weighed and approximately 200 μl of blood was obtained through tail-bleeding under diethylether anaesthesia. Some 70 μl of individual serum was immediately frozen in liquid nitrogen and stored at -80°C until lipid analysis. The remainder was used for lipoprotein analysis.

For determination of *in vivo* VLDL-apoB and VLDL-triglyceride turnover (methods see below), indicated parts of the experiment were repeated under identical conditions using mice with comparable

Table I. Composition of the Experimental Fish Oil Diets

Diet Components	Fish Oil		
	0 % w/w	3% w/w	6% w/w
		g/100 g diet	
Cocoa butter	15	12	9
Fish oil	-	3	6
Cholesterol	0.25	0.25	0.25
Sucrose	40.5	40.5	40.5
Cornstarch	10	10	10
Corn oil	1	1	1
Cellulose	5.95	5.95	5.95

In addition, all diets contained 20% casein, 1% choline chloride, 0.2% methionine, and 5.1% mineral mixture. All percentages are in weight/weight. Total energy and metabolic energy content of the three diets were 4620 and 4160 kcal/kg, respectively.

genetic background and age.

Lipid and lipoprotein analysis

Total serum cholesterol, triglycerides (without measuring free glycerol), free fatty acids were measured enzymatically using commercially available kits: #236691 (Boehringer Mannheim, Germany), #337-B (Sigma, Mo.), #994-75409 (Wako Chemicals GmbH, Neuss, Germany), respectively. Serum ß-hydroxybutyrate was determined as decribed (16).

For determination of the serum fatty acid composition some 100 μl of pooled serum was analyzed by gaschromatography as described. Total lipids from pooled serum samples were extracted according to standard procedure (17, 18). After saponification and methylation (19) the proportion of individual fatty acids in the diet, and in the serum phospholipids were determined with a Autosystem Perkin Elmer gaschromatograph fitted with a 50 m CP Sil 88 capillary column with an inner diameter of 0.25 mm and 0.20 μm film thickness. (Chrompack, Middelburg, the Netherlands). Helium (130 kPa) was used as a carrier gas. The oven temperature was programmed to stay at 160°C for ten minutes, then to rise to 190°C with a rate of 2.5°C/min, to keep this temperature for 20 min, to increase again at a rate of 4°C/min to 230°C and then kept constant. The temperature of the injector and the flame ionization detector were set at 300°C, while a split ratio of 1:20 was used. A standard mixture was used to identify the fatty acid methylesters by means of the retention times. Results were expressed as a proportion of total identified fatty acids. Butylated hydroxy-toluene (0.005 %, w/v, Sigma B1378) was added to all organic solvents to prevent oxidation of the polyunsaturated fatty acids.

For determining the serum lipoprotein distribution, some 100 μl of pooled serum (from 10 mice) was subjected to density gradient ultracentrifugation according to Redgrave et al. (20). After ultracentrifugation, the volume was fractionated in fractions of 0.5 ml and density was measured using a DMA 602M densitometer (Paar, Germany). Fractions with density $d < 1.006$, 1.006-1.019, 1.019-1.063, and 1.063-1.120 g/ml correspond to VLDL, IDL, LDL and HDL, respectively. After dialysis against PBS, containing 1 mM EDTA (pH 7.4), lipoprotein fractions were analyzed enzymatically for cholesterol and triglyceride content, using kits #236691 and #701904 (Boehringer Mannheim, Germany), respectively.

VLDL-apoB turnover studies: Isolation and labelling of VLDL

After a 5 hour fasting period, mice anaesthetized by diethylether and blood was collected from the retroorbital plexus. VLDL was isolated from serum of 6 control fed or 12 fish oil fed (3% w/w) female transgenic mice by density gradient ultracentrifugation. VLDL cholesterol and triglyceride were determined enzymatically as described above, and VLDL protein was determined using the method of Lowry (21). To determine apoB(100+48) content of the VLDL, some 5 μg of VLDL protein was subjected to a 4-20% gradient SDS-PAGE. After staining with Coomassie Brilliant Blue R and destaining in 30% methanol/10% acetic acid the gels were scanned using a HP ScanJet Plus, Hewlett Packard, Santa Clara, CA. and the amount of apoB(100+48) relative to total protein was calculated. For both VLDL samples (control and fish oil fed mice), the percentage of total apoB was app. 30% of total VLDL protein. VLDL was radiolabeled with ^{125}I by the iodine monochloride method (22). The fraction of ^{125}I-label present in apoB was determined by isopropanol precipitation (23, 24) and ranged from 30 to 40%.

VLDL-apoB turnover studies: In vivo removal of ^{125}I-labeled VLDL-apoB

Fasted mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 10 μg of ^{125}I-labeled VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t=2, 5, 10, 30, 60 and 90 minutes after injection. The serum content of ^{125}I-labeled apoB(100+48) was measured by isopropanol precipitation followed by counting ^{125}I-label. A bi-exponential model was used to estimate the area under the ^{125}I-apoB decay curve and subsequently to calculate VLDL-apoB kinetics. VLDL secretion rate was calculated from VLDL fractional catabolic rate and serum VLDL-apoB pool. Since more than 90% of serum triglycerides were associated with the d < 1.006 lipoprotein fraction, we calculated the serum VLDL-apoB pool by multiplying serum triglyceride levels at t=2 min after injection with the apoB/triglyceride ratio in the isolated d < 1.006 lipoproteins (VLDL) of mice from the same group (autologous).

VLDL-triglyceride turnover studies: Preparation of endogenously labeled VLDL.

^{3}H-palmitate dissolved in ethanol (Amersham) was evaporated under nitrogen and redissolved in 0.9% NaCl containing 2 mg/ml BSA. Fasted mice were injected intravenously via the tail vein with 100 μCi of the prepared ^{3}H-palmitate. 25 minutes after injection mice were anaesthetized by diethylether and bled from the retroorbital plexus. Radiolabeled VLDL used for clearance studies was isolated from serum of 6 control fed and 10 fish oil (3% w/w) fed mice by ultracentrifugation. Obtained VLDL samples were dialysed against PBS, pH 7.4 at 4°C. In both VLDL samples used, more than 95% of the radioactive label was bound to triglycerides.

VLDL-triglyceride turnover studies: In vivo removal of ^{3}H-triglyceride labeled VLDL

Fasted mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 80.000 dpm of ^{3}H-triglyceride labeled VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t=1, 2.5, 5, 7.5, 10, 12.5, 15, 30, and 60 minutes after injection. Total plasma radioactivity was used to represent VLDL-triglyceride radioactivity, since pilot study showed that the disappearance of radioactivity as measured after lipid extraction followed by TLC analysis did not differ from disappearance of total plasma radioactivity (not shown). As for VLDL apoB-turnover, a bi-exponential model was used to estimate the area under the ^{3}H-triglyceride decay curve and subsequent calculation of VLDL-triglyceride kinetics. VLDL secretion rate was calculated from VLDL fractional catabolic rate and serum triglyceride pool as measured in each mouse during the experiment.

Statistical analysis

The data were analyzed with the General Linear Models procedure of the Statistical Analysis System (SAS; SAS Institute Inc. SAS User's guide: Statistics Version 5 Edition. Cary, NC: SAS Institute Inc., 1985.). The outcome variables consisted of the changes between the start and the end of the study. When the analysis indicated a significant effect of diet ($P < 0.05$), the Tukey method was

used to adjust for multiple comparisons. As a results, only two-tailed P-values of less than 0.02 were considered significant. For the VLDL-kinetic studies, data were analyzed using non-parametric Mann-Whitney rank sum tests. P-values less than 0.05 were regarded as significant.

RESULTS

The effect of dietary fish oil on serum lipids and lipoprotein levels

The effects of dietary fish oil on serum cholesterol and triglyceride levels are presented in table II. Compared to control fed mice (*no fish oil*), fish oil fed mice showed a significant dose-dependent decrease in serum total cholesterol levels (up to -43%). In addition, a strong significant decrease in serum triglyceride levels (up to -60%) was observed. Unlike cholesterol levels, plasma triglyceride levels did not further decrease when the amount of fish oil in the diet was increased from 3 to 6% w/w.

The distribution of serum cholesterol and triglycerides over the lipoproteins was determined by density gradient ultracentrifugation (figure 1). The marked reduction in serum cholesterol on the fish oil diets was mainly confined to the VLDL fraction (-80%). However, a reduction in IDL-cholesterol was also observed, while LDL- and HDL-cholesterol levels were not affected by fish oil feeding. As expected, fish oil only reduced triglyceride levels in the VLDL fraction.

Serum cholesterol, triglycerides and lipoprotein distribution were also determined after two weeks of feeding. Results were not significantly different from those of the four-week data (data not shown).

Parallel to the decrease in serum triglyceride concentrations fish oil resulted significantly lowered serum free fatty acids levels by 30% (table III). Serum free glycerol levels were not significantly different between fish oil and control fed group. However, a slight but significant difference in serum free glycerol levels is observed for the 3 and 6% fish oil group (table III). Fish oil lowered the level of serum ß-hydroxybutyrate (table III). This

*Table II. The Effect of Dietary Fish Oil on Plasma Lipid Levels in APOE*3-Leiden Transgenic Mice*

	Serum Cholesterol			Serum Triglycerides		
Fish oil	0 wk	4 wk	$\Delta_{0\to4\ wk}$	0 wk	4 wk	$\Delta_{0\to4\ wk}$
	mmol/l			mmol/l		
0% w/w	9.43 ± 1.51	10.34 ± 1.28	0.91 ± 1.61	1.42 ± 0.41	1.50 ± 0.59	0.08 ± 0.57
3% w/w	8.99 ± 2.00	6.26 ± 1.08	-2.73 ± 1.20[a]	1.28 ± 0.49	0.63 ± 0.20	-0.65 ± 0.45[a]
6% w/w	9.44 ± 1.11	5.78 ± 0.46	-3.66 ± 0.95[a]	1.33 ± 0.32	0.61 ± 0.13	-0.73 ± 0.35[a]

$\Delta_{0\to4\ wk}$, difference in serum levels between 0 and 4 wk data. Mice were fed for 4 weeks a western (table I) diet to which was added indicated amount of fish oil. Fasted mice were bled and serum triglycerides and cholesterol were determined. Values are the mean of 10-12 mice ± SD. [a]$P<0.020$ indicating significantly different from control fed mice, using ANOVA with correction for multiple comparisons.

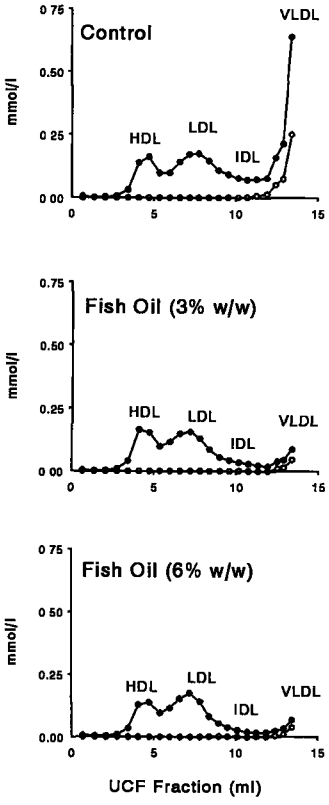


Figure. 1. Distribution of serum cholesterol and triglycerides among lipoprotein fractions. Lipoprotein fractions were separated by density gradient ultracentrifugation, and fractions were analyzed for cholesterol (●) and triglycerides (○). Lipoprotein profiles are shown for female APOE*3-Leiden mice fed a control diet (panel A), or a diet supplemented with 3% w/w fish oil (panel B) or 6% w/w dietary fish oil (panel C). Each run is performed with a fasted pool serum of at least 10 mice of the same group. Fractions with density d < 1.006, 1.006-1.019, 1.019-1.063, and 1.063-1.120 g/ml correspond to VLDL, IDL, LDL and HDL, respectively.

effect was already maximal at the 3% fish oil diet.

The fatty acid composition of the diets was reflected by the composition of the total serum lipids. Compared to control fed animals, the proportion of n-3 polyunsaturated fatty acids increased by 11.7% in the 3% fish oil and by 13.2% on the 6% fish oil fed group. The increase in n-3 polyunsaturated fatty acids was mainly compensated by decreases in n-6 poluunsaturated fatty acids (-8.9 and -11.2%, respectively) and monounsaturated fatty acids (-4.3 and -5.3%, respectively).

During the 4-week of experimental period, body weight increase were higher in the fish oil groups as compared to the control group (3.1 ± 1.8 (3% fish oil) and 2.0 ± 0.8 (6% fish oil) versus 1.4 ± 1.4 gram (control)). However, only the difference in changes between control and 3% fish reached statistical significance (P=0.007).

*Table III. The Effect of Dietary Fish Oil on Serum Free Fatty Acid, Free Glycerol and ß-Hydroxybutyrate Levels in APOE*3-Leiden Transgenic mice*

Fish oil	Free Fatty acids			Free Glycerol			ß-Hydroxybutyrate		
	0 wk	4 wk	$\Delta_{0\text{-}4\ wk}$	0 wk	4 wk	$\Delta_{0\text{-}4\ wk}$	0 wk	4 wk	$\Delta_{0\text{-}4\ wk}$
	mmol/l			*mmol/l*			*mmol/l*		
0% w/w	1.72 ± 0.18	1.74 ± 0.49	0.03 ± 0.50	0.67 ± 0.09	0.55 ± 0.11	-0.11 ± 0.11	4.49 ± 1.71	8.47 ± 3.28	3.97 ± 2.93
3% w/w	1.71 ± 0.13	1.27 ± 0.22	-0.44 ± 0.24[a]	0.60 ± 0.08	0.40 ± 0.08	-0.19 ± 0.08	4.08 ± 1.44	2.74 ± 0.96	-1.34 ± 0.99[a]
6% w/w	1.62 ± 0.17	1.17 ± 0.12	-0.44 ± 0.17[a]	0.51 ± 0.08	0.43 ± 0.06	-0.07 ± 0.06[b]	3.53 ± 0.98	3.12 ± 1.30	-0.41 ± 1.20[a]

$\Delta_{0\text{-}4\ wk}$, difference in serum levels between 0 and 4 wk data. Mice were fed for 4 weeks a western diet supplemented with indicated amount of fish oil. Fasted mice were bled and serum free fatty acids, free glycerol and ß-hydroxybuyrate were determined. Values are the mean of 10-12 mice ± SD. [a]$P<0.020$ indicating significantly different from control fed mice, using ANOVA with correction for multiple comparisons. [b]$P<0.020$, indicating significant difference between 3 and 6% w/w fish oil fed group, using ANOVA with correction for multiple comparisons.

*Table IV. The Effect of Dietary Fish Oil on VLDL-apoB and VLDL-Triglyceride Turnover in APOE*3-Leiden Transgenic Mice*

Fish oil	VLDL-apoB turnover			VLDL-triglyceride turnover			Lipolytic ratio*
	VLDL-apoB FCR	VLDL-apoB pool	VLDL-apoB SR	VLDL-TG FCR	VLDL-TG pool	VLDL-TG SR	
	pool/hr	*µg/g mouse*	*µg/hr/g mouse*	*pool/hr*	*µmol/g mouse*	*µmol/hr/g mouse*	
0% w/w	8.8 ± 1.3	3.4 ± 0.9	28.9 ± 5.7	36.1 ± 6.7	0.087 ± 0.037	3.2 ± 1.7	4.0
3% w/w	22.2 ± 4.6[a]	1.6 ± 0.9[a]	32.5 ± 17.1	60.4 ± 13.3[a]	0.033 ± 0.015[a]	1.3 ± 0.8[a]	2.7

FCR, fractional catabolic rate; SR, secretion rate; TG, triglyceride. VLDL kinetic studies were performed in female mice fed a control or a 3% w/w fish oil diet for two weeks (maximal reduction in serum VLDL levels were observed already under these conditions (not shown)). After 5 hour fasting period female mice were injected with either 10 µg of autologous labeled ^{125}I-labeled VLDL protein or 10^5 dpm autologous ^{3}H-TG-VLDL. ^{125}I-apoB and ^{3}H-TG-VLDL disappearance from circulation was determined and FCR and SR were calculated (see Methods). Values are the mean±SD of five mice per group. [a]$P<0.05$, indicating significant difference between control and fish oil fed mice, using nonparametric non-parametric Mann-Whitney tests. *Lipolytic ratio, as calculated from the mean VLDL-apoB FCR and VLDL-TG FCR.

Changes in serum cholesterol levels correlated positively with those in triglyceride levels ($r=0.64$, $P<0.001$), which reflected the strong reduction in VLDL (cholesterol+triglyceride) levels (figure 1). The changes in cholesterol and triglyceride levels correlated also positively with the changes in both free fatty acid levels ($r=0.54$, $P=0.0013$ and $r=0.51$, $P=0.003$) and ß-hydroxybutyrate levels ($r=0.70$, $P<0.001$ and $r=0.76$, $P<0.001$). In addition, a positive correlation was found between the decrease in serum free fatty acid and ß-hydroxybutyrate levels ($r=0.49$, $P=0.005$).

The effect of dietary fish oil on VLDL kinetics in APOE*3-Leiden mice

To investigate whether the strong reduction in VLDL cholesterol and triglycerides upon fish oil feeding was related to increased VLDL catabolism and decreased VLDL production rate, VLDL turnover studies were performed. The amount of fish oil in the diet was 3% (w/w), and feeding period was two weeks, as effects were already maximal under these conditions.

As shown in figure 2 (left panel) and presented in table IV, fish oil fed mice showed a significant more than 2-fold increase in VLDL-apoB clearance rate (VLDL-apoB FCR) as compared to control fed animals. Feeding a fish oil diet did not affect the VLDL-apoB secretion rate, indicating that the increase in VLDL clearance rate is the sole factor contributing to the observed 2-fold reduction in the VLDL-apoB pool upon fish oil feeding.

To study VLDL-triglyceride turnover, autologous VLDL was in vivo labeled in the triglyceride core using ^{3}H-palmitate. Fish oil fed mice showed a significant 60% reduction in VLDL-triglyceride production rate (table IV). Combining the respective mean VLDL-apoB

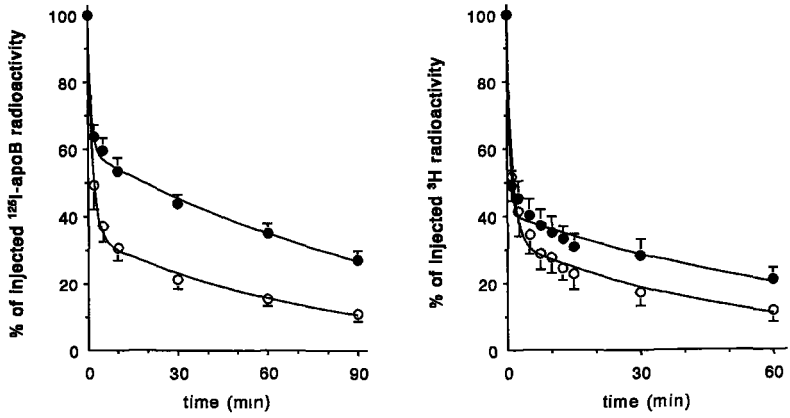


*Figure. 2. VLDL-apoB and VLDL-triglyceride removal in control and fish oil fed APOE*3-Leiden transgenic mice.* After a 5-hour fasting period, female APOE*3-Leiden transgenic mice fed two weeks a control (●) or fish oil (3% w/w) diet (○) were injected with autologous ^{125}I-labeled VLDL (left panel) or ^{3}H-palmitate labeled VLDL (right panel). 25 µl of blood was drawn at each time point and the respective ^{125}I-apoB(100+48) or ^{3}H radioactivity of the serum sample was measured. Values are the mean ± S.D. of five mice. Curves were calculated from the mean data using a bi-exponential curve fit model.

SR and mean VLDL-TG SR data, fish oil feeding resulted in the production of relative triglyceride-poor VLDL particles as compared to control feeding (0.04 versus 0.11 μmol TG/μg apoB), which is in line with the relative low-triglyceride content of VLDL isolated from fish oil fed mice compared to control mice, under fasted conditions (0.027 versus 0.032 μmol TG/μg apoB).

As illustrated in figure 2 (right panel) and table IV, VLDL-triglyceride FCR was significantly higher (1.7x) in fish oil fed mice as compared to control fed mice. The increase on the FCR of VLDL-triglycerides could have been secondary to the secretion of triglyceride poor VLDL and the consequent decrease in VLDL-triglyceride pool (-62%). Alternatively, the increase in VLDL-triglyceride FCR may partly also result from a stimulation of a VLDL-triglyceride clearance mechanism i.e. lipoprotein lipase activity. As calculated from the mean VLDL-apoB FCR and VLDL-triglyceride FCR, the triglyceride label was cleared 4-times faster than the apoB-label in the control group, whereas in fish oil group, the triglyceride-label was cleared only 2.7-times faster than the apoB-label (lipolytic ratio of 4.0 and 2.7 for control and fish oil fed group, respectively; table IV). Thus, fish oil feeding decreased the rate of VLDL-triglyceride clearance relative to the apoB clearance, indicating that fish oil does not extra stimulate the lipolytic rate.

DISCUSSION

In the present study, the effects of dietary fish oil on VLDL metabolism were investigated using hyperlipidemic APOE*3-Leiden transgenic mice. Besides a genetic homogenous background (inbred C57BL/6J), these mice have a "human-like" lipoprotein profile and are extremely sensitive to small changes in VLDL production and clearance rate (13, 14). Consequently, these mice may serve as a suitable animal model for studying the mechanisms underlying the effects of fish oil under highly standardized conditions.

Feeding a fish oil diet to APOE*3-Leiden transgenic mice resulted in a dramatic lowering of serum cholesterol and triglyceride levels, mainly due to a strong reduction in serum VLDL levels. This strong reduction in serum VLDL upon fish oil feeding was related to i) an increased clearance rate of the VLDL particles (figure 2, table IV), and ii) a reduction in hepatic VLDL-triglyceride production (table IV).

The decreasing effect of dietary fish oil on hepatic VLDL triglyceride production observed in APOE*3-Leiden has been reported previously for humans (2-5). Fish oil reduces hepatic fatty acid synthesis and stimulates hepatic fatty acid catabolism thereby reducing the amount of fatty acids available for VLDL synthesis (5). In addition, a reduced flux of fatty acids to the liver, as reflected in the reduced serum free fatty acid levels observed in fish oil fed APOE*3-Leiden mice (table III), may also contribute to the decrease in hepatic VLDL triglyceride production as well. Nestel et al. (2) showed in a study with five normolipidemic and one hypertriglyceridemic subjects that besides VLDL triglyceride also VLDL apoB production decreased upon fish oil feeding. Remarkably, in our study a concomitant

reduction in hepatic VLDL-apoB production rate was not observed. Whether this differential effect of fish oil on VLDL-apoB production in our mouse study as compared to the human study of Nestel et al. (2) is the result of the large difference in the amount of fish oil supplemented to the diet (3% versus 20-30%, respectively) remains to be established. Our results indicate that fish oil feeding at low dose, leads to production of normal amounts of VLDL particles, however the VLDL produced is triglyceride-poor. VLDL isolated from pool serum fish oil fed mice indeed had a reduced triglyceride to apoB ratio when compared to particles isolated from control fed mice (0.027 versus 0.032 μmol TG/μg apoB). Since, this difference was determined using VLDL particles isolated from fasted pool serum, it can be expected that analysis of nascent VLDL will yield larger differences.

Another reported effect of dietary fish oil is modulation of LDL receptor expression. Several *in vitro* (7,9) and *in vivo* studies (8) showed that n-3 fish oils reduce expression of LDL receptors in hepatic cells, suggesting a decrease in hepatic uptake of lipoproteins via the LDL receptor pathway upon fish oil feeding. In contrast, we show that fish oil feeding increases clearance rate of VLDL-apoB by 60%, suggesting that a possible decrease in hepatic LDL receptor expression does not affect VLDL-apoB clearance rate in APOE*3-Leiden mice. The mechanisms of how fish oil increases VLDL-apoB clearance rate is not clear. Possibly, the relative triglyceride-poor VLDL, as produced upon fish oil feeding, forms a better substrate for uptake via hepatic receptors than normal VLDL. This can be either via the (downregulated) LDL receptor or LDL receptor-related protein (LRP), the second receptor involved in chylomicron and VLDL remnant uptake. In addition, a stimulatory effect of dietary fish oil on the expression level of this LRP cannot be excluded.

It has been suggested that fish oil feeding may lower plasma triglycerides and increase plasma LDL levels by increasing lipolytic activity (6). The observation that VLDL-triglyceride fractional catabolic rate increases in fish oil fed mice (figure 2, table IV), may indicate a fish oil-mediated stimulation of lipolytic activity also in these mice. However, in our study, the increase in VLDL triglyceride FCR at least partly results from the observed decrease in serum triglyceride pool which primarily results from the observed decrease in VLDL triglyceride production. The serum VLDL-triglyceride removal independent from that of the VLDL-particle (=VLDL-apoB) was not increased by fish oil feeding (table IV), indicating that fish oil had no extra stimulatory effect on lipolytic activity. This was corroborated by the fact that we did not observe an increase in serum free fatty acid levels levels upon fish oil feeding. Treatment of APOE*3-Leiden mice with Gemfibrozil (van Vlijmen, unpublished) resulted in a strong reduction in serum VLDL cholesterol and triglycerides and a concomitant increase in LDL cholesterol due to a stimulation of the lipoplytic activity by this drug. The absence of such an increase of plasma LDL in APOE*3-Leiden mice fed the fish oil diet also argues against a stimulatory effect of fish oil on lipolytic activity.

Fish oil feeding often results in increased ketogenesis due to induction of ß-oxidation of fatty acids (25-27). Surprisingly, in APOE*3-Leiden mice fish oil feeding resulted in

decreased levels of serum ß-hydroxybutyrate, reflecting a decrease in ketogenesis. Our present data do not provide an explanation for this difference. However, interestingly, the decrease in serum ß-hydroxybutyrate levels strongly correlated with to the decrease in serum cholesterol, triglycerides and free fatty acid levels, suggesting that there may be causal relation. Future research focussing on the relation between serum ß-hydroxybutyrate levels and serum cholesterol, triglycerides and free fatty acid levels will clarify this.

The present study showed that the serum lipid levels of APOE*3-Leiden mice are highly responsive to dietary fish oil. The effects of fish oil observed for APOE*3-Leiden mice largely resembles the effects observed in (hyperlipidemic) humans. Our study shows that the hypolipidemic effect of fish oil primarily results from a decreasing effect on hepatic VLDL-triglyceride production, concomitant with an improved VLDL-apoB clearance. Our results do not provide evidence for a stimulatory effect of dietary fish oil on lipolytic activity.

The responsiveness of VLDL metabolism of APOE*3-Leiden mice to dietary fish oil feeding combined with the use of these mice as a sensitive atherosclerosis-model as reported elsewhere (13, 28), makes the APOE*3-Leiden mouse a suitable model for evaluating the effects of dietary fish oil on development of atherosclerosis.

ACKNOWLEDGEMENTS

This research was supported by the Netherlands Heart Foundation and the Netherlands Organisation for Scientific Research (projects #900-504-092 and #900-539-117). Financial contribution of the specific RTD Programme of the European commission (BIOMED-2, #BMH4-CT96-0898) is gratefully acknowledged. Dr. Marten Hofker is an Established Investigator of the Netherlands Heart Foundation.

REFERENCES

1. Harris, W.S. 1989. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. *J. Lipid. Res.* 30:785-807.
2. Nestel, P.J., W.E. Connor, M.F. Reardon, S. Connor, S. Wong, and R. Boston. 1984. Suppression by diets rich in fish oil of very low density lipoprotein production. *J. Clin. Invest.* 74:82-89.
3. Sanders, T.A.B., D.R. Sullivan, J. Reeve, and G.R. Thompson. 1985. Triglyceride-lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. *Atheriosclerosis.* 5:459-465.
4. Stacpoole, P.W., J. Alig, L.L. Kilgore, C.M. Ayala, P.N. Herbert, L.A. Zech, and W.R. Fisher. 1988. Lipodystrophic diabetes mellitus. Investigations of lipoprotein metabolism and effects of omega-3 fatty acid administration in two patients. *Metabolism.* 37:944-951.
5. Harris, W.S., W.E. Connor, D.R. Ilingworth, D.W. Rothrock, and D.M. Foster. 1990. Effects of fish oil on VLDL triglyceride kinetics in humans. *J. Lipid. Res.* 31:1549-1558.
6. Kasim-Karakas, S.E. 1995. Impact of n-3 fatty acids on lipoprotein metabolism. *Curr. Opin. Lipidol.* 6:167-171.
7. Wong, S., and P.J. Nestel. 1987. Eicosapentaenoic acid inhibits the secretion of triacylglycerol and of apoprotein B and the binding of LDL in HepG2 cells. *Atherosclerosis* 64:139-146.
8. Roach, R., D.A. Kambouris, D.L. Topping, R.P. Trimble, and P.J. Nestel. 1987. The effects of dietary fish oil on hepatic high density and low density lipoprotein receptor activities in the rat. *FEBS Lett.* 222:159-162.

9. Lindsey, S, A. Pronczuk, and K.C. Hayes. 1992. Low density lipoprotein from humans supplemented with n-3 fatty acids depresses both LDL receptor activity and LDLr mRNA abundance in HepG2 cells. *J. Lipid Res.* 33:647-658.
10. Linga, V., M.A. Leight, L.K. Curtiss, Y.L. Marcel, R.W. St. Clair, and J.S. Parks. 1994. Dietary fish oil-induced decrease in low density lipoprotein binding to fibroblasts is mediated by apolipoprotein E. *J. Lipid. Res.* 35:491-500.
11. Huff, M.W., and D.E. Telford. 1989. Dietary fish oil increases conversion of very low density lipoprotein apolipoprotein B to low density lipoprotein. *Arteriosclerosis 9*:58-66.
12. van den Maagdenberg, A.M.J.M., M.H. Hofker, P.J.A. Krimpenfort, I.H. de Bruijn, B.J.M. van Vlijmen, H. van der Boom, L.M. Havekes, and R.R. Frants. 1993. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 268:10540-10545.
13. van Vlijmen, B.J.M, A.M.J.M. van den Maagdenberg, M.J. Gijbels, H. van der Boom, H. HogenEsch, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1994. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* 93:1403-1410.
14. van Vlijmen, B.J.M., H.B. van 't Hof, M.J.T.M. Mol, H. van der Boom, A. van der Zee, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1996. Modulation of very low density lipoprotein production and clearance contributes to age- and gender dependent hyperlipoproteinemia in apolipoprotein E3-Leiden tansgenic mice. *J. Clin. Invest.* 97:1184-1192.
15. Nishina, P.M., J. Verstuyft, and B. Paigen. 1990. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J. Lipid Res.* 31:859-869.
16. Moore, J.J., M. Marcus, and S.M. Sax. 1982. Kinetic assay of ß-hydroxybutyrate in plasma with CobasBio centrifugal analyzer. *Clin. Chem.* 73:1334-1339.
17. Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497-509.
18. Bligh, E.D., W.J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-917.
19. Metcalfe, L.D., A.A. Schmitz, and J.R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* 38:514-515.
20. Redgrave T.G., Roberts D.C.D. and West C.E. Separation of plasma lipoproteins by density gradient centrifugation. *Anal. Biochem.* 1975; 65:42-49
21. Lowry, O.H. N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 249:5133-5162.
22. Bilheimer, D.W., S.Eisenberg, and R.I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* 260:212-221.
23. Kita, T., M.S. Brown, D.W. Bilheimer, and J.L. Goldstein. 1982. The metabolism of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. *Proc. Natl. Acad. Sci. USA.* 79:5693-5697.
24. Holmquist, L., K. Carlson, and L.A. Carlson. 1978. Comparison between the use of isopropanol and tetramethylurea for the solubilization and quantitation of human serum very low density apolipoproteins. *Anal. Biochem.* 88:457-460.
25. Wong, S.H., P.J. Nestel, R.P. Trimble, G.B. Storer, R.J. Illman, and D.L. Topping. The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. *Biochim. Biophys. Acta.* 792:103-109.
26. Bergseth, S., E.N. Christiansen, and J. Bremer. 1986. The effect of feeding fish oils, vegetable oils and clofibrtae on the ketogenesis from long chain fatty acids in hepatocytes. *Lipids.* 21:508-514.
27. Yamazaki, R.K., T. Shen, and G.B. Schade. 1987. A deit rich in n-3 fatty acids increases peroxisomal beta-oxidation activity and lowers plasma triacylglycerols without inhibiting glutathione-dependent detoxification activities in the rat liver. *Biochim. Biophys. Acta.* 920:62-67.
28. Groot, P.H.E., B.J.M. van Vlijmen, G.M. Benson, M.H. Hofker, R. Schiffelers, M. Vidgeon-Heart, and L.M. Havekes. 1996. Quantitative assessment of aortic atherosclerosis in APO E3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Ath. Tromb. Vasc. Biol.* 16:926-933.

CHAPTER 8

IN THE ABSENCE OF ENDOGENOUS MOUSE APOLIPOPROTEIN E, APOLIPOPROTEIN E*2(ARG158→CYS) TRANSGENIC MICE DEVELOP MORE SEVERE HYPERLIPOPROTEINEMIA THAN APOLIPOPROTEIN E*3-LEIDEN TRANSGENIC MICE.

Bart J.M. van Vlijmen[1], Ko Willems van Dijk[2], H. Belinda van 't Hof[1], Patrick J.J. van Gorp[2], André van der Zee[2], Hans van der Boom[1], Marco L. Breuer[3], Marten H. Hofker[2], and Louis M. Havekes[1,4]

[1]TNO Prevention and Health, Gaubius Laboratory, Leiden, [2]Department of Human Genetics, [3]Department of Molecular Carcinogenesis, Leiden University, Leiden, [4]Departments of Cardiology and Internal Medicine, Academic Hospital Leiden, Leiden, The Netherlands.

Reproduced from
The Journal of Biological Chemistry, 1996, 271:30595-30602
by copyright permission of the
American Society for Biochemistry and Molecular Biology

SUMMARY

Apolipoprotein E*2(Arg158→Cys) (APOE*2) transgenic mice were generated and compared to the previously generated apolipoprotein E*3-Leiden (APOE*3-Leiden) transgenic mice to study the variable expression of hyperlipoproteinemia associated with these two APOE variants. In the presence of the endogenous mouse *Apoe* gene, the expression of the APOE*3-Leiden gene resulted in slightly elevated levels of serum cholesterol as compared to control mice (2.7 ± 0.5 versus 2.1 ± 0.2 mmol/l, respectively), whereas the expression of the APOE*2(Arg-158→Cys) gene did not affect serum cholesterol levels, even after high/fat cholesterol feeding. The extreme cholesterol level usually found in apoE-deficient mice (*Apoe-/-* mice; 23.6 ± 5.0 mmol/l) could be rescued by introducing the APOE*3-Leiden gene (APOE*3-Leiden·*Apoe-/-*; 3.6 ± 1.5 mmol/l), whereas the expression of the APOE*2(Arg-158→Cys) gene in *Apoe-/-* mice minimally reduced serum cholesterol levels (APOE*2·*Apoe-/-*; 16.6 ± 2.9 mmol/l). *In vivo* very low density lipoprotein (VLDL) turnover studies revealed that APOE*2·*Apoe-/-* VLDL and APOE*3-Leiden·*Apoe-/-* VLDL display strongly reduced fractional catabolic rates (FCR) as compared to control mouse VLDL (4.0 and 6.1 versus 22.1 pools/hr). *In vitro* low density lipoprotein (LDL) receptor binding studies using HepG2 and J774 cells showed that APOE*2·*Apoe-/-* VLDL is completely defective in binding to the LDL receptor, whereas APOE*3-Leiden·*Apoe-/-* VLDL still displayed a considerable binding activity to the LDL receptor. After transfection of APOE*2·*Apoe-/-* and APOE*3-Leiden·*Apoe-/-* mice with adenovirus carrying the gene for the receptor associated protein (AdCMV-RAP), serum lipid levels strongly increased (15.3 to 42.8 and 1.4 to 15.3 mmol/l for cholesterol and 5.0 to 35.7 and 0.3 to 20.7 mmol/l for triglycerides, respectively). This indicates that RAP-sensitive receptors, possibly the LDL receptor-related protein (LRP), mediate the plasma clearance of both APOE*2·*Apoe-/-* and APOE*3-Leiden·*Apoe-/-* VLDL.

We conclude that *in vivo* the APOE*2 variant is completely defective in LDL receptor binding but not in binding to LRP, whereas for the APOE*3-Leiden mutant both LRP and LDL receptor binding activity are only mildly affected. As a consequence of this difference, APOE*2·*Apoe-/-* develop more severe hypercholesterolemia than APOE*3-Leiden·*Apoe-/-* mice.

INTRODUCTION

Apolipoprotein E is one of the major structural components of chylomicron and very low density lipoprotein (VLDL) remnants and serves as a ligand in the receptor mediated uptake of these particles from the blood by the liver (for review (1-3)). Mutant forms of apoE can lead to an impaired clearance and subsequent accumulation of remnant lipoproteins in the circulation. This condition is known as Familial Dysbetalipoproteinemia (FD) or Type III hyperlipoproteinemia (for review (2,4)), and can be inherited either as a recessive trait or as

a dominant trait. The recessive inheritance pattern occurs in FD patients carrying the APOE*2(Arg-158→Cys) mutation. Although about 1% of the population is homozygous for this defective APOE*2 allele, only a small percentage (4%) of these homozygous carriers develop hyperlipidemia indicating that secondary metabolic or genetic factors are required for clinical expression of disease. Several rare mutations show a dominant inheritance pattern, including APOE*3(Arg142→Cys), APOE*2(Arg145→Cys), APOE*1(Lys146→Glu), APOE*2(Lys146→Gln) and the APOE*3-Leiden mutation (a 7-amino acid tandem repeat of residues 120-126). Also in the case of the dominantly inherited forms of FD, additional environmental and genetic factors do modulate the severity of the disease (4,5).

Several groups have studied the biochemical characteristics of the different mutant forms of apoE. *In vitro* studies showed that apoE2(Arg158→Cys) was characterized by defective binding to the LDL receptor (2% of normal apoE3 binding activity), while binding to heparan sulphate proteoglycans (HSPG) and the LDL receptor related protein (LRP) were conserved (3,4,6). In contrast, mutant forms of apoE associated with the dominant mode of inheritance, showed different binding affinities to the LDL receptor (20-100% of normal E3 binding activity), but were defective in binding to HSPG and LRP (6). These *in vitro* studies suggested a correlation between the mode of inheritance of the specific apoE mutation and the binding to HSPG and LRP.

Previously, we reported the generation of apolipoprotein E*3-Leiden (APOE*3-Leiden) transgenic mice. These mice proved to be very useful in studying the (variable) expression of hyperlipoproteinemia associated with this dominant APOE variant *in vivo* (7-9). In the present paper we report the generation of APOE*2(Arg158→Cys) transgenic mice. We compared the *in vivo* functions of the recessive APOE*2 mutation with the dominant APOE*3-Leiden mutation using transgenic mice expressing these apoE variants either on a wild type apoE (*Apoe*+/+) or an apoE-deficient (*Apoe*-/-) background. We found that in the presence of the endogenous *Apoe* gene, the APOE*2 and APOE*3-Leiden gene indeed behave like a recessive and dominant mutation, respectively, like in humans. However, on an apoE deficient background, APOE*2 expressing mice displayed a much more severe hyperlipidemic phenotype than APOE*3-Leiden expressing mice. *In vivo* RAP adenovirus transfection experiments and *in vitro* LDL receptor binding studies showed that APOE*2 remnant lipoproteins were cleared via a RAP-sensitive receptor pathway only, most likely the LRP, whereas in APOE*3-Leiden mice remnant lipoproteins were cleared via both the RAP-sensitive pathway and the LDL receptor pathway.

METHODS

DNA construct

The APOE*2-HCR construct (Figure 1A) was generated from plasmid pJS276 kindly provided by Dr J.D. Smith (The Rockefeller University, New York, USA), carrying both the APOE gene (from the -650 bp *Bgl*II-site to the +1.9 kb *Hind*III-site) and a 5.5 kb *BamHI* fragment from the region adjacent to APOC-I', including the hepatic control region (HCR) (10). The APOE*2 gene was introduced into pJS276 by exchanging a 2 kb *EcoRI* fragment encompassing exon 4 of the APOE*3 gene with the similar fragment from a cosmid carrying APOE*2. The resulting insert (APOE*2-HCR) was excised from the plasmid using the restriction enzymes *Kpn*I and *Hind*III.

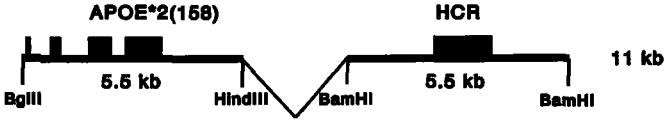


*Figure 1A. Schematic representation of the APOE*2-HCR construct used for microinjection.* 11-kb DNA construct used for microjection. APOE*2 and HCR sequences (solid black boxes) and relevant restriction sites are indicated (see methods).

Generation and analysis of transgenic mice

Transgenic mice expressing human APOE*3-Leiden were generated previously (11). These mice carry the human APOE*3-Leiden including an HCR fragment and are different from APOE*3-Leiden transgenic mice of the earlier described line 2 which co-express human apoCI (7-9). Transgenic and non-transgenic littermates were obtained by breeding with C57BL/6J mice (The Broekman Institute bv, Someren, The Netherlands). Mice of the F4 generation, were included in the experiments. Transgenic mice were identified by sandwich ELISA for the presence of human apoE in the serum (9). Transgenic mice, expressing human APOE*2 were generated according to Hogan et al. (12). Transgenic offspring were identified by polymerase chain reaction analysis and Southern-blot analysis on genomic tail-derived DNA (7). Three founders were obtained from which one line with high liver expression of the APOE*2 transgene was bred with C57BL/6J mice. Mice of the F3 generation have been used for the current experiments.

ApoE-deficient (*Apoe-/-*) mice were created as described previously (13-15). LDL Receptor-deficient (*Ldlr-/-*) mice were purchased from the Jackson laboratory (Bar Harbor, Maine). APOE*3-Leiden and APOE*2 transgenic mice were cross-bred with *Apoe-/-* mice to obtain APOE*2·*Apoe-/-* and APOE*3-Leiden·*Apoe-/-* mice. The resulting breeding offspring was analyzed for the presence of the transgenic human apoE protein by ELISA and the endogenous *Apoe-/-* genotype through tail tip DNA analysis, as described earlier (13).

For experiments, female mice 8-12 weeks of age were included. Mice were housed under standard conditions in conventional cages and given free access to food and water.

Diets

Mice were fed a regular mouse diet (SRM-A: Hope Farms, Woerden, The Netherlands). In case of dietary treatment, mice were fed for four weeks a semi-synthetic high fat/cholesterol diet (HFC/0.5%). This HFC/0.5% diet (purchased from Hope Farms) is a basic semi-synthetic diet, which was composed essentially according to Nishina et al. (16), supplemented with cocoa butter (15%, by weight), cholesterol (1%, by weight) and cholate (0.5%, by weight).

Human ApoE mRNA measurements

Total RNA was isolated from brain, heart, kidney, liver, muscle, skin and spleen using the

RNAZOL procedure (Cinna/Biotecx, Houston, TX). RNA samples (10 μg per lane) were separated by electrophoresis through a denaturing agarose gel (1.2% w/v) containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond N+, Amersham) according to the manufacturer's recommendations. Blots were subsequently hybridized with a ^{32}P-labeled probe of human APOE cDNA (17) and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA at 55°C in a solution containing 50% formamide. For liver tissue, the intensity of the hybridization signal was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amounts of APOE mRNA were related to the level of GAPDH mRNA.

Lipid, lipoprotein and apoE measurements

Mice were fasted from 8 am to 1 pm, weighted and approximately 150 μl of blood was obtained from each individual mouse through tail-bleeding. Total serum cholesterol and triglyceride levels (without measuring free glycerol) were measured enzymatically using commercially available kits: #236691 (Boehringer Mannheim, Mannheim, Germany) and #337-B (Sigma Chemicals Co., St. Louis, MO.).

Lipoprotein fractions were separated by FPLC chromatography using a 25-ml Superose 6B column as described previously (9).

For determination of serum mouse apoE concentrations, some 2 μl of serum was subjected to a 4-20% gradient SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) followed by incubation with polyclonal rabbit antisera against mouse apoE. Donkey anti-rabbit ^{125}I-IgG (Amersham, Litle Chalfont, UK) was used as a secondary antibody, and detection was performed by scanning the blots with a PhosphorImager. Mouse serum apoE level is expressed relative to the mouse apoE level of pooled serum of normal mice fed the standard chow diet. Human apoE concentrations were measured by sandwich ELISA as described previously (9).

Isolation, characterization and labeling of VLDL

After a 5-hour fasting period, blood was collected from 7-15 female mice (60 female mice in case of a wild type mice). Sera were pooled and ultracentrifuged to obtain the VLDL fraction ($d < 1.006$ g/ml). Total and free cholesterol, triglyceride (without glycerol) and phospholipid content of the VLDL were measured enzymatically, using commercially available kits (#236691 and #310328: Boehringer Mannheim, Mannheim, Germany; #337-B:Sigma Chemicals Co., St. Louis, MO; and 990-54009: Wako Chemicals GmbH, Neuss, Germany, respectively). VLDL protein was determined using the method of Lowry (18). To determine apolipoprotein composition of the VLDL, some 4.5 μg of VLDL protein was subjected to a 4-20% gradient SDS-PAGE. Proteins were either stained with Coomassie Brilliant Blue R or transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) followed by incubation with polyclonal rabbit antisera against mouse apoB, human apoE and mouse apoE. Donkey anti-rabbit ^{125}I-IgG (Amersham, Litle Chalfont, UK) was used as a secondary antibody, and detection was performed by scanning the blots with a PhosphorImager. The polyclonal rabbit anti-human apoE shows some cross-reaction with mouse apoE, at least in western blotting experiments. VLDL was radiolabeled with ^{125}I by the iodine monochloride method (19). The fraction of ^{125}I-label present in apoB(100+48) was determined by isopropanol precipitation (20,21) and ranged from 30 to 65% of total label. The isopropanol method, precipitates apoB100 and apoB48 completely, whereas other VLDL apolipoproteins are not precipitated. This was confirmed for the various mouse VLDL samples by apolipoprotein analysis of post-precipitation supernatant, using SDS-PAGE and subsequent Coomassie staining of the proteins as described above.
Human VLDL and LDL were isolated from fasted serum of healthy volunteers by density gradient ultracentrifugation as described by Redgrave et al (22).

VLDL kinetic studies

After a 5-hour fasting period, SRM-A fed female mice were intravenously injected with 0.2 ml

of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 10 μg of ^{125}I-labeled autologous VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t= 5, 10, 30, 60 and 90 minutes after injection. The serum content of ^{125}I-labeled apoB(100+48) was measured by isopropanol precipitation followed by counting ^{125}I-label in the pellet. A bi-exponential model was used to estimate the area under the ^{125}I-apoB decay curve and subsequent calculation of VLDL-apoB fractional catabolic rate (FCR).

Production rate of hepatic VLDL was determined by intravenous injection of Triton WR 1339 as described (9).

Binding of VLDL to HepG2 and J774 cells

HepG2 cells were cultured in 24-well plates as described (23). Twenty-four hours prior to each experiment, the cells were washed with DMEM containing 1% (v/v) human serum albumin (HSA) and further incubated with DMEM containing 5% (v/v) of lipoprotein deficient serum (LPDS d < 1.21 g/ml) instead of FCS.

The receptor-mediated binding of ^{125}I-labeled VLDL to the cells was determined after a 3 hour incubation at 4°C with indicated amounts of ^{125}I-labeled lipoprotein, either in the presence or in the absence of a 200 μg/ml excess of unlabeled lipoprotein or human VLDL, exactly as described earlier (23).

To study whether the respective VLDL samples bind the LDL receptor, competition experiments were performed using J774 cells (24). Therefore, J774 cells were incubated for 3 hours at 4°C with 10 μg/ml ^{125}I-labeled human LDL in the presence of indicated amounts of unlabeled VLDL lipoprotein samples. Thereafter, cells were washed and binding at 4°C was measured as described (24).

Adenovirus transfections

The generation of the recombinant adenoviral vectors expressing RAP (AdCMV-RAP) and LacZ (AdCMV-LacZ) under control of the CMV promotor has been described and were kindly provided by Dr. T. Willnow and Dr. J.Herz (25). The recombinant adenovirus was propagated and titrated on the Ad5 E1-transformed human embryonic kidney cell line 911 as described (26). For storage, the virus was supplemented with mouse serum albumin (0.2 %) and glycerol (10%), and aliquots were flash frozen in liquid N_2, and stored at -80°C. Routine virus titers of the stocks varied from 1 to $5x10^{10}$/ml.

For *in vivo* adenovirus transfection, on day zero, $1.5x10^9$ plaque forming units in a total volume of 200 μl (diluted with PBS) were injected into the tail vein of the SRM-A fed female mice. Fasted blood samples were drawn from the tail vein of fasted mice at 2, 3 and 4 days after virus injection.

RESULTS

Generation of transgenic mice

Three strains of APOE*2 transgenic mice were generated of which two strains showed high level expression of human apoE mRNA in the liver. One strain was used for further studies. Analysis of a series of different tissues by Northern blotting demonstrated that the expression of the APOE*2 transgene was mainly confined to the liver (figure 1B, top panel). Hepatic human APOE mRNA levels were about 30% higher in de APOE*2 transgenic mice as compared to the previously generated APOE*3-Leiden mice (100.0 ± 19.7 versus 67.9 ± 10.5 %). In addition to transgene expression in the liver, APOE*3-Leiden mice also express the transgene at high level in brain and at lower levels in the other tissues examined (figure 1B, bottom panel).

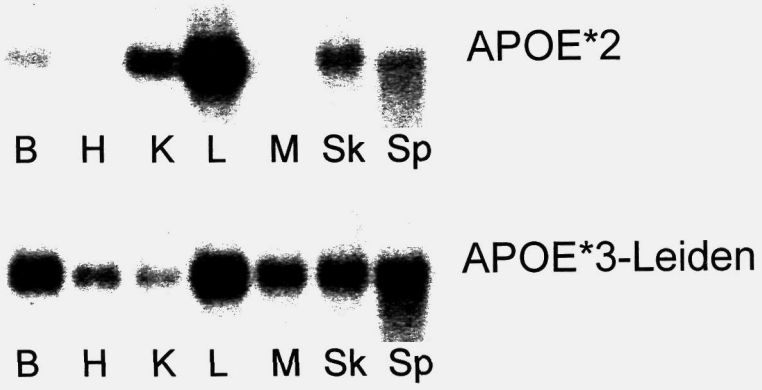


*Figure 1B. Tissue transgene expression pattern in APOE*2 and APOE*3-Leiden mice.* Total RNA was isolated from brain (B), heart (H), kidney (K), liver (L), muscle (M), skin (Sk) and spleen (Sp) of SRM-A fed female APOE*2·*Apoe*-/- (top panel) and APOE*3-Leiden·*Apoe*-/- mice (bottom panel). Some 10 μg was used for northern blot analysis followed by hybridization with a probe of human APOE cDNA and rat GAPDH cDNA as a reference (not shown).

Serum lipid, lipoprotein and apoE levels in various apoE transgenic mice

As presented in table I, APOE*3-Leiden mice show significantly elevated levels of serum cholesterol levels as compared to non-transgenic mice. This increase in serum cholesterol was confined to the VLDL/LDL-sized lipoprotein fractions (not shown). On the regular chow diet (SRM-A), APOE*2 transgenic mice did not show elevated serum cholesterol levels as compared to non-transgenic mice.

On a high/fat cholesterol diet, APOE*3-Leiden transgenic mice had two-fold higher serum cholesterol level as compared to non-transgenic mice, which was mainly due to increased levels of VLDL/LDL-sized lipoproteins (not shown). In contrast, serum cholesterol levels in cholesterol fed APOE*2 transgenic were similar to non-transgenic mice. Hence, in the presence of the mouse *Apoe* gene, APOE*2 transgenic mice are normolipidemic, even under dietary stress, whereas APOE*3-Leiden transgenic mice develop (diet-induced) hypercholesterolemia.

For studying the *in vivo* functional properties of the mutant apoE forms in absence of a functional mouse *Apoe* gene, APOE*2 and APOE*3-Leiden transgenic mice were cross-bred with *Apoe*-/- mice (13-15) (designated as APOE*2·*Apoe*-/- and APOE*3-Leiden·*Apoe*-/- mice, respectively).

On a regular SRM-A diet, the expression of the APOE*3-Leiden transgene resulted in an almost complete rescue of the extremely hypercholesterolemic phenotype usually found in *Apoe*-/- mice (table I). While in *Apoe*-/- mice the cholesterol was confined to the VLDL/LDL fractions, APOE*3-Leiden·*Apoe*-/- mice showed an equal distribution of

*Table I. The Effect of the Human APOE*3-Leiden and the APOE*2 Transgene on Serum Lipids, Lipoproteins and Apolipoprotein E Levels in Mice* With

		endogenous *Apoe* genotype						
		Apoe+/+				*Apoe-/-*		
diet	APOE transgene	TC	TTG	mouse apoE*	human apoE	TC	TTG	Human apoE
		mmol/l	*mmol/l*	*R.U.*	*mg/dl*	*mmol/l*	*mmol/l*	*mg/dl*
SRM-A	-	2.1 ± 0.2	0.5 ± 0.2	1.0 ± 0.2	-	23.6 ± 5.0	0.5 ± 0.3	-
SRM-A	APOE*3-Leiden	2.7 ± 0.5‡	0.8 ± 0.4	1.0 ± 0.3	2.7 ± 0.4	3.6 ± 1.5‡	0.3 ± 0.2	0.5 ± 0.1
SRM-A	APOE*2	2.1 ± 0.2§	0.6 ± 0.2	1.0 ± 0.3	0.9 ± 0.2§	16.5 ± 2.9‡§	2.4 ± 0.8‡§	9.2 ± 0.8§
HFC/0.5%	-	6.5 ± 1.1	0.1 ± 0.0	1.6 ± 0.6	-	nd	nd	nd
HFC/0.5%	APOE*3-Leiden	13.7 ± 2.5‡	0.2 ± 0.1‡	2.4 ± 0.7	4.5 ± 0.4	nd	nd	nd
HFC/0.5%	APOE*2	6.8 ± 1.5§	0.1 ± 0.1‡	1.6 ± 1.0	1.1 ± 0.2§	nd	nd	nd

Female mice of 2-3 months of age were fed a SRM-A diet or a HFC/0.5% diet. After 4 weeks of feeding, mice were fasted and bloodsamples were drawn from the tail vein. Total serum cholesterol (TC), triglycerides (TTG) and ApoE(mouse and human) values are the mean ± S.D. of 4-7 in case of mice with the *Apoe+/+* background or 15-18 mice in case of the *Apoe-/-* background. R.U., relative units; nd, not determined. *Mouse apoE levels are expressed relative to mouse apoE levels present in pool serum of normal female mice fed the regular SRM-A diet. ‡$P<0.05$, significantly different from non-transgenic mice fed the same diet, using nonparametric Mann-Whitney tests. §$P<0.05$, indicating significant difference between APOE*3-Leiden and APOE*2 transgenic on the same diet and the same Apoe genotype, using non-parametric Mann-Whitney tests.

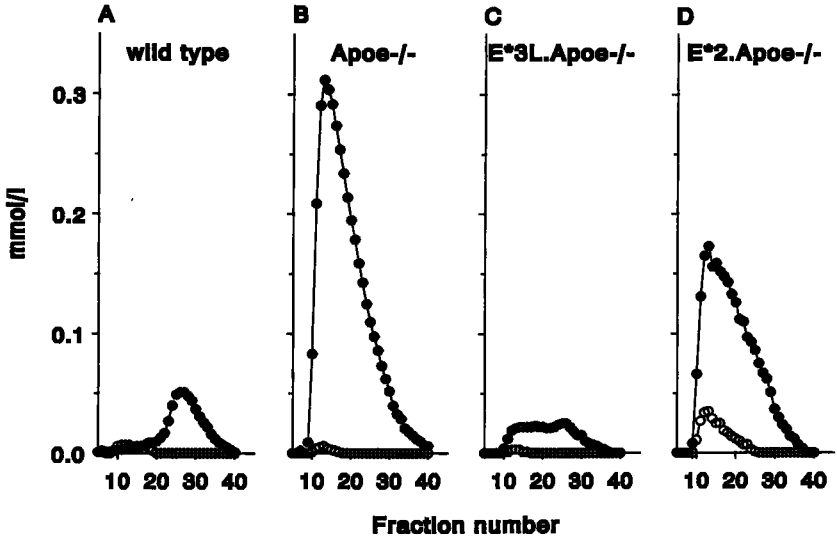


Figure 2. Distribution of serum cholesterol and triglycerides among lipoprotein fractions. Lipoprotein fractions were separated by FPLC permeation chromatography using a 25-ml Superose 6B column, and fractions were analyzed for cholesterol (●) and triglycerides (○). Lipoprotein profiles are shown for SRM-A fed female wild type (panel A), *Apoe-/-* (panel B), APOE*3-Leiden·*Apoe-/-* (panel C) and APOE*2·*Apoe-/-* SRM-A fed mice (panel D). Each run is performed with a fasted pool serum of at least 12 mice of the same group. Fraction numbers 10-23 and 24-40 correspond to VLDL/LDL and HDL, respectively.

cholesterol in both VLDL/LDL and HDL-sized lipoprotein fractions (figure 2). However, APOE*2·*Apoe-/-* mice were severely hypercholesterolemic and, in addition, showed a relatively mild hypertriglyceridemia. The increased levels of serum cholesterol and triglycerides were confined to the VLDL/LDL-sized lipoprotein fractions (figure 2). Strikingly, serum human apoE levels in APOE*2·*Apoe-/-* mice were much higher than in APOE*2·*Apoe*+/+ mice (9.2 versus 0.9 mg/dl). Opposite, APOE*3-Leiden·*Apoe-/-* mice had lower human apoE levels than APOE*3-Leiden·*Apoe*+/+ mice (0.5 versus 2.7 mg/dl).

Composition of d < 1.006 lipoproteins isolated from the apoE transgenic mice

From the various SRM-A fed female apoE transgenic mice the d < 1.006 g/ml (VLDL) were isolated by density gradient ultracentrifugation and lipid and apolipoprotein compositions were determined. As shown in table II, VLDL isolated from hyperlipidemic APOE*3-Leiden·*Apoe*+/+ mice were 2-fold higher in free and esterified cholesterol and phospholipids as compared to VLDL isolated from *Apoe*+/+ (wild type) and APOE*2·*Apoe*+/+ mice. Strikingly, in the absence of endogenous mouse apoE, all VLDL samples were strongly enriched in cholesterol (free plus esterified) and phospholipids and contained less triglycerides. Although reduced, triglyceride content of APOE*2·*Apoe-/-*

Table II. The Lipid Composition of the D < 1.006 Lipoproteins

human APOE transgene	endogenous *Apoe* genotype									
	Apoe+/+					*Apoe-/-*				
	TC	CE	FC	TTG	PL	TC	CE	FC	TTG	PL
	μmol/mg protein					*μmol/mg protein*				
-	2.0	0.7	1.3	9.1	1.3	20.0	14.8	5.2	0.3	3.0
APOE*3-Leiden	4.9	2.6	2.3	9.2	2.3	22.3	17.0	5.2	2.2	3.9
APOE*2	2.3	1.1	1.2	7.7	1.2	20.6	14.5	6.1	5.3	4.7

SRM-A fed female mice (>10) were fasted and bled via orbital puncture. D < 1.006 lipoproteins were isolated from pool serum by density gradient ultracentrifugation. D < 1.006 fraction was analyzed for total, free and esterified cholesterol, triglycerides and phospholipids. TC, total cholesterol; CE, cholesterol ester; FC, free cholesterol; TTG, total triglycerides; PL, phospholipids.

VLDL was still high when compared to *Apoe-/-* and APOE*3-Leiden·*Apoe-/-* VLDL and is in line with the observed high serum triglyceride levels observed in these mice (table I).

As shown in figure 3, VLDL isolated from the different *Apoe+/+* (transgenic) mice all contained both apoB100 and apoB48. After crossbreeding to endogenous *Apoe* gene deficiency, VLDL contained no (*Apoe-/-*), hardly (APOE*3-Leiden·*Apoe-/-*) or little (APOE*2·*Apoe-/-*) apoB100 as compared to mice with the respective *Apoe+/+* background.

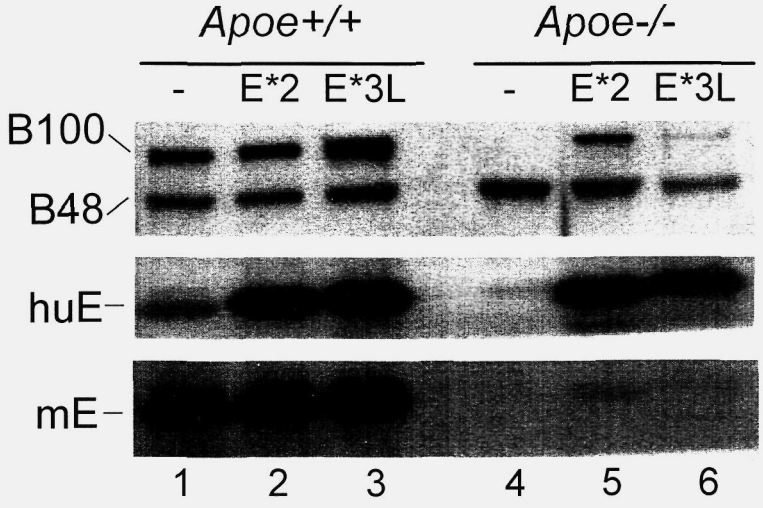


Figure 3. Western blot analysis of the d < 1.006 lipoproteins of the various apoE transgenic mice. D < 1.006 lipoproteins were isolated from fasted pool serum of SRM-A fed female wild type (*Apoe+/+*, lane 1), APOE*2·*Apoe+/+* (lane 2), APOE*3-leiden·*Apoe+/+* (lane 3), *Apoe-/-* (lane 4), APOE*2·*Apoe-/-* (lane 5) and APOE*3-Leiden·*Apoe-/-* mice (lane 6). 4.5 μg of VLDL-protein was subjected to SDS-gel electrophoresis (4-20% gradient gels) and transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antisera against mouse apoB (B100 and B48), human apoE (huE) and mouse apoE (mE). Note: The polyclonal rabbit anti-human apoE shows some cross-reaction with mouse apoE.

APOE*2·*Apoe-/-* VLDL was relatively rich in human apoE when compared to APOE*3-Leiden·*Apoe-/-* VLDL.

VLDL-apoB kinetics in various apoE transgenic mice.

To study the underlying mechanism of the different hyperlipoproteinemias in the SRM-A fed female *Apoe-/-*, APOE*3-Leiden·*Apoe-/-* and APOE*2·*Apoe-/-* mice, *in vivo* VLDL-apoB kinetic studies were performed. Mice were injected with 10 μg of autologous ^{125}I-labeled VLDL, and the ^{125}I-apoB disappearance from the circulation was determined. VLDL-apoB clearance rate was clearly reduced in all apoE transgenic mice (figure 4, table III) in the

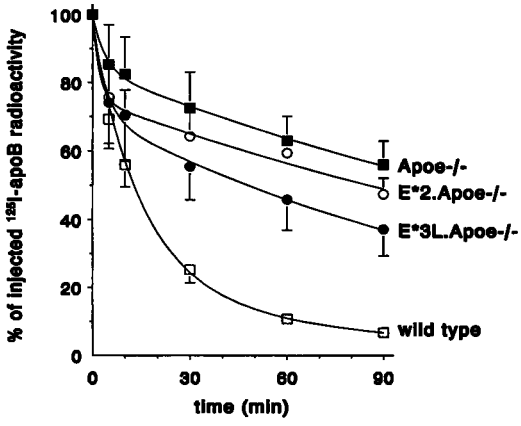


*Figure 4. VLDL-apoB removal in wild type, Apoe-/-, APOE*3-Leiden·Apoe-/- and APOE*2·Apoe-/- mice.* After a 5-hour fasting period, SRM-A fed female wild type (□), *Apoe-/-* (■), APOE*3-Leiden·*Apoe-/-* (●) and APOE*2·*Apoe-/-* (○) mice were injected with autologous ^{125}I-labeled VLDL. 25 μl of blood was drawn at each time point and ^{125}I-apoB(100+48) radioactivity of the serum sample was measured. Values are the mean ± S.D. of seven mice. Curves were calculated from the mean data using a bi-exponential curve fit model.

order: wild type >> APOE*3-Leiden·*Apoe-/-* > and APOE*2·*Apoe-/-* > *Apoe-/-* mice. To investigate whether an increase in VLDL production contributes to the observed accumulation of VLDL-sized lipoproteins, we determined hepatic VLDL-triglyceride production rate directly from serum triglyceride increase after injection of Triton WR 1339. *Apoe-/-* and APOE*3-Leiden·*Apoe-/-* mice had a significant two-fold reduction in hepatic VLDL triglyceride production rate as compared to wild type mice, whereas APOE*2·*Apoe-/-* mice had a hepatic VLDL production rate comparable to wild type mice (table III).

The above described results indicate that the accumulation of VLDL-sized lipoproteins in APOE*2·*Apoe-/-* mice was due to a strong decreasing effect on VLDL clearance. In contrast, the mild accumulation of VLDL-sized lipoproteins observed in APOE*3-Leiden·*Apoe-/-* mice seems to be due to a reduced VLDL clearance that is partly compensated by a reduction in VLDL production rate.

*Table III VLDL-apoB Fractional Catabolic Rates (FCR) and In Vivo Hepatic VLDL Triglyceride Production Rate (PR) in APOE*3-Leiden and APOE*2 Transgenic Mice Without Endogenous Mouse Apoe Alleles*

mouse	VLDL-apoB FCR	VLDL-triglyceride PR
	pool/hr	*mmol/hr/kg mouse*
wild type	22.1 ± 3.4‡	0.136 ± 0.044‡
Apoe-/-	3.2 ± 0.7*	0.076 ± 0.023*
APOE*3-Leiden·*Apoe-/-*	6.1 ± 1.9*,‡	0.077 ± 0.017*
APOE*2·*Apoe-/-*	4.0 ± 0.5*,‡	0.128 ± 0.027‡

After 5 hour fasting period SRM-A fed female mice were injected 10 μg of autologous labeled ^{125}I-labeled VLDL protein. ^{125}I-apoB(100+48) disappearance from circulation was determined and FCR was calculated (see Methods). For determining hepatic VLDL production rate fasted SRM-A fed female mice were injected with Triton WR1339. Fasted serum triglycerides were determined just before injection (0 min) and at 30 and 60 min after Triton injection. Production of hepatic triglyceride production rate was calculated from the slope of the curve and is expressed as mmol/h/kg mouse. Values are the mean±SD of 6-7 mice per group. *$P<0.05$, significantly different from mice with the wild type mouse *Apoe* allele, using nonparametric Mann-Whitney tests. ‡$P<0.05$, significantly different from *Apoe-/-* mice, using nonparametric Mann-Whitney tests.

Binding of VLDL isolated from the various apoE transgenic mice to HepG2 and J774 cells.

To study whether the observed decrease in VLDL FCR in the apoE transgenic mice was due to a reduced binding efficiency of VLDL to hepatic cells, we determined the receptor-mediated binding of the VLDL lipoproteins to HepG2 cells. As shown in figure 5, the specific binding of the VLDL was reduced in all transgenic mice as compared to VLDL isolated from wild type mice. Binding efficiency was in the order: wild type > APOE*3-Leiden·*Apoe-/-* > APOE*2·*Apoe-/-* = *Apoe-/-*, and corresponds with the order observed for VLDL FCR (figure 4, table III) and level of hypercholesterolemia (table I).

The interaction of remnant lipoproteins with hepatic cells include the LDL receptor and the LRP. Figure 6 shows that unlabeled VLDL isolated from wild type mice was most efficient in competing with ^{125}I-labeled human LDL for the binding to J774 cells, whereas VLDL isolated from APOE*3-Leiden·*Apoe-/-* mice was a less efficient in this respect. VLDL isolated from *Apoe-/-* and APOE*2·*Apoe-/-* mice did not compete with ^{125}I-labeled LDL for binding to the J774 cells at all. Thus, apoE deficient VLDL and VLDL containing apoE2 as the sole apoE protein cannot bind to the LDL receptor.

Treatment of various apoE transgenic mice with adenovirus containing RAP cDNA

To investigate whether remnant lipoproteins in the respective apoE transgenic mice were cleared via the LRP, RAP was overexpressed via injection of recombinant adenovirus

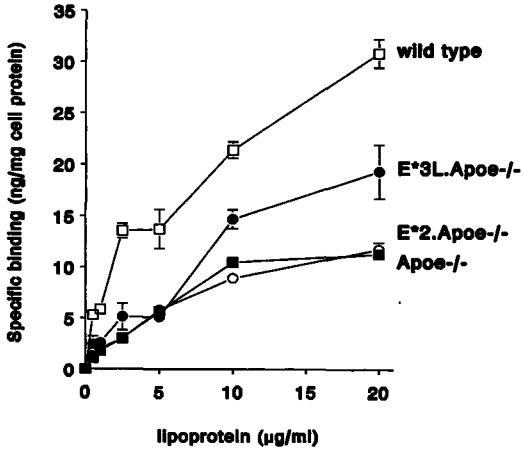


*Figure 5. Binding of VLDL isolated from wild type, Apoe-/-, APOE*3-Leiden·Apoe-/-and APOE*2·Apoe-/- mice.* The binding of VLDL isolated from fasted serum of SRM-A fed female wild type (□), *Apoe-/-*(■), APOE*3-Leiden·*Apoe-/-* (●) and APOE*2·*Apoe-/-* (○) mice to HepG2 cells was measured upon incubation of the cells with indicated amounts of labeled lipoprotein at 4°C for a period of 3 hours. Binding was determined as described in 'Methods'. Values represent the mean ± S.D. of three measurements..

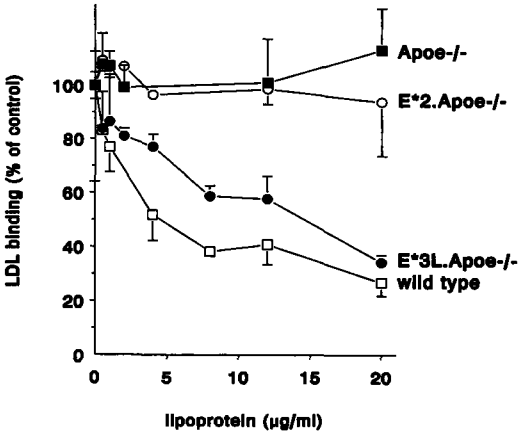


*Figure 6. Competition for binding of ^{125}I-labeled human LDL to J774 cells by VLDL isolated from wild type, Apoe-/-, APOE*3-Leiden·Apoe-/- and APOE*2·Apoe-/-mice.* Competition studies were performed by incubating J774 cells with 10 µg/ml of ^{125}I-labeled LDL for 3 hours at 4°C in the presence of the indicated amounts of VLDL isolated from fasted serum of SRM-A fed female wild type (□), *Apoe-/-* (■), APOE*3-Leiden·*Apoe-/-* (●) and APOE*2·*Apoe-/-* (○) mice. The binding is expressed as percentage of the value in the absence of competitor, and is determined as described in 'Methods'. Values represent de mean ± S.D. of four measurements.

containing RAP cDNA (AdCMV-RAP) in the various mice. It has previously been shown that injection of AdCMV-RAP efficiently blocks ligand clearance via the LRP (25). Injections of adenovirus containing LacZ (AdCMV-LacZ) were used as control. Four days after AdCMV-RAP injections, LDL receptor deficient (*Ldlr-/-*) mice showed a strong hypercholesterolemic and mild hypertriglyceridemic response, indicating a strong RAP-mediated inhibition of the RAP-sensitive receptors, likely the LRP (Table IV). Both APOE*3-Leiden·*Apoe-/-*and APOE*2·*Apoe-/-* and not *Apoe-/-* mice displayed a strong hypercholesterolemic response to the AdCMV-RAP injection, whereas all three lines showed

Table IV. Serum Lipids and Human ApoE Levels in LDL Receptor-deficient and Various ApoE Transgenic Mice Before and After Adenovirus-mediated Overexpression of Receptor Associated Protein (RAP)

mouse	AdCMV-LacZ				AdCMV-RAP			
	n	TC	TG	apoE	n	TC	TG	apoE
		mmol/l	*mmol/l*	*mg/dl*		*mmol/l*	*mmol/l*	*mg/dl*
Ldlr-/-	2	9.1 ± 0.0	1.5 ± 0.2	-	5	23.4 ± 10.8	3.1 ± 1.0	-
Apoe-/-	2	25.6 ± 2.4	0.8 ± 0.2	-	5	27.5 ± 4.2	21.5 ± 5.1	-
APOE*3-Leiden·*Apoe-/-*	2	1.4 ± 0.1	0.3 ± 0.0	1.7 ± 0.1	3	15.3 ± 2.5	20.7 ± 4.5	19.1 ± 6.0
APOE*2·*Apoe-/-*	2	15.3 ± 0.1	5.0 ± 0.2	21.8 ± 4.2	5	42.8 ± 6.0	35.7 ± 7.2	53.3 ± 3.8

SRM-A fed female *Ldlr-/-* and apoE transgenic mice were intravenously injected with AdCMV-RAP (± 1.5 x 10^9 PFU) or AdCMV-LacZ (± 1.5 x 10^9 PFU). Four days after injection mice were bled and fasted serum lipids and apoE were determined. Values are the mean ± SD of indicated number of mice. TC, total serum cholesterol; TG, total serum triglycerides; apoE, human apolipoprotein E; n, number of mice.

a strong hypertriglyceridemic effect upon AdCMV-RAP transfection. In addition, the transgenic mice displayed a strong increase in serum human apoE levels upon AdCMV-RAP transfection. These results indicate that RAP-sensitive receptors recognize both apoE2 and apoE3-Leiden proteins.

DISCUSSION

Previously, we used transgenic mice to study the dominant APOE*3-Leiden mutation (7-9). APOE*3-Leiden transgenic mice exhibited a hyperlipoproteinemic phenotype and proved to be very useful in studying the role of subtle environmental and genetic factors in the expression of hyperlipidemia (8,9) and the development of atherosclerosis (8,27). In the present study, APOE*2(Arg158→Cys) mice were generated and compared to the previously generated APOE*3-Leiden transgenic mice, both when expressed on a wild type apoE or on an apoE deficient background. A detailed *in vivo* characterisation of both the APOE*2(Arg158→Cys) and APOE*3-Leiden mutant in mice may help to better understand the differential expression pattern of FD associated with both APOE variants.

In the present study we showed that APOE*3-Leiden expression in mice can lead to a hyperlipidemic phenotype, already in the presence of normal functioning mouse *Apoe* genes, whereas APOE*2 expressing mice exhibit a hyperlipidemic phenotype only in the complete absence of the normal mouse *Apoe* gene. This implies that in mice the APOE*3-Leiden and APOE*2 mutation behave as a dominant and recessive mutation, respectively, as they do in humans.

The present *in vivo* and *in vitro* data show that apoE2 is unable to bind to the LDL receptor, whereas apoE3-Leiden still exhibits a considerable binding activity to this receptor. These observations are in line with earlier results from *in vitro* studies (2-4,6). Ji et al. (6) concluded that apoE2 binds to the LRP *in vitro* comparable to the wild type isoform apoE3, whereas apoE3-Leiden appeared to be rather defective in this respect (20% of apoE3 binding). Our present data using adenovirus-RAP transfections indicated that *in vivo* both apoE3-Leiden and apoE2 do bind to the LRP. Thus, in spite of a considerable LRP binding, the loss of LDL receptor binding activity of the APOE*2 variant leads to a severely impaired remnant lipoprotein clearance and, consequently, a massive hyperlipidemia. For the APOE*3-Leiden variant both LRP and LDL receptor binding activity are largely conserved, leading to only a mild hyperlipidemia.

The APOE*2·*Apoe-/-* mice can be compared with E2E2 homozygous subjects as far as their apoE genotype is concerned. However, at least two remarkable differences in hyperlipidemic phenotype became apparent: (i) in humans only a small proportions (4%) of E2E2 homozygotes become hyperlipidemic (2), whereas in the present study all APOE*2·*Apoe-/-* mice exhibited hyperlipidemia, and (ii) in APOE*2·*Apoe-/-* mice the level of plasma cholesterol and triglyceride is extremely high as compared to their hyperlipidemic human counterparts. ApoE-deficient mice also exhibit extreme hypercholesterolemia (13-15)

as compared to apoE-deficient humans (28-30). For apoE-deficient mice this extreme hyperlipidemia is assumed to be due to the hepatic editing of apoB100 that occurs in mice and not in humans (31-34). Indeed, an important role of apoB100 as alternative ligand mediating hepatic remnant clearance is clearly illustrated by the complete absence of apoB100 in VLDL accumulating in the plasma of apoE-deficient mice (figure 3, (13-15)). Such a reduced availability of apoB100 could also explain the extreme phenotype found in APOE*2·*Apoe-/-* mice relative to E2E2 humans. However, VLDL isolated from APOE*2·*Apoe-/-* transgenic mice did contain relatively high levels of apoB100 (figure 3), indicating that in APOE*2·*Apoe-/-* mice apoB100 is not effective as alternative ligand for remnant clearance (figure 4, table III) via binding to LDL receptor (figure 6). Since in these mice, the VLDL remnants contain relatively high amounts of apoE, we hypothesise that a high apoE2 content per remnant particle somehow hampers the action of apoB100 as alternative ligand in remnant clearance. Such a mechanism might also be an important aggravating factor in the clinical expression of FD in E2E2 subjects.

Several lines of evidence emerged arguing for an important inhibitory role of apoE in the process of triglyceride lipolysis *in vitro* (35-36) and *in vivo* (37). The current observation that APOE*2·*Apoe-/-* transgenic mice display hypertriglyceridemia in addition to high plasma VLDL cholesterol and apoE levels (table I), suggests that high levels of apoE2 per VLDL particle prevents efficient VLDL-triglyceride lipolysis *in vivo* as well. This is in line with the earlier findings in humans that the metabolic conversion of VLDL into LDL is hampered in E2E2 subjects (38,39).

Serum human apoE levels in APOE transgenic mice strongly relate to the level of hyperlipidemia in these mice (table I), since it is a major constituent of the accumulating remnants (figure 3). Similar is observed for APOE*3-Leiden/CI transgenic mice (9) and FD subjects. Remarkably, for APOE*3-Leiden·*Apoe-/-* mice serum apoE levels are very low despite a considerable accumulation of remnant lipoproteins. Until now we do not have an explanation for this striking observation.

Ji et al (6) studied the interaction of several mutant forms of apoE with heparan sulphate proteoglycans (HSPG). HSPG may facilitate the interaction of remnant lipoproteins with the LRP for internalization by hepatic cells (40). It was found that dominant apoE mutants, including APOE*3-Leiden, were defective in binding to HSPG. However, the current observation that APOE*3-Leiden mice exhibit only a mild hyperlipidemia, irrespective of the presence or absence of endogenous mouse apoE, suggests that the impaired interaction of APOE*3-Leiden with HSPG is not of major importance *in vivo*, as far as the development of hyperlipidemia is concerned. In *Apoe-/-* mice, the plasma clearance of remnants via binding to HSPG cannot occur because of complete absence of apoE. Thus, the fact that APOE*2·*Apoe-/-* mice exhibit extreme hypercholesterolemia comparable to *Apoe-/-* mice, whereas apoE2 itself does bind to HSPG (6), also argues against an important role of HSPG in plasma clearance of VLDL remnants.

We found that APOE*3-Leiden·*Apoe-/-* mice and *Apoe-/-* mice had a decreased

production rate of hepatic VLDL-triglyceride (table III). Whether the APOE*3-Leiden mutant or the absence of hepatic apoE synthesis affect the production of VLDL in the liver will be subject for further investigation. Remarkably, APOE*2·*Apoe-/-*, APOE*3-Leiden·*Apoe-/-* and *Apoe-/-* mice displayed a dramatic increase in serum triglyceride levels after adenovirus-mediated RAP overexpression (table IV). In *Apoe-/-* mice neither the LDL receptor nor the LRP are involved in the clearance of the VLDL remnants accumulated in the plasma. The observation that RAP transfection leads to an extreme hypertriglyceridemia in these *Apoe-/-* mice suggests that RAP, somehow, inhibits VLDL-triglyceride lipolysis independent of both the LDL receptor and LRP pathway. RAP overexpression in LDL receptor-deficient mice also leads to mice in which both the LDL receptor and LRP activity are eliminated. The observation that these mice do not display hypertriglyceridemia upon RAP treatment, suggests that endogenous mouse apoE is able to circumvent the suggested RAP-mediated inhibition of lipolysis. Such an escape then could not be obtained by either apoE3-Leiden or apoE2 (table IV). An inhibitory effect of RAP on VLDL-triglyceride lipolysis has not been reported before and is currently under further investigation.

Since APOE*3-Leiden and APOE*2 appeared to behave as a dominant and recessive mutation, respectively, in transgenic mice as well, we conclude that the differential expression of FD associated with these apoE mutants can also be studied in mice. From the present results it is strongly suggested that a difference between the two apoE mutants in binding efficiency to HSPG does not represent a major cause for the different expression patterns of FD associated with these apoE mutants in humans. Experimental evidence is accumulating for an important role of apoE in *in vivo* lipolysis of VLDL triglyceride. It is striking that in mice the E2E2 genotype displays a complete penetrance of hyperlipoproteinemia, which is in strong contrast to humans where E2E2 homozygosity exhibits a low penetrance for hyperlipidemia (4%). Further analyses of the differences between humans and mice in this respect might help finding the factors involved in VLDL remnant clearance, a process which is commonly assumed to be of major importance in the western societies regarding the risk of development of early atherosclerosis. More specifically, these analyses might help finding major aggravating factors involved in the clinical expression of FD.

ACKNOWLEDGEMENTS

We are grateful to drs T. Willnow and J. Herz for providing the recombinant adenoviral vectors expressing RAP and LacZ. We thank Mr A. van der Zee for technical assistance and Mr. J.L.S. van Rijn, Mrs I. Nieuwenhuizen and I. Rietbroek for animal care.

This research was supported by the Netherlands Heart Foundation and the Netherlands Organisation for Scientific Research (projects #900-504-092 and #900-539-117). Financial contribution of the specific RTD Programme of the European commission (BIOMED-2, #BMH4-CT96-0898) is gratefully acknowledged. Dr. Marten Hofker is an Established

Investigator of the Netherlands Heart Foundation.

REFERENCES

1. Mahley, R.W. (1988) *Science.* **240,** 622-630
2. Mahley, R.W. and Rall, S.C., Jr. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., eds) 6th Ed., pp. 1195-1213, McGraw-Hill, Inc., New York
3. Mahley, R.W., Innerarity, T.L., Rall, S.C.,Jr., Weisgraber, K.H., and Taylor, J.M. (1990) *Curr. Opin. Lipid.* **1,** 87-95
4. Rall, S.C., Jr., and Mahley, R.W. (1992) *J. Intern. Med.* **231,** 653-659
5. de Knijff, P., van den Maagdenberg, A.M.J.M., Stalenhoef, A.F.H., Gevers Leuven, J.A., Demacker, P.N.M., Kuyt, L.P., Frants, R.R., and Havekes, L.M. (1991) *J. Clin. Invest.* **88,** 643-655
6. Ji, Z.-S., Fazio, S., and Mahley, R.W. (1994) *J. Biol. Chem.* **269,** 13421-13428
7. van den Maagdenberg, A.M.J.M., Hofker, M.H., Krimpenfort, P.J.A., de Bruijn, I.H., van Vlijmen, B.J.M., van der Boom, H., Havekes, L.M., and Frants, R.R. (1993) *J. Biol. Chem.* **268,** 10540-10545
8. van Vlijmen, B.J.M, van den Maagdenberg, A.M.J.M., Gijbels, M.J., van der Boom, H., HogenEsch, H., Frants, R.R., Hofker, M.H., and Havekes, L.M. (1994) *J. Clin. Invest.* **93,** 1403-1410
9. van Vlijmen, B.J.M., van 't Hof, H.B, Mol, M.J.T.M., van der Boom, H., van der Zee, A., Frants, R.R., Hofker, M.H., and Havekes, L.M. (1996) *J. Clin. Invest.* **97,** 1184-1192
10. Shachter, N.S., Zhu, Y., Walsh, A., Breslow, J.L., and Smith, J.D. (1993) *J. Lipid. Res.* **34,** 1699-1707
11. Jong, M.C., Dahlmans, V.E.H., van Gorp, P.J.J., Breuer, M.L., Mol, M.J.T.M., van der Zee, A., Frants, R.R., Hofker, M.H., and Havekes, L.M. (1996) *Ath. Thromb. Vasc. Biol.* **16,** 934-940.
12. Hogan, B., Constantini, F., and Lacey, E. (1986) *Manipulating the Mouse Embryo: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
13. van Ree, J.H., van den Broek, J.A.A., Dahlmans, V.E.H., Groot, P.H.E., Vidgeon-Hart, M., Frants, R.R., Wierenga, B., Havekes, L.M., and Hofker, M.H. (1994) *Atherosclerosis.* **111,** 25-37
14. Zhang, S.H., Reddick, R.L., Piedrahita, J.A., and Maeda, N. (1992) *Science* **258,** 468-471
15. Plump, A.S., Smith, J.D., Hayek, T., Aalto Setälä, K., Walsh, A., Verstuyft, J.G., Rubin, E.M., and Breslow, J.L. (1992) *Cell* **71,** 343-353
16. Nishina, P.M., Verstuyft, J., and Paigen, B. (1990) *J. Lipid Res.* **31,** 859-869
17. Zannis, V.I., McPherson, J., Goldberger, G., Krathanasis, S.K., and Breslow, J.L. (1984) *J. Biol. Chem.* **259,** 5495-5499
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* **249,** 5133-5162
19. Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) *Biochim. Biophys. Acta.* **260,** 212-221
20. Holmquist, L., Carlson, K., and Carlson, L.A. (1978) *Anal. Biochem.* **88,** 457-460
21. Kita, T., Brown, M.S., Bilheimer, D.W., and Goldstein, J.L. (1982) *Proc. Natl. Acad. Sci. USA.* **79,** 5693-5697
22. Redgrave T.G., Roberts, D.C.D., and West, C.E. *Anal. Biochem.* (1975) **65,** 42-49
23. Mulder, M., de Wit, E., and Havekes, L.M. (1991) *Biochim. Biophys. Acta* **1081,** 308-314.
24. Hendriks, W.L., van der Boom, H., van Vark, L.C., and Havekes, L.M. (1996) *Biochem. J.* **314,** 563-568.
25. Willnow, T.E., Sheng, Z., Ishibashi, S., and Herz, J. (1994) *Science.* **264,** 1471-1474
26. Fallaux, F.J., Kranenburg, O., Cramer, S.J., Houweling, A., van Ormondt, H., Houben, R.C., and van der Eb, A.J. (1996) *Hum. Gene Ther.* **7,** 215-222
27. Groot, P.H.E., van Vlijmen, B.J.M., Benson, G.M., Hofker, M.H., Schiffelers, R., Vidgeon-Heart, M., and Havekes, L.M. (1996) *Ath. Tromb. Vasc. Biol.* **16,** 926-933.
28. Ghiselli, G., Schaefer, E.J., Gascon, P., and Brewer, H.B., Jr. (1981) *Science.* **214,** 1239-1241
29. Brewer, H.B., Jr., Zech, L.A., Gregg, R.E., Schwartz, D., and Schaefer, E.J. (1983) *Ann. Intern. Med.* **98,** 623-640
30. Schaefer, E.J., Gregg, R.E., Ghiselli, G., Forte, T.M., Ordovas, J.M., Zech, L.A., and Brewer, H.B., Jr. (1986) *J. Clin. Invest.* **78,** 1206-1219
31. Lusis, A.J., Taylor, B.A., Quon, D., Zollman, S., and LeBoeuf, R.C. (1987) *J. Biol. Chem.* **262,** 7594-7604
32. Higuchi, K., Kitagawa, K., Kogishi, K., and Takeda, T. (1992) *J. Lipid. Res.* **33,** 1753-1764

33. Chen, S.H., Habib, G., Yang, C.Y., Gu, Z.W., Lee, B.R., Weng, S., Silberman, S.R., Cai, S.J., Deslypere, J.P., Rosseneu, M., Gotto, A.M., Jr., Li, W.H., and Chan, L. (1987) *Science.* **238,** 363-366
34. Powell, L.M., Wallis, S.C., Pease, R.J., Edwards, Y.H., Knott, T.J., and Scott, J. (1987) *Cell.* **50,** 831-840
35. McConathy, W.J., Wang, C.-S. (1989) *FEBS Letters* **251,** 250-252
36. Mulder, M., van der Boom, H., de Knijf, P., Braam, C., van den Maagdenberg, A., Gevers Leuven, J.A., and Havekes, L.M. (1994) *Atherosclerosis.* **108,** 183-192
37. Rensen, P.C.N., and van Berkel, Th.J.C. (1996) *J. Biol. Chem.* **271,** 14791-14799.
38. Enholm, C., Mahley, R.W., Chappel, D.A., Weisgraber, K.H., Ludwig, E., and Witztum, J.L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5566-5570
39. Demant, T., Bedford, D., Packard, C.J., and Sheperd, J. (1991) *J. Clin. Invest.* **88,** 1490-1501
40. Ji, Z.-S., Fazio, S., Lee, Y.-L., and Mahley, R.W. (1994) *J. Biol. Chem.* **269,** 2764-2772.

CHAPTER 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In Western societies people live under non-fasting conditions for most of their lifespan. Therefore, it is commonly assumed that in these societies an increased level of remnant lipoproteins is a main contributor to the high risk of atherosclerosis.

In the present thesis we investigated the environmental and genetic factors that may influence remnant lipoprotein metabolism. A better insight into the mechanism of remnant lipoprotein metabolism will eventually lead to new strategies in lowering the prevalence of cardiovascular diseases.

Since humans are heterogenous in both genetic and environmental background (nutrition), we decided to perform our studies with the use of transgenic mice. However, since mice display very low plasma lipid levels and the metabolism of remnant lipoproteins is very rapid in these animals, we decided to generate mice that were less efficient in remnant lipoprotein clearance. Defective remnant lipoprotein clearance was achieved in mice by introducing human APOE variants (like APOE*3-Leiden and APOE*2) associated with Familial Dysbetalipoproteinemia. As presented in this thesis, these mutant APOE transgenic mice proved to be valuable tools for studying factors that modulate remnant lipoprotein metabolism.

The results described in this thesis show that overproduction of lipoproteins is a major determinant of the expression of hyperlipidemia in mutant APOE transgenic mice (chapter 2 and 4). High fat/cholesterol intake was certainly expected to stimulate intestinal lipoprotein production and to contribute to the accumulation of remnant lipoproteins. However, (over)production of VLDL, under normal feeding conditions, stressed remnant lipoprotein metabolism as well. In mice the level of VLDL production was age-and gender-related. Although in humans the role of (over)production of hepatic VLDL is less clear, body weight gain, uncontrolled diabetes mellitus and excessive alcohol consumption, which are known to stimulate VLDL production in humans, are strong aggravating factors in the accumulation of VLDL remnants (Mahley and Rall, 1989). In the light of this evidence, it is worthwhile to investigate all aspects of VLDL production in more detail, in order to develop new drugs that modulate VLDL production. At present, the assembly and secretion of VLDL by the liver is only partly understood. The assembly of VLDL is thought to involve the synthesis of apoB and its subsequent association with lipids, like triglycerides, cholesterol and phospholipids. Initially, a small HDL-like VLDL precursor is obtained, which is further

filled up with triglycerides through the action of microsomal triglyceride transport protein (MTP) (for review see Yao, 1994 and Dixon, 1993). Therefore, apoB and MTP, are commonly assumed to be important targets for direct therapeutic intervention. In addition, elucidation of new factors that are important in chylomicron and VLDL production may provide additional new targets for therapeutic intervention. A probable candidate is apoE, which has been reported to play a role in VLDL secretion as well (Ellsworth, 1986; Gretch; 1995; Fazio, 1995, Kuipers, 1995).

Lipolytic processing of chylomicrons and VLDL is an important first step in their catabolism leading to the formation of remnants lipoproteins and to LDL. Several lines of evidence emerged indicating an important inhibitory role for apoE in the process of triglyceride lipolysis *in vitro* (McConnathy, 1989; Mulder, 1994) and *in vivo* (Rensen, 1996). In chapter 8 we describe that APOE*2·*Apoe-/-* transgenic mice display hypertriglyceridemia in addition to high plasma VLDL cholesterol and apoE levels, suggesting that high levels of apoE2 per VLDL particle prevents efficient VLDL-triglyceride lipolysis *in vivo* as well. This is in line with the earlier findings in humans that the metabolic conversion of VLDL into LDL is hampered in E2E2 subjects (Enholm, 1991; Demant, 1991). Jong et al (1996a) reported that apoE*3-Leiden affects *in vivo* triglyceride lipolysis as well. Saxena (1992; 1993; 1995) suggested that apoE inhibits lipolysis by substrate dissociation from LPL rather than inactivation of substrate-associated LPL. In conclusion, numerous sets of data indicate that apoE inhibits lipolysis irrespective of the presence of a mutation in apoE. The mechanism of how apoE inhibits lipolysis needs further investigation and could lead to a better understanding of the mechanism(s) underlying hypertriglyceridemia.

The initial removal of chylomicron and VLDL remnant particles from the circulation is suggested to be independent of the LDL receptor and the LRP. Cell surface heparan sulphate proteoglycans (HSPG) may play an important role in this receptor independent sequestration of remnant in the liver (Ji, 1994a, 1995). Ji et al (1994b) studied the interaction of several mutant forms of apoE with HSPG. It was found that dominant apoE mutants, including APOE*3-Leiden, were defective in binding to HSPG. In chapter 8, we show that APOE*3-Leiden mice exhibit only a mild hyperlipidemia, suggesting that the impaired interaction of APOE*3-Leiden with HSPG is not of major importance *in vivo*, as far as the development of hyperlipidemia is concerned. In *Apoe-/-* mice, the plasma clearance of remnants via binding to HSPG cannot occur because of the complete absence of apoE. Thus, the fact that APOE*2·*Apoe-/-* mice exhibit extreme hypercholesterolemia similarly to *Apoe-/-* mice, whereas apoE2 itself does bind to HSPG (Ji, 1994b), also argues against an important role of HSPG in the plasma clearance of VLDL remnants. Hence, whether HSPG does play a major role in remnant lipoprotein clearance and whether LPL or hepatic lipase are involved in this HSPG role are, at present, far from clear and should be investigated in more detail.

After sequestration of the remnant lipoproteins, apoE serves as the ligand for receptor mediated uptake by the LDL receptor and LRP (Mahley, 1988; Ishibashi, 1994; Willnow, 1994). The amount of apoE per particle is an important determinant for receptor binding

affinity (Windler, 1980, Eisenberg, 1988; Mahley, 1988). Introduction of apoE in apoE-deficient mice by adenoviral transfection (Kashyap, 1995; Stevenson, 1995) or bone marrow transplantation (Linton, 1995) showed that only a small fraction (12.5%) of normal apoE levels is sufficient to achieve almost complete normalization of plasma lipoprotein levels. However, this does not necessarily imply that mice with a low expression level of apoE are protected against diet-induced hyperlipidemia. We presented evidence that subnormal expression of the apoE gene leads to hyperlipidemia if the metabolic system is stressed by a mild atherogenic diet (chapter 5). In line with this, van Ree (1994) showed that heterozygous apoE-deficient mice developed hypercholesterolemia when challenged with a severe high/fat cholesterol diet. Reciprocally, Shimano (1992) showed that for resistance to diet-induced hypercholesterolemia in wild type mice additional apoE gene expression by transgenesis was required. Thus, the amount of apoE required for protection against hyperlipidemia largely depends on the dietary status of the animal. In the Western society, people live under non-fasting conditions with continuous release of chylomicron and VLDL in the circulation for most of their life-span. Thus, under these metabolically stressful conditions apoE gene expression may become a limiting factor. Further studies on the role of apoE in the protection against hyperlipidemia in relation to diet will provide valuable information for strategies to lower plasma lipid levels and, consequently, the risk of atherosclerosis in humans.

Studies by Herz (1995) with LDLR-/- mice showed that endocytosis of remnants in mice is primarily mediated by the LDL receptor. In the absence of the LDL receptor, endocytosis proceeds by means of a backup pathway involving LRP, albeit at a much slower rate. The observation that the apoE2 variant with defective LDL receptor binding activity but intact LRP binding activity leads to severe hyperlipidemia in APOE*2.*Apoe-/-* mice (chapter 8), indicates that the LRP cannot function as a complete backup system for apoE-mediated remnant lipoprotein clearance in these mice. Remarkably, in contrast to E2E2 mice, most E2E2 humans are normolipidemic (Mahley and Rall, 1989). This could mean that in most humans LRP can function as a complete backup clearance pathway. Thus, the difference in hyperlipidemia between E2E2 mice and E2E2 humans may be due to a difference in LRP activity between these two species. Similarly, a difference in LRP activity could also explain the metabolic difference between hyperlipidemic and normolipidemic E2E2 humans. Recent studies on mice overexpressing human APOCI on an LDL receptor-deficient background revealed that apoCI modulates binding of lipoproteins to LRP (Jong, 1996b). Whether the expression of the APOCI gene may play a role in the activity of LRP and thus in the development of hyperlipidemia in E2E2 humans remains to be investigated. Crossbreeding APOE*2.*Apoe-/-* mice with APOCI overexpressing mice (Jong, 1996b; Shachter, 1996) or *Apoc1* deficient mice (van Ree, 1995) might shed more light on the possible involvement of LRP activity in the development of hyperlipidemia in E2E2 subjects.

ApoB100 can serve as an alternative ligand mediating hepatic clearance of VLDL remnant particles via the LDL receptor, when apoE-mediated clearance is hampered. This

is, amongst others, clearly illustrated by the complete absence of apoB100 in the VLDL-fraction of apoE-deficient mice (Plump, 1992; Zhang, 1992; van Ree, 1994;). However, VLDL isolated from APOE*2·*Apoe-/-* transgenic mice with extreme levels of plasma lipids (chapter VIII) did contain relatively high levels of apoB100, indicating that in APOE*2·*Apoe-/-* mice apoB100, is not effective as an alternative ligand for VLDL remnant clearance via binding to LDL receptor. Since in these mice, the VLDL remnants contain relatively high amounts of apoE, we hypothesise that a high apoE2 content per remnant particle somehow hampers the action of apoB100 as an alternative ligand in remnant clearance. Such a mechanism of balance between apoE2 and apoB100 might also be an important factor in the developing hyperlipidemia in E2E2 subjects.

The accumulation of remnant lipoproteins in the plasma was shown to be a highly atherogenic condition in APOE*3-Leiden transgenic mice (chapter 2 and 3), as it is in humans (Mahley and Rall, 1989). Similarly, lipoprotein remnant lipoprotein accumulation in APOE*3(Arg142→Cys) transgenic mice (Fazio, 1993; 1994) and apoE-deficient mice (Zhang, 1992; Plump, 1992; van Ree, 1994) also leads to development of atherosclerosis. However, APOE*3-Leiden and APOE*3(Arg142→Cys) mice appeared to be less susceptible to atherosclerosis than apoE-deficient mice. In contrast to apoE-deficient mice, APOE*3-Leiden and APOE*3(Arg142→Cys) mice synthesize functional endogenous apoE. Although this functional endogenous apoE may not be effective as a ligand on remnant particles for uptake via lipoprotein receptors, because of the dominant behaviour of the exogenous mutant apoE, other functions may not be affected. For instance, lipid-laden macrophages synthesize large quantities of apoE (Basu, 1983) and this process may be associated indirectly or directly with cholesterol efflux from these cells (Basu, 1983; Rosenfeld, 1993; Schmitz, 1988). Presumably, this process is still functional in APOE*3-Leiden and APOE*3(Arg142→Cys) mice but not in apoE-deficient mice. In this context, Shimano (1995) and Bellosta (1995) have recently reported that low-level expression of apoE in the arterial wall of apoE-deficient mice leads to protection from atherosclerosis, demonstrating a role for apoE in lesion development irrespective of its ability to influence plasma lipoprotein levels. We postulate that the less severe atherogenic phenotype of APOE*3-Leiden and APOE*3(Arg142→Cys) mice compared with apoE-deficient mice is at least partly due to its unaltered ability to synthesize functional apoE in extrahepatic cells, including vascular macrophages.

Although apoE2 is defective as a ligand for LDL receptor mediated remnant removal (chapter 8), it still may be functional in mediating cholesterol efflux from (vascular) cells, like normal apoE. If so, APOE*2.*Apoe-/-* will be protected from atherosclerosis in spite of the extremely high plasma levels of atherogenic lipoproteins as observed in apoE-deficient mice. Future experiments are needed to see whether mutant apoE2 has the same anti-atherogenic effect as wild type apoE has in this respect. In particular, studies regarding the effect of regulation of apoE in macrophages might be helpful in better understanding the risk of atherosclerosis development and/or regression given a certain level of plasma cholesterol.

At present most known genes involved in lipoprotein metabolism have been overexpressed or disrupted in the mouse. Thus, the evaluation of the role of a gene of interest, or a set of genes in the expression of the hyperlipidemia becomes feasible upon crossbreeding the respective transgenic mice. Studies on these mice will undoubtly lead to more knowledge about the gene-gene and gene-environment interaction relevant in remnant lipoprotein metabolism. This will in its turn lead to new strategies in lowering the prevalence of cardiovascular disease, especially in Western societies.

REFERENCES

Basu, S. K., J. L. Goldstein, and M. S. Brown. 1983. Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science.* 219:871-873

Bellosta, S., R. W. Mahley, D. A. Saana, J. Murata, D. L. Newland, J. M. Taylor, and R. E. Pitas. 1995. Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. *J. Clin. Invest.* 96::2170-2179.

Demant, T., D. Bedford, C. J. Packard, and J. Sheperd. 1991. Influence of apolipoprotein E polymorphism on apoliprotein B-100 metabolism in normolipidemic subjects. *J. Clin. Invest.* 88:1490-1501

Dixon, J. L., and H. N. Ginsberg. 1993. Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J. Lipid Res.* 34:167-179

Eisenberg, S. G. Friedman, and T. Vogel. 1988. Enhanced metabolism of normolipidemic human plasma very low densiity lipoprotein in cultered cells by exogenous apolipoprotein E-3 *Arteriosclerosis* 8:480-487.

Enholm, C., R. W. Mahley, D. A. Chappel, K. H. Weisgraber, E. Ludwig, and J. L. Witztum. 1984. Role of apolipoprotein E in the lipolytic conversion of ß-very low density lipoproteins to low density lipoproteins in type III hyperlipoproteinemia. *Proc. Natl. Acad. Sci. USA* 81:5566-5570.

Ellsworth, J. L., S. K. Erickson, and A. D. Cooper. 1986. Very low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acids. *J. Lipid. Res.* 27:858-874.

Fazio, S., Y. L. Lee, Z. S. Ji, and S. C. Rall Jr. 1993. Type III Hyperlipoproteinemic phenotype in transgenic mice expressing dysfunctional apolipoprotein E. *J. Clin. Invest.* 92:1497-1503.

Fazio, S., D. A. Sanan, Y-L. Lee, Z-S. Ji, R. W. Mahley and S. C. Rall Jr. 1994. Susceptibility to diet-induced atherosclerosis in transgenic mice expressing a dysfunctional human apolipoprotein E(Arg 112,Cys142). *Arteriosclerosis and Thrombosis* 14:1873-1879.

Fazio, S., and Z. Yao. 1995. The enhanced association of apolipoprotein E with apolipoprotein B-containing lipoproteins in serum-stimulated hepatocytes occurs intracellularly. *Arterioscler. Thromb. Vasc. Biol.* 15:593-600

Gretch, D. G., S. L. Sturley, and A. D. Attie. 1995. Human apolipoprotein E mediates processive buoyant lipoprotein formation in insect larvae. *Biochemistry.* 34:545-552

Herz. J, S. Q. Qiu, A. Oesterle, H. V. DeSilva, S. Shafi, and R. J. Havel. 1995. Initial hepatic removal of chylomicron remnants is unaffected but endocytosis is delayed in mice lacking the low density lipoprotein receptor. *Proc. Natl. Acad. Sci. U. S. A.* 92:4611-4615

Ishibashi, S., J. Herz, N. Maeda, J. L. Goldstein, and M. S. Brown. 1994. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc. Acad. Sci. U. S. A.* 91:4431-4435.

Ji, Z-S, S. Fazio, Y-L Lee, and R. W. Mahley. 1994a. Secretion-Capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 269:2764-2772.

Ji, Z-S, S. Fazio, and R. W. Mahley. 1994b. Varaiable heparan sulfate proteoglycan binding of apolipoprotein E variants may modulate the expression of type III hyperlipoproteinemia. *J. Biol. Chem.* 269:13421-13428.

Ji, Z-S, D. A. Sana, and R. W. Mahley. 1995. Intravenous heparinase inhibits remnant lipoprotein clearance from the plasma and uptake by the liver: in-vivo role of heparan sulphate proteoglycans. *J. Lipid. Res.*

36:583-592.

Jong, M. C., V. E. H. Dahlmans, P. J. J. van Gorp, M. L. Breuer, M. J. T. M. Mol, A. van der Zee, R. R. Frants, M. H. Hofker, and L. M. Havekes. 1996a. Both lipolysis and hepatic uptake of very low density lipoproteins are impaired in transgenic mice co-expressing human apolipoprotein E*3-Leiden and human apolipoprotein C1. *Ath. Thromb. Vasc. Biol.* 16:934-940.

Jong, M. C. V. E. H. Dahlmans, P. J. J. van Gorp, K. Willems van Dijk, M. L. Breuer, M. H. Hofker, and L. M. Havekes. 1996b. In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor associated protein (RAP)-sensitive pathway. *J. Clin. Invest.* in press.

Kashyap, V. S., S. Santamarina-Fojo, D. R. Brown, C. L. Parrott, D. Applebaum-Bowden, S. Meyn, G. Talley, B. Paigen-B, N. Maeda, and H. B. Brewer Jr. 1995. Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. *J. Clin. Invest.* 96:1612-1620

Kuipers, F., R. Havinga, M. H. Hofker, R. J. Vonk, H. M. G. Princen, L. M. Havekes. 1995. Role of apolipoprotein E (apoE) in intracellular lipid metabolism and very low density lipoprotein (VLDL) secretion by mouse hepatocytes. *Hepatology* 22:239A.

Linton, M. F., J. B. Atkinson, and S. Fazio. 1995. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science.* 267:1034-1037

Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 240:622-630.

Mahley, R. W. and S. C. Rall. 1989. Type III hyperlipoproteinemia (dysbetalipoproteinemia): The role of apolipoprotein E in normal and abnormal metabolism. In: *The metabolic basis of inherited disease*, edited by Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. New York: McGraw-Hill, p. 1195-1213.

McConathy, W. J., C-S Wang. 1989. Inhibition of lipoprotein lipase by the receptor binding domain of apolipoprotein E. *FEBS Letters* 251:250-252

Mulder. M., H. van der Boom, P. de Knijf, C. Braam, A. van den Maagdenberg, J. A. Gevers Leuven, and L. M. Havekes. 1994. Triglyceride-rich lipoproteins of subjects heterozygous for apolipoprotein E2(Lys 146→Gln) are inefficiently converted to cholesterol-rich lipoproteins. *Atherosclerosis.* 108:183-192

Plump. A. S., J. D. Smith, T. Hayek, K. Aalto Setälä, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71:343-353.

Rensen, P. C. N., and Th. J. C. van Berkel. 1996. Apolipoprotein E effectively inhibits lipoprotein lipase-mediated lipolysis of chylomicron-like triglyceride-rich lipid emulsions in vitro and in vivo. *J. Biol. Chem. 271:14791-14799.*

Rosenfeld, M. E., S. Butler, V. A. Ord, B. A. Lipton, C. A. duer, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1993. Abundant expressuion of apolipoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arterioscler. Thromb.* 13:1382-1389

Saxena, U., M. G. Klein, T. M. Vanni, and I. J. Goldberg. 1992. Lipoprotein lipase increases low density lipoprotein retention by subendothelial cell matrix. *J. Clin. Invest.* 89:373-380

Saxena, U., E. Ferguson, and C. L. Bisgaier. 1993. Apolipoprotein E modulates low density lipoprotein retention by lipoprotein lipase anchored to the subendothelial matrix. *J. Biol. Chem.* 268:14812-14819

Saxena, U., B. J. Auerbach, E. Ferguson, J. Wolle, Y. L. Marcel, K. H. Weisgraber, R. A. Hegele, and C. L. Bisgaier. 1995. Apolipoprotein B and E basic amino acid clusters influence low-density lipoprotein association with lipoprotein lipase anchored to the subendothelial matrix. *Arterioscler. Thromb. Vasc. Biol.* 15:1240-12477

Schmitz, G., H. Robenek, R. Krause, A. Schurek, and R. Niemann. 1998. Ca^{2+} antagonists and ACAT inhibitors promote cholesterol efflux from macrophages by different mechanisms, I: characterization of cellular lipid metabolism. *Arteriosclerosis* 8:46-56

Shachter, N. S., T. Ebara, R. Ramakrishnan, G. Steiner, J. L. Breslow, H. N. Ginsberg, and J. D. Smith. 1996. Combined hyperlipidemia in transgenic mice overexpressing human apolipoprotein C1. *J. Clin. Invest.* 98:846-855.

Shimano, H., N. Yamada, M. Katsuki, M. Shimada, T. Gotoda, K. Harada, T. Murase, C. Fukazawa, F. Takaku, and Y. Yazaki. 1992. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet- induced hypercholesterolemia. *Proc. Natl. Acad. Sci. U. S. A.* 89:1750-1754.

Shimano, H., J. Ohsuga, M. Shimada, Y. Namba, T. Gotoda, K. Harada, M. Katsuki, Y. Yazaki, and N.

Yamada. 1995. Inhibition of diet-induced atheroma formation in transgenic mice expressing apolipoprotein E in the arterial wall. *J. Clin. Invest.* 95:469-476.

Stevenson, S. C., J. Marshall-Neff, B. Teng, C. B. Lee, S. Roy, and A. P. McClelland. 1995. Phenotypic correction of hypercholesterolemia in apoE-deficient mice by adenovirus-mediated in vivo gene transfer. *Arterioscler. Thromb. Vasc. Biol.* 15:479-484

van Ree, J. H., J. A. A. van den Broek, V. E. H. Dahlmans, P. H. E. Groot, M. Vidgeon-Hart, R. R. Frants, B. Wierenga, L. M. Havekes, and M. H. Hofker. 1994. Diet-Induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.* 111:25-37.

van Ree, J. H., M. H. Hofker, W. J. van den Broek, J. M. van Deursen, H. van der Boom, R. R. Frants, B. Wieringa, and L. M. Havekes. 1995. Increased response to cholesterol feeding in apolipoprotein C1-deficient mice. *Biochem. J.* 305:905-911

Willnow, T. E., Z. Sheng, S. Ishibashi, and J. Herz. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor anatagonist. *Science.* 264: 1471-1474.

Windler, E., Y. Chao, and R.J. Havel. 1980. Regulation of hepatic uptake of triglyceride-rich lipoproteins in the rat. *J.Biol.Chem.* 255:8303-8307

Yao, Z. and R. S. McLeod. 1994. Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim. Biophys. Acta.* 1212:152-166.

Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258:468-471.

SUMMARY

Increased plasma levels of chylomicron and VLDL remnant lipoproteins predispose to atherosclerosis. This increase can be caused by disease such as Familial Dysbetalipoproteinemia (FD), due to an impaired hepatic uptake of these particles. However, also a Western lifestyle may lead to increased plasma levels of these atherogenic lipoproteins. In the present thesis we investigated the environmental and genetic factors that influence remnant lipoprotein metabolism. Therefore, transgenic mice were generated that express human APOE mutants that are associated with FD. These mice offered the possiblity of studying these APOE mutants in a homogenous environmental and genetic background. Moreover, these mice were used to identify and characterize the genetic and/or environmental factors that modulate remnant lipoprotein metabolism in general.

Like humans, transgenic mice expressing the dominant APOE*3-Leiden variant showed a hyperlipidemic phenotype, despite the presence of normal functional mouse apoE (chapter 2). The hyperlipidemia resulted from an accumulation of of VLDL/LDL-sized lipoproteins, and was related to the expression level of the APOE*3-Leiden transgene. On high fat/high cholesterol (HFC) diet, the high expressing lines #2 and #181 developed severe hypercholesterolemia, mainly due to an increase in the VLDL/LDL-sized lipoproteins. The low expressing line #195 reacted only mildly upon HFC diet. On HFC diets, the high expressor APOE*3-Leiden mice developed atherosclerotic lesions in the aortic arch, the descending aorta and the carotid arteries, varying from fatty streaks containing foam cells to severe atherosclerotic plaques containing cholesterol crystals, fibrosis and necrotic calcified tissue. The severity of the atherosclerosis is positively correlated with the serum levels of cholesterol-rich VLDL/LDL-sized lipoproteins and the level of expression of the APOE*3-Leiden transgene. Quantitative assesment of aortic atherosclerosis (chapter 3) revealed that high expressing line #2 mice had 5 to 10 times greater lesion area compared to the non-trangenic controls. In addition, highly significant positive correlations were found between the log-transformed data on lesion area and serum cholesterol exposure.

The hyperlipidemic phenotype of APOE*3-Leiden transgenic mice was strongly influenced by age (chapter 4). Transient elevated levels of VLDL/LDL sized lipoproteins occurred in transgenic mice at early age. At this age in the APOE*3-Leiden mice an enhanced production of hepatic VLDL was observed, whereas the plasma clearance of VLDL was not age-dependent. In addition, APOE*3-Leiden transgenic mice displayed a marked effect of gender on the expression of hyperlipidemia. Increased serum lipid levels were observed in females, and after estrogen administration in males, and were explained by a relative high rate of VLDL production. We conclude that introducing a defective apoE, like in the APOE*3-Leiden transgenic mice, leads to a high responsiveness of the serum lipid levels to relative mild changes in chylomicron -and VLDL production rate.

The role of the endogenous apoE gene in determining the expression level of hyperlipoproteinemia in APOE*3-Leiden transgenic mice was investigated in detail (chapter 5). Therefore, the level of functional apoE in APOE*3-leiden mice was either reduced by

crossbreeding with apoE-deficient mice. One functional Apoe gene was clearly not enough to protect APOE*3-Leiden mice from hyperlipidemia when fed a mild hypercholesterolemic diet. Upon feeding a mild atherogenic diet, lack of one of the two endogenous apoE alleles exaberated hyperlipidemia in high expressing APOE*3-Leiden mice, while low expressing APOE*3-Leiden were nolonger normolipidemic and develop also a hyperlipidemic phenotype. The data in this study indicate that the quantitative ratio between apoE3-Leiden and endogenous apoE protein, both in plasma and on VLDL particles, determines the VLDL clearence rate and consequently, the level of hyperlipidemia.

The "human-like" lipoprotein profile, the extreme sensitivity of plasma lipid levels to changes in lipoprotein metabolism and the clear relation between aortic lesion size and cholesterol exposure suggested to us that APOE*3-Leiden mice may serve as a suitable animal model for the testing of lipid lowering and anti-atherosclerotic effects of hypolipidemic drugs. In chapter 6, APOE*3-Leiden mice were used for testing the hypolipidemic effect of two marketed agents, Lovastatin and Gemfibrozil, as well as a novel compound SB 204990. Dose-related decreases in plasma cholesterol (Lovastatin and SB 204990) and triglyceride levels (Gemfibrozil and SB 204990) were observed. Although the effects of these drugs were not simply explained by changes in a single determinant of VLDL metabolism, APOE*3-Leiden mice responded to hypolipidemic therapy, which makes this mouse an attractive model for the testing of hypolipidemic drugs.

APOE*3-Leiden mice were also used to study the strong modulating effect of n-3 fish oils on remnant lipoprotein levels (chapter 7). APOE*3-Leiden mice were highly responsive to dietary fish oil. A strong reduction in serum VLDL is observed, primarily due to the decreasing effect of fish oil on hepatic VLDL triglyceride production rate, and an increasing effect on VLDL-apoB clearance rate.

Finally, transgenic mice expressing the common recessive apoE2(Arg158→Cys) variant became available. These mice were used to compare the *in vivo* functions of the recessive APOE*2 mutation with the dominant APOE*3-Leiden mutation using transgenic mice expressing these apoE variants either in the presence or in the absence of wild type endogenous apoE ((*Apoe*+/+) or (*Apoe*-/-) background, respectively) (chapter 8). In the presence of the endogenous *Apoe* gene, the APOE*2 and APOE*3-Leiden gene indeed behave like a recessive and dominant mutation, respectively, like in humans. However, on an *Apoe* deficient background, APOE*2 expressing mice displayed a much more severe hyperlipidemic phenotype than APOE*3-Leiden expressing mice. *In vivo* transfection experiments with an adenovirus carrying the gene for the receptor associated protein (RAP) and *in vitro* LDL receptor binding studies showed that APOE*2 remnant lipoproteins were cleared via a RAP-sensitive receptor pathway only, most likely the LRP, whereas in APOE*3-Leiden mice remnant lipoproteins were cleared via both the RAP-sensitive pathway and the LDL receptor pathway. The remarkable complete penetrance of hyperlipoproteinemia in APOE*2 transgenic mice suggests that these mice may serve as an important model for finding major aggravating factors involved in the development of hyperlipidemia in E2E2 subjects.

NEDERLANDSE SAMENVATTING

Een verhoging van de plasma niveaus van chylomicron en VLDL 'remnant' lipoproteïnen, vergroot de kans op atherosclerose. Een verhoogd niveau kan voorkomen bij ziekte zoals Familiaire Dysbetalipoproteïnemie (FD), als gevolg van een gestoorde leverklaring van deze deeltjes. Echter, ook als gevolg van de Westerse levensstijl, kunnen verhoogde plasma niveaus van deze atherogene deeltjes ontstaan. In dit proefschrift zijn genetische en omgevingsfactoren onderzocht die het 'remnant' lipoproteïne metabolisme kunnen beïnvloeden. Daarom zijn transgene muizen gegenereerd die aan FD-gerelateerde apoE mutanten tot expressie brengen. Deze muizen maakten het mogelijk om apoE mutanten nader in een *in vivo* systeem te bestuderen, in een constante omgeving tegen een homogene genetische achtergrond. Daarnaast zijn deze muizen gebruikt voor het identificeren en karakteriseren van genetische en omgevingsfactoren die het 'remnant' lipoproteïne metabolisme beïnvloeden.

Transgene muizen die de dominante APOE*3-Leiden mutant tot expressie brengen vertonen, net als de menselijke dragers van deze APOE*3-Leiden mutatie, een hyperlipidemisch fenotype, ondanks de aanwezigheid van normaal goed functionerend endogeen apoE (hoofdstuk 2). De hyperlipidemie is het gevolg van een accumulatie van 'remnant' lipoproteïnen in het plasma. De mate van hyperlipidemie is gerelateerd aan het niveau van transgen expressie. APOE*3-Leiden muizen reageren sterk op een hoog vet/cholesterol (HVC) dieet. Als hoge expressor muizen voor APOE*3-Leiden een HVC dieet gevoerd worden, dan zien we een sterke toename van de plasma cholesterol waarden. Daarentegen lage expressor muizen van lijn 195, reageren matig op het een HVC dieet. Op een HVC dieet, ontwikkelen hoge expressor muizen, atherosclerose in de aortaboog, de dalende aorta en de carotiden. Het type atherosclerotische lesie varieert van "fatty streaks" met schuimcellen tot "atherosclerotische plaques" met cholesterol kristallen, bindweefselvorming, necrose en verkalking. De ernst van de atherosclerose was gecorreleerd aan de hoeveelheid VLDL-deeltjes in het serum en aan het niveau van transgen expressie. Na kwantitatieve evaluatie van de atherosclerose in de aortaboog bleek dat hoge expressor muizen na 3 maanden op een HVC dieet, een 5 tot 10 keer zo groot lesie-oppervlak hadden als controle muizen (hoofdstuk 3). Er bleek een verband te bestaan tussen de log-getransformeerde data van lesiegrootte en blootstelling aan serum cholesterol.

De hyperlipidemie in APOE*3-leiden transgene muizen werd sterk beïnvloed door leeftijd -en geslacht afhankelijke factoren (hoofdstuk 4). Op jonge leeftijd, hadden APOE*3-Leiden muizen hogere VLDL niveaus in het serum. Op deze leeftijd hadden de muizen een verhoogde produktiesnelheid van VLDL door de lever, terwijl de klaring van VLDL niet veranderde met de leeftijd. Verhoogde lipide waarden werden ook waargenomen in vrouwtjes muizen en in mannetjes na oestrogenen behandeling. Ook hier bleek dat een verhoging van de serum lipide waarden, verklaard kon worden door een toename in de produktie van VLDL. We concluderen dat aanwezigheid van het defecte APOE*3-Leiden, leidt tot

verhoogde gevoeligheid van de serum lipiden voor veranderingen in chylomicron en VLDL produktie.

De rol van endogeen normaal apoE in de expressie van hyperlipidemie van APOE*3-Leiden muizen is nader onderzocht (hoofdstuk 5). Door APOE*3-Leiden muizen te kruisen met apoE-deficiënte muizen kon het endogene apoE niveau worden verlaagd. De beschikbaarheid van slechts één normaal apoE allel had duidelijk consequenties; bij hoge expressor muizen van lijn #2 leidde dit tot extra verhoogde plasma lipiden waarden na het voeren van een cholesterol-rijk dieet, terwijl bij lage expressor muizen van lijn 195 nu ook een accumulatie van 'remnant' lipoproteïnen te zien was. De hoeveelheid apoE*3-Leiden, ten op zichte van normaal apoE, zowel op het VLDL-deeltje als in het plasma, bepaalde de klaringssnelheid van VLDL en daarmee de ernst van de hyperlipidemie in deze muizen.

APOE*3-leiden muizen zouden geschikt kunnen zijn als model voor het testen lipide-verlagende farmaca en hun potentiële anti-atherogene werking: i) het lipoproteïne profiel lijkt op dat van de mens, ii) de plasma lipiden zijn gevoelig voor veranderingen in het VLDL metabolisme en iii) er is een sterk verband tussen blootstelling aan cholesterol en de grootte van de atherosclerotische lesies. In hoofdstuk 6 hebben wij het lipiden verlagende effect in APOE*3-Leiden muizen van twee geregistreerde farmaca, Lovastatine en Gemfibrozil, en een nieuwe verbinding genaamd SB 204990 getest. Effecten van deze farmaca op een van de determinanten van het VLDL metabolisme, zoals produktie, klaring en lipolyse, waren niet waarneembaar. Desalniettemin, zijn dosis-afhankelijke verlagingen van het plasma cholesterol (Lovastatine en SB 204990) en triglyceriden (Gemfibrozil en SB 204990) waargenomen na behandeling met deze farmaca. We concluderen dat APOE*3-Leiden muizen een geschikt model zijn voor het testen van de lipid verlagende farmaca.

APOE*3-Leiden muizen zijn ook gebruikt om de effecten van n-3 visolie op het 'remnant' lipoproteïne metabolisme te bestuderen (hoofdstuk 7). APOE*3-Leiden muizen waren erg gevoelig voor visolie in de voeding. Een sterke verlaging van VLDL in het serum werd waargenomen, hetgeen het gevolg was van een verminderde produktie van VLDL-triglyceriden en een toegenomen klaring van VLDL-apoB.

Tenslotte, kwamen er transgene muizen beschikbaar die de veelvoorkomende APOE*2 mutatie tot expressie brengen. Deze muizen werden gebruikt om de *in vivo* functie van de apoE2 te vergelijken met die van de bovenvermelde apoE3-Leiden variant, zowel in aan- als afwezigheid van de beide endogene apoE allelen (hoofdstuk 8). In de aanwezigheid van muis apoE, gedroegen APOE*2 en APOE*3-Leiden mutatie zich respectievelijk als een recessieve en dominante mutatie, zoals dat ook in de mens het geval is. In afwezigheid van het muis apoE, vertoonde APOE*2 expresserende muizen ernstige hyperlipidemie, veel meer dan APOE*3-Leiden muizen. Uit *in vivo* experimenten, waarbij het "receptor associated protein" (RAP) middels een adenovirus tot overexpressie gebracht werd, en *in vitro* LDL receptor bindingsstudies, bleek dat de apoE2 variant alleen nog via de LRP geklaard kan worden, terwijl apoE3-Leiden zowel via de LRP als LDL receptor route geklaard wordt. De opmerkelijk hoge penetrantie van het hyperlipemische fenotype in APOE*2 transgene

muizen, maakt deze muizen tot een belangrijk model voor het identificeren en karakteriseren van factoren die de ontwikkeling van hyperlipidemie bij mensen met het E2E2 genotype beïnvloeden.

LIST OF PUBLICATIONS

van den Maagdenberg, A.M.J.M., M.H. Hofker, P.J.A. Krimpenfort, I.H. de Bruijn, B.J.M. van Vlijmen, H. van der Boom, L.M. Havekes, and R.R. Frants. 1993. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 268:10540-10545.

van Vlijmen, B.J.M., A.M.J.M. van den Maagdenberg, M.J.J. Gijbels, H. van der Boom, H. HoogenEsch, R.R. Frants, M.H. Hofker, and L.M. Havekes. Diet-Induced Hyperlipoproteinemia and Atherosclerosis in Apolipoprotein E3-Leiden Transgenic Mice. 1994. *J. Clin. Invest.* 93:1403-1410.

Havekes, L.M., B.J.M. van Vlijmen, R.R. Frants and M.H. Hofker. 1994. Rare mutations in the *Apo*E gene and their effect on chylomicron and VLDL remnant metabolism in familial dysbetalipoproteinemic patients and in transgenic mice. *In* Multiple Risk Factors in Cardiovascular Disease. A. Yamamoto, editor. Churchill Livingstone. Tokyo, Japan. 167-171.

van Ree, J.H., B.J.M. van Vlijmen, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1995. Hyperlipidemia and atherosclerosis in ApoE transgenic mice. *In* Advances in lipoprotein and atherosclerosis research, diagnostics and treatment. [Proceedings of the 8th International Dresden Lipid Symposium]. W. Jaross, S. Bergmann, M. Hanefeld, and H. Dude, editors. Fischer, Jena, Germany. 272-283.

Havekes, L.M., B.J.M. van Vlijmen, J.H. van Ree, R.R. Frants, and M.H. Hofker. 1995. Effect of expression of apoE mutants in transgenic mice. *In* Atherosclerosis X. F.P. Woodford, J. Davignon, and A. Sniderman, editors. Elsevier, Amsterdam, The Netherlands. 656-661.

van Vlijmen, B.J.M., M.J.T.M. Mol, H. van der Boom, H.B. van 't Hof, A. van der Zee, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1996. Modulation of very low density lipoprotein production and clearance contributes to age- and gender-dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* 97:1184-1192

van Vlijmen, B.J.M., K. Willems van Dijk, H.B. van t Hof, P.J.J. van Gorp, H. van der Boom, M.L. Breuer, M.H. Hofker, and L.M. Havekes. 1996. In the absence of endogenous mouse apolipoprotein E, apolipoprotein E*2(Arg158→Cys) transgenic mice develop more severe hyperlipoproteinemia than apolipoprotein E*3-Leiden transgenic mice. *J. Biol. Chem.* 271:30595-30602.

van Vlijmen, B.J.M., J.H. van Ree, R.R. Frants, M.H. Hofker, and L.M. Havekes. 1996. Elevated levels of chylomicron-and VLDL-remnants leads to atherosclerosis in apoE transgenic mice. *Z. Gastroenterology* 34:113-115.

Groot, P.H.E., B.J.M. van Vlijmen, G.M. Benson, M.H. Hofker, R. Schiffelers, M. Vidgeon-Hart, and L.M. Havekes. 1996. Quantitative assessment of aortic atherosclerosis in transgenic mice, expressing a dysfunctional apo E variant (apoE3 Leiden) and its relation to serum cholesterol exposure. *Arterioscler. Thromb. Vasc. Biol.* 16:926-933.

Havekes, L.M., B.J.M. van Vlijmen, M.C. Jong, P.H.E. Groot, R.R. Frants, and Hofker M.H. 1996. The metabolism of VLDL remnants in APO*3-Leiden transgenic mice. *In* Drugs affecting lipid metabolism. A.M. Gotto, editor. Kluwer, Dordrecht, The Netherlands. 10: 263-270.

Hendriks, W.L., F. van der Sman-de Beer, B.J.M. van Vlijmen, L.C. van Vark, M.H. Hofker, and L.M. Havekes. Uptake by J774 macrophages of very low density lipoproteins isolated from ApoE-deficient mice is mediated by a distinct receptor and stimulated by lipoprotein lipase. *Arterioscler. Thromb. Vasc. Biol.* in press

ABBREVIATIONS

Ad	adenoviral vector
Apo	apolipoprotein
CETP	cholesterylester transfer protein
CMV	cytomegalo virus
FCR	fractional catabolic rate
FD	familial dysbetalipoproteinemia
HDL	high density lipoproteins
HFC	high fat/cholesterol diet
HPS	hematoxylin-phloxine-saffron
HSPG	heparan sulphate proteoglycans
IDL	intermediate low density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	LDL receptor
LFC	low fat/cholesterol
LPL	lipoprotein lipase
LRP	LDL receptor related protein
RAP	receptor associated protein
SR	secretion rate
SRM-A	standard rat mouse diet
VLDL	very low density lipoproteins
WHHL	Watanabe-heritable-hyperlipidemic

Curriculum Vitae

Bart Jozef Maria van Vlijmen is geboren op 5 augustus 1967 te Delft. In 1985 behaalde hij zijn VWO diploma aan het St. Stanislas College te Delft. In dat zelfde jaar ving hij aan met zijn studie Biologie aan de Rijksuniversiteit Leiden (RUL). Na het behalen van zijn propadeuse Biologie studeerde hij van van 1986 tot 1991 Bio-Farmaceutische Wetenschappen aan de RUL met als hoofvakken Biofarmacie (prof. dr. Th.J.C. van Berkel en dr. M.K. Bijsterbosch) en Farmacochemie (dr. A.P. IJzerman en dr. A. van de Bent). In het kader van de Farmacochemie doorliep hij tevens een stage bij Organon International te Oss. In februari 1991 behaalde hij het doctoraalexamen. Van februari 1991 tot juni 1992 werd in het kader van de vervangende dienstplicht onderzoek verricht bij de afdeling Genetische Toxicologie van het (toenmalige) TNO-Medisch Biologisch Laboratorium te Rijswijk.

Van augustus 1992 tot augustus 1996 was hij werkzaam als onderzoeker in opleiding (o.i.o.) in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO/NHS programma 'Stimuleringsfonds' project #901-04-92) bij TNO-Preventie en Gezondheid, Gaubius Laboratorium, Leiden (prof. dr. ir. L.M. Havekes), in nauwe samenwerking met de afdeling Antropogenetica van de RUL (dr. M.H. Hofker, prof. dr. R.R. Frants). De resultaten van dit promotie onderzoek staan beschreven in dit proefschrift. Vanaf februari 1997 is hij werkzaam als postdoc bij de afdeling Biofarmacie van het Leiden/Amsterdam Center for Drug Research (prof. dr. Th.J.C. van Berkel) en TNO-Preventie en Gezondheid, Gaubius Laboratorium (prof. dr. ir. L.M. Havekes).

Nawoord

Dit proefschrift is het resultaat van vier leuke en leerzame jaren van onderzoek. De tot standkoming van dit proefschrift was natuurlijk niet mogelijk zonder de belangrijke bijdrage van anderen. De experimentele ondersteuning van Linda en Hans heb ik als heel produktief, heel leuk en heel gezellig ervaren. Onmisbaar waren de muisjes van Arn en Janine, het virus van Ko en de moleculair biologische hulp van Patrick, André, Marga. Mijn kamer- en labgenoten noem ik voor de nodige hulp, steun en plezier. Zo ook, alle andere collega's van zowel het Gaubius Laboratorium als Antropogenetica, inclusief de studentes Miranda, Femke, Thea en Ellen. De medewerkers van de Proefdier Faciliteiten van het Sylvius Laboratorium, de Transgene Faciliteit Leiden en Proefdier faciliteiten van TNO-PG, ben ik zeer erkentelijk voor hun inzet en ondersteuning. Dr. Marion Gijbels en dr. Harm HogenEsch (toenmalige afdeling Pathologie, IVVO-TNO, Leiden), dr. Pieter Groot en medewerkers (Dept. of Vascular Biology, SmithKline Beecham Pharmaceuticals, Welwyn, UK), dr. Marc Mol (Algemene Interne Geneeskunde, Academische Ziekenhuis Nijmegen), Martin Bergö (Dept. of Biochemistry and Biophysics, Umeå University, Umeå, Zweden), dr. Bart Staels (Dept. d'Athérosclerose, Institut Pasteur, Lille France) en dr. Ronald Mensink (Afd. Humane Biologie, Universiteit van Maastricht) voor de prettige en vruchtbare samenwerking. Tenslotte, noem ik mijn ouders, zussen, vrienden en vriendinnen, èn Marieke, maar hen ben ik sowieso al meer dan erkentelijk.