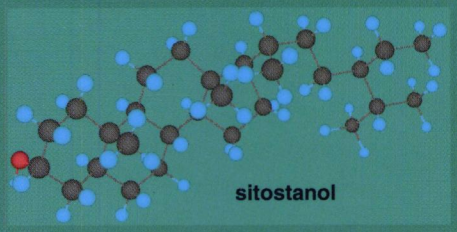
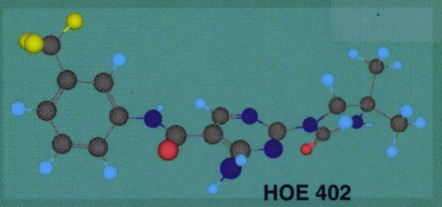
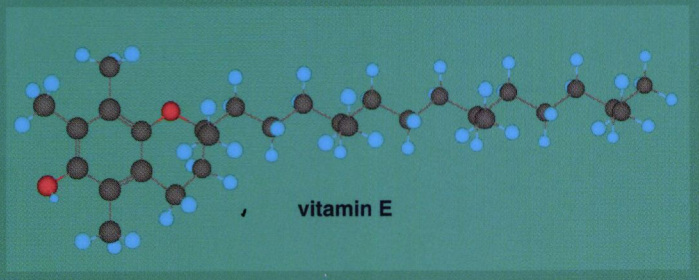
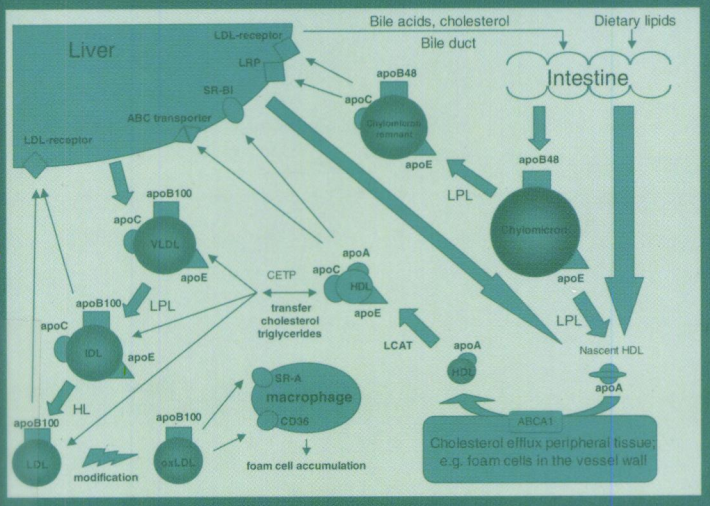
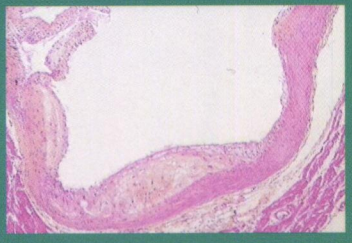
Modulation of lipoprotein metabolism and atherosclerosis by food components and drugs in hyperlipidaemic mice

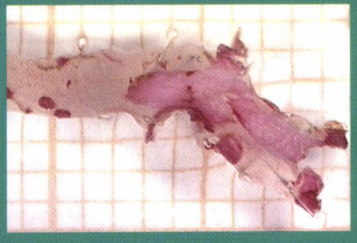












Oscar L. Volger

Modulation of lipoprotein metabolism and atherosclerosis by food components and drugs in hyperlipidaemic mice

PROEFSCHRIFT

ter verkrijging van de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
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Oscar Leonard Volger

geboren te Wormerveer in 1974

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Referent:	Prof. dr. A. van der Laarse
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Stellingen behorende bij het proefschrift:

Modulation of lipoprotein metabolism and atherosclerosis by food components and drugs in hyperlipidaemic mice

1. De waarneming dat het HMG CoA synthase mRNA in de lever niet wordt verhoogd door toediening van stanol esters betekent niet dat de endogene cholesterolsynthese onveranderd blijft. *Dit proefschrift*

2. Niet de effectiviteit maar de natuurlijke beschikbaarheid bepaalt dat sitostanol en niet campestanol het hoofdbestanddeel is van stanol esters van plantaardige oorsprong. *Dit proefschrift (H. Gylling et al. Metabolism 1999;48(5):575-80)*

3. De waarneming dat vitamine E de atherosclerotische plaque stabiliseert betekent nog niet dat vitamine E anti-atherosclerotisch werkt als gevolg van zijn antioxidatieve eigenschappen. *Dit proefschrift (JF Keany Jr, FASEB J 1999;13(9):965-75)*

4. Het feit dat bij de atherosclerotische muis de plaques niet ruptureren betekent niet dat plaquestabiliteit in deze diermodellen niet kan worden bestudeerd. *Dit proefschrift*

5. Hoewel statines anti-atherosclerotische effecten laten zien die onafhankelijk zijn van hun lipidenverlagende eigenschappen, blijft het verlagen van plasma lipiden hun belangrijkste anti-atherosclerotische werking. *(H. Chen et al. Hypertens Res 2000;23(2):187-92S; Bellosta et al. Atherosclerosis 1998;137 S101-9)*

6. Farmacologische verhoging van HDL cholesterol hoeft niet noodzakelijkerwijs tot remming van het atherosclerotische proces te leiden.

7. Vitamine E heeft evenals een onderzoeker collega's nodig voor het leveren van goede resultaten.

8. Het is nodig dat de dierenbescherming een campagne start met de boodschap dat er niet in aanwezigheid van huisdieren gerookt dient te worden. *(JF. Reif et al. Am J Epidemiol 1998;1;147(5):488-92; SW. Ueng et al J Trauma 1999;46(1):110-5)*

9. Gezien de strenge regels die gehanteerd worden bij het werken met diermodellen verdient het aanbeveling medewerkers van dierenspeciaalzaken ook de cursus proefdierkunde verplicht te laten volgen.

10. Een beetje heer in het verkeer zit tegenwoordig langer vast dan de gemiddelde tasjesdief.

11. Een structurele verbetering van de professionele kinderopvang is nodig om het aantal werkzame vrouwen van boven de 30 jaar in Nederland te laten stijgen.

12. Aangezien de Nederlandse CO_2-uitstoot niet meer verminderd kan worden door middel van het kopen van buitenlandse stroom kan de regering besluiten om de tabaksindustrie te verplichten sigaretten met katalysatorfilter te ontwikkelen.

Dankwoord

Na meer dan vier jaar hard werken is mijn proefschrift dan eindelijk klaar. Wat een lekker gevoel! Dit boekje had natuurlijk niet tot stand kunnen komen zonder de hulp van jullie.
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Oscar

Aan Aaf † '98 en Gerrit Volger
Voor Mari-Christine

Contents

Chapter 1

General introduction

Introduction

Currently, cardiovascular disease is the most common cause of mortality in Western society, and is responsible for approximately 50% of all deaths, in both men and women. Atherosclerosis, the primary cause of cardiovascular disease, is a chronic and multifactorial disease that is characterized by the accumulation of lesions in the arterial wall. Ultimately, rupture of these lesions causes thrombus formation, leading to cardiovascular events, such as myocardial infarction and stroke. Major risk factors for the development of atherosclerosis are elevated serum levels of cholesterol and triglycerides, hypertension, diabetes mellitus, abdominal obesity, smoking, sex and age (1).

Lipoprotein metabolism

The body obtains cholesterol and triglycerides via the diet and endogenous synthesis. Cholesterol plays important roles in the regulation of the fluidity and barrier function of cell membranes, and in the endogenous synthesis of bile acids and steroid hormones. Triglycerides are used as an energy source for cardiac and skeletal muscle and are stored in adipose tissue. Cholesterol and triglycerides are hydrophobic lipids that are packaged into lipoproteins for their suspension and transport in hydrophilic environments such as the bloodstream and lymph ducts.

There are five different classes of lipoproteins that can be distinguished according to their density or size (table 1). Except for the disc shape of native HDL, all these lipoproteins are spherical and consist of a non-polar core of triglycerides and esterified cholesterol, and of a polar exterior, comprising free cholesterol, phospholipids, and proteins, called apolipoproteins. The five different lipoprotein subclasses contain different apolipoproteins that have several distinct functions in lipoprotein metabolism: these structural proteins stabilize the lipoprotein particles, and are ligands for lipoprotein receptors and are co-factors/inhibitors of enzymes such as lipoprotein lipase (LPL). In addition, lp(a) is a subclass of LDL, and comprises LDL coupled to apo(a) protein (2).

Table 1. Physical properties and composition of human plasma lipoproteins (3).

	Chylomicron	VLDL	IDL	LDL	HDL
diameter (nm)	75-1200	30-80	25-35	18-25	5-12
density (g/ml)	<0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.210
mobility*	origin	Pre-β	Pre-β/β	β	α
protein	1-2	6-10	11	21	45-55
triglycerides	88	56	29	13	15
phospholipids	8	20	26	28	45
esterified cholesterol	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,CII,CIII, E

The physical properties and composition of human chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) are depicted. *According to the mobility of plasma α- and β-globulins on agarose gel electrophoresis. The values given for protein, triglyceride, phospholipid, and esterified and free cholesterol are expressed as the percentage of total weight.

The metabolism of chylomicrons, that deliver dietary lipids from the intestines to the body, is called the *exogenous pathway*. First, the intestines will absorb dietary lipids, and will package these lipids into nascent chylomicrons, that will enter the bloodstream via the lymph ducts (figure 1). In the small intestines dietary lipids are hydrolyzed into their free form (free fatty acids, free cholesterol) by pancreatic lipases. Thereafter, bile acids that originate from the liver also enter the intestines via the bile duct, where they emulsify these lipids. This results in the formation of micelles in the intestinal lumen. Micelles are spherical droplets consisting of a non-polar lipid core and a polar surface of bile acids that transport lipids to the ilial and jejunal brush border membranes, where the lipids are absorbed by the enterocytes (4-6). As depicted in figure 1, cholesterol needs to be dissolved into these micelles for its intestinal absorption. This process is inhibited by plant stanols and sterols, since these compounds are more easily dissolved into micelles than cholesterol, thereby displacing a large portion of cholesterol from the micelles, resulting in a

reduced intestinal cholesterol uptake (7,8). Currently, margarines enriched with plant stanol esters and plant sterol esters are available for consumption. Daily consumption of approximately 25 grams of these margarines, which is equivalent to a daily intake of 2.5 grams of plant stanol or sterol esters, has been shown to reduce the levels of serum LDL cholesterol by 10% on average (9).

After having released the lipids to the enterocytes, the micelles disintegrate, and approximately 95% of the bile acids are reabsorbed further downstream by the enterocytes of the terminal ileum, and are returned to the liver via the portal vein, figure 1 (10). Within the enterocyte, the newly absorbed lipids are re-esterified and packaged together with the apolipoproteins apoB48, apoA1, and apoA4 to produce nascent chylomicrons (11). Chylomicrons are the largest and most triglyceride-rich of all lipoproteins (table 1). After their excretion by the enterocytes into the mesenteric lymph ducts, the chylomicrons reach the bloodstream in the subclavian veins.

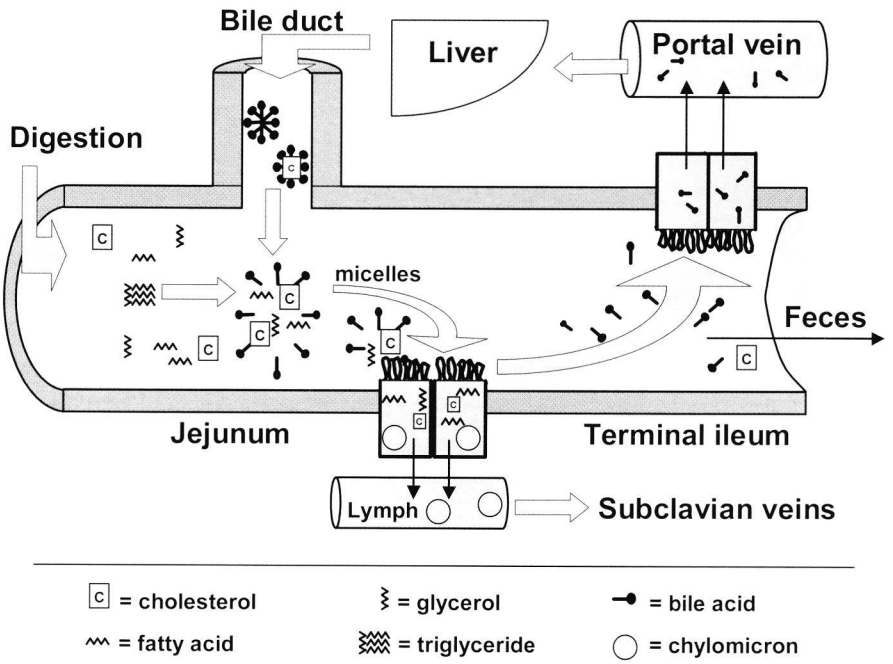

Figure 1. Schematic presentation of intestinal lipid absorption and production of nascent chylomicrons.

Once in the bloodstream, the chylomicrons acquire apoCs and apoE, and are then further metabolized (figure 2). These apolipoproteins regulate the further metabolism of the chylomicrons. Chylomicrons deliver free fatty acids to the periphery after hydrolysis of their triglycerides by the enzyme lipoprotein lipase (LPL), which is present at the surface of the capillary bed of the endothelial cells (12,13). This process leads to the formation of smaller and triglyceride-depleted chylomicron remnants. As mentioned above, the free fatty acids are used as an energy source for the muscles, or are stored in adipose tissue. In the normal situation, chylomicron remnants are rapidly cleared from the bloodstream via receptor-mediated endocytosis by the LDL receptor-related protein (LRP) and the LDL receptor (LDLR) (14,15), whereby apoE acts as the ligand for these receptors (16,17).

The metabolism of very low density lipoprotein (VLDL), which delivers lipids from the liver to the peripheral tissues, is called the *endogenous pathway* (figure 2). Hepatic cholesterol and triglycerides are incorporated into nascent VLDL particles that contain apoB100, apoCs and apoE when secreted into the circulation (18). Like chylomicrons, VLDL triglycerides are hydrolyzed to deliver free fatty acids to the periphery, resulting in the formation of smaller VLDL-remnants, called intermediate density lipoprotein (IDL). IDL can either be cleared from the bloodstream by the liver, which is a process that is also mediated by apoE, or can be further hydrolyzed by hepatic lipase (HL), and after subsequent loss of triglycerides, phospholipids, apoC and apoE, low density lipoprotein (LDL) is formed. The function of LDL is the delivery of cholesterol to the periphery, for production of steroid hormones in the adrenals and gonads. LDL particles can be cleared from the circulation by the LDL receptor, using its apB100 as ligand (19). Additionally, LDL can undergo oxidative and proteolytic modifications in the vessel wall. Several pro-atherogenic events can be initiated by oxidized modified LDL (oxLDL), including the differentiation of monocytes into macrophages and subsequently foam cells, which is mediated by its scavenger receptor- mediated uptake via SR-A and CD 36.

The transport of cholesterol from peripheral tissues to the liver and the subsequent excretion from the body via bile, mediated by high density lipoprotein (HDL) particles, is called the *reversed cholesterol pathway* (figure 2). HDL appears in the circulation after synthesis by the liver and small intestines, and after lipolysis of chylomicrons, as surface remnants. The recently discovered ATP-binding cassette transporter 1 (ABCA1) (20,21) actively transports cellular cholesterol to the interstitial fluid, where HDL serves

as a cholesterol acceptor. In the circulation HDL cholesterol is esterified by lecithin: cholesterol acyl transferase (LCAT), with apoAI, apoAIV and apoCI as co-factors. The cholesterol esters formed are translocated to the inner core of the HDL particle, thereby increasing the HDL particle size. HDL can then deliver cholesterol to the liver via three pathways (22). Firstly, cholesteryl ester transfer protein (CETP) can transfer esterified cholesterol from HDL to VLDL, IDL and LDL in exchange for triglycerides. Secondly, after acquiring apoE from other circulating lipoproteins, HDL can be cleared via apoE-mediated receptor uptake, and thirdly, cholesteryl esters can specifically be taken up from HDL by the scavenger receptor SR-B1 and ABC transporters on the liver (20,23,24).

Lipoproteins have several important links with the development of atherosclerosis. Elevated levels of the apoB-containing lipoproteins VLDL, IDL and LDL, are prominent risk factors for the development of atherosclerosis (25), and the above-mentioned modification of LDL is considered to be an important pro-atherogenic process (26-28). Additionally, the reverse transport of cholesterol from cells of the arterial wall to the liver is believed to be an important anti-atherosclerotic process (29).

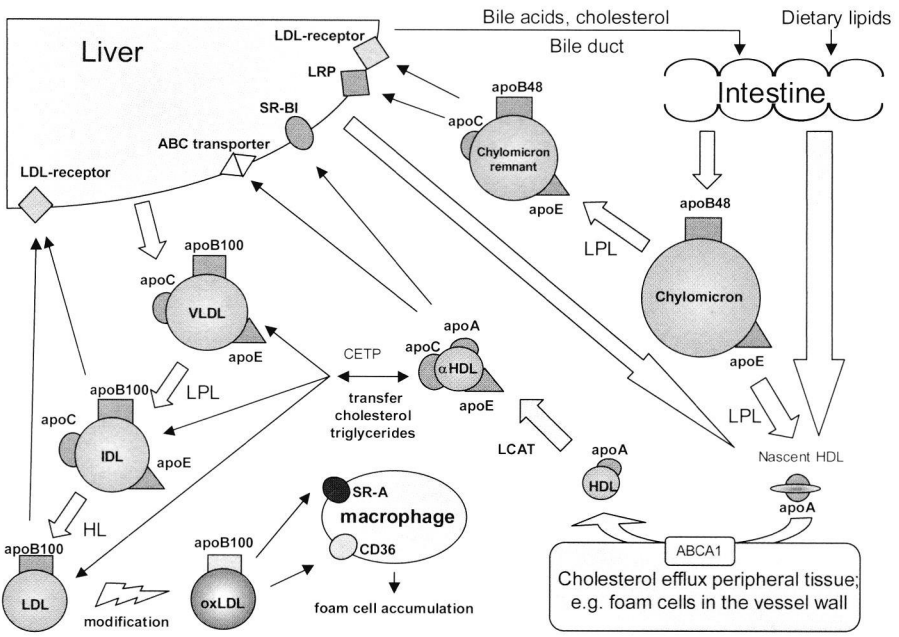

Figure 2. Schematic representation of lipoprotein metabolism.

Atherosclerosis development

The arterial wall consists of three layers, the tunica intima, media and adventitia. The intima is situated at the luminal side of the vessel wall, and mainly comprises endothelial cells that separate the vessel wall from the bloodstream, and underlying extracellular matrix components. The intima is separated from the media by the lamina elastica interna, the elastic fibre that is closest to the arterial lumen. The media mainly consists of layers of smooth muscle cells that are separated by longitudinal layers of elastic fibres. The number of layers depends on the diameter of the artery. The adventitia surrounds the media, and comprises a layer of loose connective tissue that merges with surrounding connective tissue, and elastic fibres that are less abundant in the media.

Initiation of atherosclerosis

The initiation of atherosclerosis is depicted in figure 3A. In a healthy arterial wall, there is a balance between the uptake of LDL from the circulation and the excretion of LDL from the vessel wall towards the circulation. The development of atherosclerosis is initiated when this balance is shifted towards an increased uptake and/or decreased excretion of LDL, leading towards an increased retention of LDL by the arterial wall. Extracellular matrix proteins are responsible for the retention of LDL. These proteins are situated in the sub-endothelial space of the vessel wall, where oxidative modification of LDL occurs, proceeding via minimally modified LDL (mmLDL) to oxidized LDL (oxLDL) (30,31). LDL can be oxidized non-enzymatically by hydroxyl radicals formed from the superoxide anion via metal ions (32), or enzymatically by myeloperoxidase (33), nitric oxide produced by inducible nitric oxide synthase (iNOS) (34-36), 15-lipoxygenase (37-40), and NADPH oxidase (41). In addition, all these enzymes, except NADPH oxidase (42), have been found to accelerate atherosclerosis development in genetically modified mice. This process is stimulated by mmLDL and inflammatory cytokines. Thereafter, chemotactic molecules such as monocyte chemotactic protein-1 (MCP-1) and CC chemokine receptor-2 (CCR-2), are produced. After recruiting cellular adhesion factors, such as vascular cell adhesion molecule-1 (VCAM-1), selectins (E, P), and intracellular adhesion molecule-1 (ICAM-1), new monocytes and T lymphocytes adhere to endothelial cells. These chemotactic molecules mediate adherence and migration of new monocytes and T lymphocytes through the endothelial barrier to the sub-endothelial space.

Several growth factors and pro-inflammatory cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), tumour necrosis factor α (TNFα) and interferon-γ, produced by macrophage foam cells, T lymphocytes, endothelial cells and smooth muscle cells also play a central role during all phases of atherosclerosis. In the early phases of atherosclerosis, these pro-inflammatory factors induce the differentiation of monocytes into macrophages. Thereafter these macrophages will start loading lipids via scavenger receptor (SRA and CD36)-mediated uptake of oxLDL (43). In contrast to LDL receptor-mediated uptake of LDL, SR-mediated uptake of oxLDL is not down regulated feed back by intracellular cholesterol levels via activation of the sterol regulatory element binding protein (SREBP) (44). Therefore these macrophages can rapidly expand by storing large quantities of oxLDL-derived lipids, and transform into macrophage foam cells to form initial atherosclerotic lesions. Accumulations of either individual or small groups of macrophage-derived foam cells, or small groups of macrophage-derived foam cells and T lymphocytes in the intima of the vessel wall, characterize these lesions. Using the characterization method of the American Heart Association (AHA) (45), in which the severity of atherosclerotic lesions is classified into different types (I-VI), these initial lesions are classified as type I. The process of foam cell formation can be inhibited and even reversed via apoE and ABCA1-mediated efflux of cholesterol to HDL (20). When the reverse cholesterol transport is impaired, due to the absence of functional ABCA1, as is the case in Tangier disease patients (46,47), plasma HDL is almost completely absent (48,49) and the prevalence of atherosclerotic disease is increased (29).

Somewhat larger accumulations of T lymphocytes and foam cells in the intima of the arterial wall, are called fatty streaks, and are classified as type II. Fatty streaks are the first physical sign of atherosclerosis, and in man, these can already appear from early childhood onwards (50). Characteristic type III lesions consist of intimal macrophages and foam cells, extracellular lipid droplets that are scattered just below these cells, and have a media with a diminished coherence of the intimal smooth muscle cell layers. Until the stage of type III lesions is reached, the process of atherosclerosis development is fully reversible. For instance, intervention with lipid-lowering therapy can lead to the complete disappearance of these lesions.

Progression of atherosclerosis

The further progression of atherosclerosis (figure 3B) leads to the development of more complex (type IV-VI) lesions, and is irreversible, because intervention does not lead to the disappearance of these lesions. However, intervention, like lipid-lowering therapy, can stabilize such complex lesions.

Lesion progression starts with the migration of smooth muscle cells from the media to the intimal fatty streaks. The above-mentioned cytokines and growth factors stimulate this process. Subsequently the smooth muscle cells can start to proliferate, differentiate into foam cells, or start producing extracellular matrix components such as collagen and proteoglycans (51), thereby forming a fibrous cap on top of the fatty streaks. These lesions are called fibrolipid plaques, and are classified as type IV. In later stages of atherosclerosis, the growth factors and pro-inflammatory cytokines, produced by T helper cells (Th1 and Th2) can evoke thinning of the fibrous cap in two ways. First, collagen synthesis can be inhibited by interferon-γ, and second, by foam cell-derived macrophages, T lymphocytes, and smooth muscle cells can start producing active matrix metalloproteinases (MMPs). These MMPs cooperatively degrade extracellular matrix components, thereby destabilizing the structural strength of the plaque (52). Additionally, proteolytic processes can induce degradation of the elastic fibres of the tunica media, and can induce necrosis and bursting of foam cells within the plaque. The latter leads to deposition and crystallization of foam cell-derived cholesterol, known as cholesterol clefts, and to a further pro-inflammatory response, which subsequently accelerates the atherosclerotic process. Atherosclerotic lesions with disrupted elastic fibres are severe, and are classified as type V. Several different forms of type V lesions exist. For instance, many of these lesions have a large necrotic core beneath a thick fibrous cap (type V), whereas others are largely calcified (type Vb), and some consist mainly of fibrous connective tissue and have little or no accumulated lipid or calcium (type Vc). Further destabilization of type V lesions can lead to rupture. A typical plaque that is prone to rupture has a thin fibrous cap and a large and soft lipid-rich core with inflammatory cell infiltrates, and is frequently depleted of smooth muscle cells (52-54). Rupture of such an unstable (type VI) lesion is the primary cause of cardiovascular events, because this leads to the exposure of lipids and pro-coagulant tissue factor to the circulation, resulting in thrombus formation, and subsequently, in cardiovascular events. These cardiovascular events most frequently appear around the fifth decade in men, and around the sixth decade in women (1).

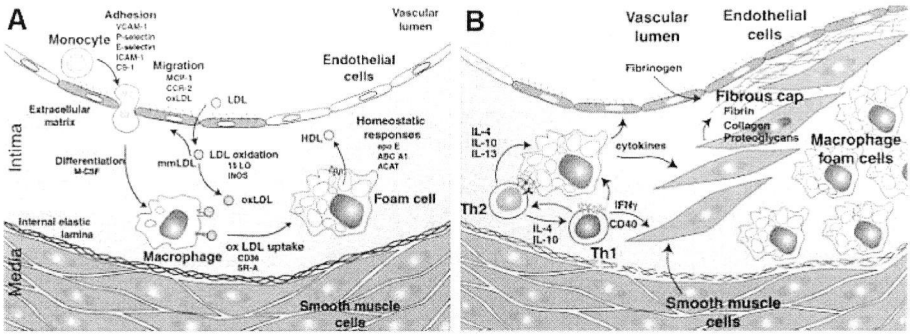

Figure 3. Schematic representation of the initiating events in fatty streak formation (**A**) and lesion progression (**B**). Reprinted from Cell (55), by copyright permission of Elsevier Science.

Treating atherosclerosis

Since the role of oxidative modification of LDL in the onset of atherosclerosis became more clear (26), and epidemiological studies found a link between antioxidant status and the occurrence of cardiovascular disease (56,57), the effects of different antioxidants on the develop of atherosclerosis has been evaluated in many clinical trials. These trials revealed that antioxidant therapies, such as with vitamin E (58-62) and beta carotene (63,64), were less effective in attenuating atherosclerotic disease than lipid-lowering therapies.

Lipid-lowering therapy with statins, due to inhibition of endogenous HMG-CoA reductase, has shown to be the most effective way of treating cardiovascular disease (65,66). In addition, activation of the nuclear peroxisome proliferator-activated receptor-α (PPAR-α) by treatment with fibric acid derivates, which leads to lowered serum triglyceride and cholesterol levels, is a promising target for the secondary prevention of atherosclerosis (67,68). Other lipid-lowering strategies, being inhibition of the intestinal uptake of bile acids by nicotinic acid and derivatives, inhibitors of acyl coenzyme A:cholesterol acyltransferase (ACAT), and compounds that inhibit the intestinal cholesterol absorption such as ezetimibe, might be promising options for the future prevention of atherosclerotic disease, if it will be found that these compounds have beneficial effects on atherosclerotic disease (69).

Certain natural ingredients of nutrition have also been found to lower cholesterol levels by modulation of the some of the above-mentioned targets. Plant stanol and sterol esters reduce LDL-cholesterol levels by inhibition of the

intestinal cholesterol absorption (9), and red yeast rice, which is a fermented rice product, reduces LDL cholesterol and triglyceride concentrations (70). The hypocholesterolaemic mechanism of red yeast rice is not completely understood, but is partly explained by its content of lovastatin, which is a natural fermentation product that inhibits endogenous HMG-CoA reductase activity (71). Furthermore, certain soy-derived proteins, being the isoflavones daidzein and genistein, have estrogen-like activities that lead to cholesterol-lowering effects (72). In addition, *in vitro* studies reported antioxidant properties of these isoflavones, but this has not been consistently confirmed *in vivo* (73,74). Future trials will reveal whether or not these natural cholesterol-lowering compounds are effective in reducing the risk for cardiovascular disease.

Mouse models of atherosclerosis

In humans, investigation of the effects of certain environmental factors on the development of atherosclerosis is difficult, because genetic variability and inter-individual differences in environmental factors and life style often interfere with the development of atherosclerosis. These interfering factors can be minimized in mice, since inbred strains are used, and similar housing and dietary conditions are applied. In addition, *in vivo* quantification of atherosclerosis is difficult in humans, whereas in mice any given stage of atherosclerosis can be determined in a standardized way. However, wild-type mice do not develop atherosclerosis on a chow diet, because of their low plasma levels of cholesterol that are mainly confined to atheroprotective HDL-particles (75). When given a semi-synthetic high fat/cholesterol (HFC) and cholate-containing diet, certain strains like C3H do not respond, whereas others like the C57Bl/6 strain develop atherosclerosis (76-78). Major factors determining these strain differences are suggested to be the degree of increase in serum levels of atherogenic (VLDL/IDL/LDL) lipoproteins in response to an HFC diet (77,78), and the degree of activation of inflammatory processes leading to endothelial cell activation in response to oxidized lipoproteins (79).

It is known that humans with the lipoprotein disorders like familial dysbetalipoproteinaemia (FD) or familial hypercholesterolaemia (FH) have increased plasma cholesterol levels and an increased risk of developing cardiovascular disease. FD is associated with apoE2 homozygosity, apoE deficiency, or with rare mutations in the apoE gene leading to an impaired uptake of remnant particles (80-85). FH is caused by mutations in the LDL

receptor gene, leading to an impaired uptake of apoB-containing lipoproteins (86). By introducing mutations in the apoE or removal of apoE and LDL receptor genes in mice, increased plasma cholesterol levels are reached, which render mice into hyperlipidaemic and atherosclerosis susceptible species (87-93). Currently, many of these genetically modified mice, including apoE deficient (87,88,94), apoE*3-Leiden transgenic (92), and LDL receptor-deficient mice (91,93) are used for studying lipoprotein metabolism and atherosclerosis in a standardized way. In addition, these mice are used as test models for lipid-lowering and anti-atherosclerotic drugs or diets.

However, as model for human-like atherosclerosis, apoE deficient mice have several major disadvantages. When given a chow diet, these mice have a prominent hypercholesterolaemia of approximately 20 mmol/l (89,95), which makes the assessment of atherosclerosis under mildly hyperlipidaemic conditions impossible. Moreover, when put on a high fat diet, these mice develop skin pathologies, including massive cutaneous xanthomatosis (96). Furthermore, apoE deficiency leads to the inhibition of several metabolic processes that possibly interfere with atherogenesis, being the cellular cholesterol efflux in macrophages via SR-B1, lipoprotein binding to their receptors, such as the LDL receptor and the LDL receptor-related protein, the extracellular proteoglycan-mediated binding of lipoproteins to these receptors (97), and the production of nascent VLDL (98,99).

On a chow diet, LDL receptor-deficient mice have only two-fold elevated serum cholesterol levels, and these mice do not develop atherosclerosis. Early studies showed that when these mice were given a semi synthetic cholate-containing high-fat/cholesterol (HFC) diet (78), their serum cholesterol levels increased more than 10-fold, confined to LDL/IDL cholesterol levels, and they developed severe atherosclerotic lesions and massive cutaneous xanthomatosis (91,100,101). This led to the belief that this mouse model was too extreme, and not relevant to human atherosclerosis (102). However, in this model the extreme hypercholesterolaemia, and cutaneous xanthomatosis can possibly be ascribed to the extreme diet conditions and the potentially toxic effects of dietary cholate (78,103). Moreover, on semi-synthetic diets that are devoid of, or lowered in cholate, serum cholesterol levels of homozygous and heterozygous LDL receptor-deficient mice can be adjusted to any desired level by varying the dietary fat and cholesterol content (90). However, serum lipid levels of heterozygous LDL receptor-deficient mice are less responsive to changes in dietary contents of fat, cholesterol and cholate than apoE*3-Leiden mice (this thesis). Furthermore, these LDL receptor-deficient mice can develop

early and advanced atherosclerotic lesions, resembling the human situation (90). These features make LDL receptor-deficient mice suitable for assessing atherosclerosis at lipid levels, and lipoprotein profiles that are comparable to the human situation.

In addition to mice models that are devoid of specific gene products that modulate lipoprotein metabolism, transgenic mice expressing the mutated human apoE variant apoE*3-Leiden, have been generated (92). ApoE*3-Leiden is a dominant human mutation, consisting of a tandem duplication of codons 120-126 of the apoE gene, leading to the insertion of seven amino acids in the apoE protein. In humans, this mutation causes impaired binding of apoE to the LDL receptor (60% of normal), and consequently to a decreased clearance of apoB-containing lipoproteins and elevation of serum lipid levels (104,105). When given a chow diet, apoE*3-Leiden transgenic mice have a mild phenotype with 1.5-fold and 5-fold elevations in the serum levels of cholesterol and triglycerides, respectively (106). Furthermore, on semi-synthetic diets, the degree of hypercholesterolaemia can be adjusted to any desired level by varying the dietary contents of fat, cholesterol and cholate (93,106,107). In apoE*3-Leiden transgenic mice, the phenotype of hypercholesterolaemia which is confined to elevated levels of apoB-containing lipoproteins, is primarily caused by a mild defect in the receptor-mediated clearance of these remnant particles from the circulation (108). Furthermore, depending on the serum cholesterol exposure, apoE*3-Leiden mice develop atherosclerosis, ranging from fatty streaks to severe lesions that are well characterized and in many aspects similar to human atherosclerosis (106,107,109-111). Moreover, apoE*3-Leiden mice have successfully been used as a test model for investigating the effects of lipid-lowering and anti-atherosclerotic drugs and dietary interventions under mildly hyperlipidaemic conditions (112-116). Additionally, in contrast to wild-type and apoE-deficient mice, apoE*3-Leiden transgenic mice respond well to treatments with statins and fibrates (112). Furthermore, these mice are the only known model that responds to the cholesterol-raising factor in unfiltered coffee, cafestol (114).

In summary, hyperlipidaemic LDL receptor-deficient and apoE*3-Leiden transgenic mice are useful models for investigating genetic and environmental factors, and the effects of drugs and dietary intervention on hyperlipidaemia and/or development of atherosclerosis. The lipid levels of LDL receptor-deficient mice are less easily modulated as those of apoE*3-Leiden mice, and apoE-deficient mice are a considerably extreme model.

However, it should be noted that at present mice are not useful subjects for investigating all aspects of human atherosclerosis, because there are some important species differences in the aetiology of atherosclerosis. First, in mice the development of atherosclerosis has not been shown to be influenced by the coagulation and fibrinolytic systems (117,118), whereas in humans, variations in coagulation factors are important risk factors of cardiovascular disease (55). Second, in mouse models of atherosclerosis, plaque rupture does not occur spontaneously, which is the most important cause for cardiovascular events in humans (52,53).

Scope of this thesis

In humans, daily consumption of margarine with 2-3 g of plant stanol esters has proven to effectively lower plasma cholesterol levels by 10% on average, due to the lowering of circulating LDL cholesterol levels by approximately 15%. The mechanism behind this cholesterol-lowering effect is inhibition of the intestinal cholesterol absorption. In chapter 2, we investigated the cholesterol-lowering efficacy of plant stanol esters in apoE*3-Leiden transgenic mice. To further elucidate the mechanism of the effects of plant stanol esters on lipid metabolism, we primarily assessed the effects of plant stanol esters on the hepatic production and composition of VLDL and bile.

In humans, LDL cholesterol-lowering drug treatment has shown to reduce cardiovascular disease-related mortality. Since plant stanol esters have also been proven to effectively lower the levels of LDL cholesterol in humans, their consumption is suggested to be promising for reducing cardiovascular disease risk. However, plant stanol ester-enriched margarines have only recently become available for human consumption, and have, therefore, not yet been demonstrated to diminish cardiovascular disease. In this light, we investigated whether plant stanol esters attenuated the progression of the atherosclerotic plaques (chapter 3). For this purpose we also used apoE*3-Leiden transgenic mice, since these mice develop atherosclerotic lesions with a composition that resembles human atherosclerosis. We also compared the cholesterol-lowering and anti-atherosclerotic efficacies of plant stanol esters derived from pinewood and vegetable oils.

HOE402 is a synthetic compound that is known to exert a potent cholesterol-lowering effect in hamsters and rabbits, possibly through induction of hepatic LDL receptor mediated clearance of apoB-containing lipoproteins from plasma. However, the precise mechanism of action was not clear. To further investigate the involvement of the LDL receptor and the underlying mechanism of the hypocholesterolaemic potency of this compound, we used wild-type, heterozygous and homozygous LDL receptor-deficient mice (chapter 4).

Since vitamin E is a potent antioxidant *in vivo*, and the oxidation of LDL plays an important role in the early onset of atherosclerosis, many studies assessed whether vitamin E can inhibit the progression of atherosclerosis. Epidemiological studies have presented evidence for the potentially beneficial effects of vitamin E on atherosclerosis and cardiovascular disease, whereas clinical intervention studies showed inconsistent results. Therefore, we investigated in heterozygous LDL receptor-deficient mice whether vitamin E

had anti-atherosclerotic properties, under human-like conditions with a mildly elevated cholesterol level and comparable lipoprotein profile (chapter 5). In addition, we investigated in apoE*3-Leiden transgenic mice whether vitamin E effectively diminished the development of atherosclerosis at a higher cholesterol level, and at a lower cholesterol level, after reducing this cholesterol level with a statin (chapter 6). Since there is clinical evidence that the composition of the atherosclerotic lesion is a more important predictor for the risk of cardiovascular disease than its size alone, we assessed whether vitamin E produced beneficial effects on the size and composition of the atherosclerotic plaques. Therefore, we quantified the severity of the plaques, and the plaque contents of collagen, smooth muscle cells and macrophages, as measures of plaque stability. In humans, an important determinant of plaque stability is the structural strength of the fibrous cap, that is mainly established by the amounts of extracellular matrix, of which collagen is an important component.

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Chapter 2

Dietary plant stanol esters reduce VLDL-cholesterol secretion and bile saturation in apoE*3-Leiden transgenic mice

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Abstract

Dietary plant stanols lower serum cholesterol levels in humans and in hyperlipidaemic rodents, mainly by inhibition of the intestinal cholesterol absorption. We used female apoE*3-Leiden transgenic mice to investigate the consequences of this effect on serum lipid levels and hepatic lipid metabolism. Five groups of 6 to 7 mice received for 9 weeks a diet containing 0.25% cholesterol and 0.0, 0.25, 0.5, 0.75 or 1.0%(wt/wt) plant stanols (sitostanol 88% wt/wt, campestanol 10% wt/wt) esterified to fatty acids. Compared with the control diet, plant stanol ester treatment dose-dependently reduced serum cholesterol levels by 10 to 33% ($P<0.05$), mainly in VLDL, IDL and LDL. Furthermore, 1.0% dietary plant stanols significantly decreased the liver contents of cholesteryl esters (–62%), free cholesterol (–31%) and triglycerides (-38%), but did not change the hepatic VLDL-triglyceride and VLDL-apolipoprotein B production rates. However, plant stanol ester feeding significantly decreased the amounts of cholesteryl esters and free cholesterol incorporated in nascent VLDL by 72% and 30% respectively, resulting in a net two-fold decreased VLDL cholesterol output. Liver mRNA levels of LDL receptor, HMG-CoA synthase, cholesterol 7α-hydroxylase and sterol 27-hydroxylase were not changed by plant stanol ester feeding. Nevertheless, serum lathosterol/cholesterol ratio was significantly increased by 23%, indicating that dietary plant stanol esters increased whole-body cholesterol synthesis. Plant stanol esters also significantly decreased the cholesterol saturation index in bile by 55%. In conclusion, in apoE*3-Leiden transgenic mice plant stanol ester feeding dose-dependently lowered serum cholesterol levels as a result of a reduced secretion of VLDL-cholesterol. This was caused by a decreased hepatic cholesterol content that also resulted in a lowered biliary cholesterol output, indicative of a reduced lithogenicity of bile in these mice.

Introduction

Plant sterols are structurally closely related to cholesterol. In contrast to cholesterol they are not synthesized by animals, but in plant cells where they are abundantly present. Plant stanols, like sitostanol and campestanol, are saturated forms of the plant sterols β-sitosterol and campesterol respectively and occur in nature only in trace amounts. However, by saturation of plant sterols, large amounts of plant stanols can be obtained. Simply, through trans-esterification with rapeseed oil fatty acids, plant stanols and sterols can be converted into more fat soluble stanol and sterol esters which makes them suitable for addition to fat-containing foods such as margarine and spreads. In several mammalian animal models the hypocholesterolaemic and anti-atherosclerotic properties of plant sterols have been shown (1-4). Plant sterols and stanols lower serum cholesterol through inhibition of the intestinal cholesterol absorption of both dietary and biliary cholesterol, leading to an increased fecal cholesterol excretion (2,5-9). In hypercholesterolaemic children, un-esterified sitostanol appears to be more effective in lowering serum cholesterol than un-esterified sitosterol (10). However, fatty acid chain esterified plant stanols (plant stanol esters) and plant sterols (plant sterol esters) are equally potent in lowering serum cholesterol levels (11,12).

The present study using apoE*3-Leiden transgenic mice was conducted to investigate the physiological mechanism underlying the reducing effects of dietary plant stanol esters on serum cholesterol levels. We chose apoE*3-Leiden mice because these mice, in contrast to non-transgenic rodents, display a human-like lipoprotein profile with abundant VLDL/LDL sized lipoproteins (13,14). When given semi-synthetic sucrose-based diets, serum lipid levels of these mice can easily be varied by changing the amount of cholesterol and fat supplied via the diets (13). Furthermore, the serum cholesterol and triglyceride levels are highly responsive to small changes in chylomicron and VLDL metabolism (13,15). apoE*3-Leiden transgenic mice respond well to hypolipidaemic drugs and diet intervention such as fish oil and the cholesterol raising factor in boiled coffee, cafestol (14,16,17).

In the present study we observed that in apoE*3-Leiden mice plant stanol ester feeding dose-dependently lowered serum cholesterol by reducing the hepatic availability of cholesterol for incorporation into nascent VLDL. This also resulted in a reduced biliary cholesterol output.

Methods

Animals, Housing and Diets

Three months old female apoE*3-Leiden transgenic mice were used for the experiments (18). Transgenic mice were identified with an ELISA for human apoE (13) and were F13 generation of breeding male apoE*3-Leiden transgenic mice with female C57BL/6Jico mice (The Broekman Institute bv, Someren, The Netherlands). All animals were housed in wire-topped Macrolon cages with sawdust as bedding and all diets and water were given ad libitum. During the study period, animals were fed semi-synthetic diets (see table 1 for composition). During the complete study period food disappearance expressed as (gram/mouse/day) was determined by periodically weighing of the diets. Dietary plant stanol esters were obtained by saturation of corresponding plant sterols and subsequent esterification with fatty acids from low erucic rapeseed oil (Raisio Group, Raisio, Finland).

TABLE 1. Composition of experimental diets

Diet components	Diet				
	Group 1	Group 2	Group 3	Group 4	Group 5
	g /100g diet				
Western fat *	11	11	11	11	11
Shortening					
Control	8	6	4	2	0
Experimental	0	2	4	6	8
Sucrose	38.5	38.5	38.5	38.5	38.5
Corn starch	10	10	10	10	10
Casein	20	20	20	20	20
Cellulose	5.95	5.95	5.95	5.95	5.95
Free stanols†	0.0	0.25	0.50	0.75	1.0
Cholesterol	0.25	0.25	0.25	0.25	0.25

* Western fat comprised 40.7% MUFA, 17.4% PUFA and 41.9% SAFA, total fat provides 37.0 energy %, sucrose 35.0 energy %, corn starch 9.2 energy % and casein 18.8 energy %. † Percentages (wt/wt) were calculated for the amount of free stanols, but were provided as stanol esters that comprised 87.6% sitostanol, 9.5% campestanol, 1.1% β-sitosterol, 0.8% campesterol, 0.5% sitostane and 0.1% campestane in the first experiment. In the second experiment, the stanol esters consisted of 73.0% sitostanol, 24.7% campestanol, 1.1% β-sitosterol, 0.9% campesterol, 0.3% sitostane and 0.1% campestane. All diets contained 1% choline chloride (50% wt/v), 0.2% methionine, 4.85% mineral mixture and 0.25% vitamin mixture (all wt/v).

Experimental Design

At the start of a 4-week run-in period diets were changed from chow (SRM-A, Hope Farms, Woerden, The Netherlands) to a semi-synthetic diet (diet group I, table 1). After this run-in period, animals were separated into five different dietary groups in a first experiment and into two different groups in a second experiment, matched for age and serum cholesterol levels.

In the first experiment, the respective dietary plant stanol contents were 0.0 (control group), 0.25, 0.50, 0.75 and 1.0% (wt/wt) (table 1) and in the second experiment, the plant stanol contents were 0.0 (control) and 1.0% (wt/wt) respectively. In experiment one, the control group comprised six mice and the dietary stanol ester groups consisted of seven mice. In the second experiment, the dietary groups each consisted of 12 mice. Blood was collected from the tail vein at weeks 0 (run-in endpoint), 4 and 8 (first experiment) or 9 (second experiment), after 4 hours of fasting.

Serum lipids, lipoproteins and non-cholesterol sterols

Levels of total serum cholesterol and triglycerides were measured enzymatically using commercially available kits (236691, Boehringer Mannheim, Germany and 337-B, GPO-Trinder kit, Sigma Chemical Co. St Louis, USA).

For determination of serum lipoprotein profiles, pooled serum was subjected to density gradient ultracentrifugation in an SW41 rotor (Beckman Instruments, Inc., Geneva, Switzerland). After fraction collection, density (using a DMA 602M densitometer, Paar, Germany) and total cholesterol and triglycerides were measured using commercial enzymatic kits (cholesterol as described above; triglycerides GPO-PAP kit, Sigma Chemical Co. St Louis, USA).

Serum non-cholesterol sterols were determined as described by Kempen et al. (19), 5α -cholestane was used as internal standard. The different sterols were separated on a 25m x 0.35mm capillary GLC column (CP Sil 5CB, Chrompack Int. Middelburg, The Netherlands) in a Varian 3800 gas chromatograph equipped with a flame ionization detector (FID). The injector temperature was raised from 240- to 280 °C in 10 minutes and the FID detector temperature was kept at 300 °C. Quantitation was based on the area ratio of the individual sterol to 5α -cholestane.

Liver lipids, mRNA analyses and cholesterol 7α-hydroxylase activity

Lipids were extracted from livers using the method of Bligh and Dyer and separated by high performance thin-layer chromatography (HPTLC) (20). Quantification was performed after charring.

Total RNA was isolated from liver using a single-step method (21). Northern-blotting and hybridization techniques were performed as described by Post et al. (17). Blots were subsequently hybridized with ^{32}P-labelled cDNA probes of LDL receptor, HMG-CoA synthase, cholesterol 7α-hydroxylase, sterol 27-hydroxylase. SB34 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were used as internal standards to correct for differences in the amount of RNA applied to the gel or filter. The intensity of the hybridization signal was quantified with a Phosphor Imager (Molecular Dynamics). Microsomal cholesterol 7α-

hydroxylase activity of freshly isolated liver microsomes was determined as described by Post et al. (17).

Bile analyses

After 4 hours of fasting and being anaesthetized as described below, gallbladders were canulated (littermates of mice used for measuring VLDL production and nascent VLDL lipid composition) and bile was immediately collected as described by Kuipers et al. (23).

Biliary bile acid concentration was determined by an enzymatic kit (Nycomed Pharma AS, Norway). After collecting gall bladder bile, newly produced bile was collected for 90 minutes in intervals of 15 minutes. Hepatic bile acid synthesis rates were determined at the nadir of individual bile acid output versus time curves (average of 60-75 and 75-90 minute intervals) (23). The biliary bile acid pool size was determined by calculating the area under the curve of individual bile acid output versus time plots, after subtraction of the hepatic bile acid synthesis rates. Biliary cholesterol and phospholipids were measured enzymatically using kits as described above. Cholesterol saturation index of bile was determined using calculations described by Carey (24).

VLDL production and nascent VLDL lipid composition

After 4 hours of fasting, mice were anaesthetized (with 2.5 ml/kg Dormicum®, Roche and 2.5 ml/kg Hypnorm, Janssen Pharmaceutica). *In vivo* hepatic VLDL-apolipoprotein B and VLDL-triglyceride production were determined after intravenous ^{35}S-methionine and Triton WR-1339 injection as described by Post et al. (17). Blood samples were taken at 30 seconds, 30-, 60- and 90 minutes after Triton WR 1339 injection and serum triglycerides were measured. Liver triglyceride production was calculated from the slope of the curve and expressed as μmol/hr/kg body weight (15).

Nascent VLDL was isolated from serum, collected 90 minutes after Triton WR 1339 injection, by density gradient ultracentrifugation. For determination of the lipid composition of nascent VLDL, total cholesterol, free cholesterol, phospholipid and triglyceride contents were measured enzymatically using kits (cholesterol and triglycerides as described above; free cholesterol MPR 1, 125512, Boehringer Mannheim, Germany; phospholipids B-kit, 990-54009, Wako Chemicals GmbH, Neuss, Germany). Cholesteryl esters were calculated as the difference between total and free cholesterol. For quantification of apolipoprotein B in nascent VLDL, apolipoprotein B was isolated by precipitation of VLDL with isopropanol (VLDL:isopropanol, 1:1 by volume) for 1 hr at room temperature, followed by centrifugation (Biofuge A, Heraeus Sepatech GmbH, Germany). Supernatants were removed and pellets were dissolved in 0.1 M NaOH at 95°C. VLDL total protein and apolipoprotein B contents were determined using the method of Lowry.

Statistical analyses

For determination of the relation between the dietary stanol content and serum cholesterol levels, regression analysis (curve estimation) was performed. For comparisons of two groups, data were analyzed non-parametrically, using the Mann-Whitney U test. P-values less than 0.05 were considered as significant. All data were
statistically analyzed using SPSS for Windows.

Results

Serum lipids, lipoproteins and non-cholesterol sterols

Food intake and body weights were not affected by stanol ester feeding (data not shown). Serum cholesterol levels decreased dose-dependently upon feeding stanol esters (figure 1 A). Maximal cholesterol-lowering was already obtained after 4 weeks of treatment in the dietary plant stanol groups treated with 0.5, 0.75 and 1.0%(wt/wt) plant stanols. Regression analysis revealed a significant negative relation between dietary stanol ester content and serum cholesterol levels (insert figure 1 A). Serum triglyceride levels remained unaffected by dietary stanol esters (data not shown). Separation of lipoproteins showed that stanol ester feeding caused a dose-dependent decrease in VLDL-, IDL- and to a lesser extent in LDL-cholesterol (figure 1B). Plant stanol ester feeding had no effect on serum HDL-cholesterol levels (figure 1B).

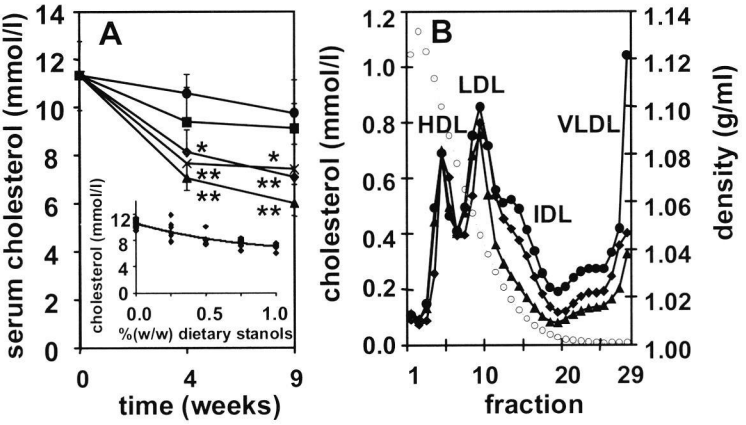

Figure 1. Effects of dietary plant stanol dose on serum cholesterol levels (means ± SD) in time (A) and on serum lipoprotein profiles after 9 weeks of treatment (B).

The percentages (wt/wt) of dietary plant stanols are indicated by the following marks: ● 0.0%, ■ 0.25%, ◆ 0.50%, × 0.75% and ▲ 1.0%. The white ○ marks indicate the density gradient of the lipoprotein profile (B). Significant differences between control diet and stanol ester diets are indicated by one asterisk * ($P<0.05$) and highly significant differences by two asterisks ** ($P<0.005$) (A). After 4 weeks of treatment, a highly significant ($P<0.005$, $R^2=0.669$) negative quadratic relation between serum cholesterol and dietary stanol ester dose ($Y = 2.2x^2 - 5.7x + 10.6$) was revealed by regression analysis (insert in 1A).

Since the effects on serum cholesterol levels were most pronounced in the groups treated with 1%(wt/wt) dietary plant stanols, we studied the underlying mechanism of this cholesterol-lowering effect at 1%(wt/wt) dose of dietary plant stanols.

The ratios of serum levels of plant sterols to cholesterol reflect the fractional intestinal absorption of (dietary and biliary) cholesterol (25). Compared with the control diet, plant stanol ester feeding lowered serum plant sterol/cholesterol ratios (table 2), indicating that the intestinal cholesterol absorption was reduced by dietary plant stanol esters. Serum plant stanol levels were also measured and were virtually undetectable in control mice (table 2). Compared with controls plant stanol levels were increased in plant stanol ester-treated mice. However, in the latter mice the plant stanol levels were still markedly lower than the levels of the plant sterols.

Lathosterol is a cholesterol precursor sterol and its serum levels relative to serum cholesterol indicate whole-body cholesterol synthesis (19). Compared with controls, plant stanol ester feeding significantly increased

serum lathosterol/cholestberol ratios (table 2), indicating that plant stanol ester feeding increased whole-body cholesterol synthesis.

TABLE 2. Effect of dietary plant stanol esters on serum levels of plant stanols and non-cholesterol sterols relative to serum cholesterol

	Control diet	Plant stanol ester diet	
	μmol/mmol cholesterol		
Plant stanols			
Campestanol	n.d.	0.21±0.02*	(840)
Sitostanol	0.08±0.05	0.20±0.03*	(253)
Plant sterols			
Campesterol	6.70±0.72	5.28±0.23*	(79)
β-Sitosterol	1.18±0.17	0.91±0.09*	(77)
Cholesterol precursor sterol			
Lathosterol	0.12±0.01	0.15±0.02*	(123)

n.d. = not detectable

Mice were treated with 1%(wt/wt) plant stanols or control diet (respective diet groups 5 and 1, table 1) for 8 weeks. Serum plant stanols and non-cholesterol sterols were determined using GLC as described in Methods and expressed as μmol/mmol cholesterol, since serum cholesterol levels influence the levels of the measured sterols (19). *Values in parentheses* represent the percentage of the value obtained in mice on control diet. Statistically significant differences between control diet and stanol ester diet are indicated by an asterisk * ($P<0.05$, Mann-Whitney U). Data are given as means±SD (n=3 pools of two mouse sera each).

Hepatic lipid and bile metabolism

Livers of stanol ester-treated mice had significantly lower cholesteryl ester, free cholesterol and triglyceride contents than livers of control mice (table 3). To investigate the consequences of the decreased hepatic cholesterol content, we measured liver mRNA levels of HMG-CoA synthase and LDL receptor.

Plant stanol ester feeding had, compared with controls, no effect on liver mRNA levels of HMG-CoA synthase and LDL receptor (table 3). In addition, dietary plant stanol esters had no influence on hepatic mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase (table 3).

TABLE 3. Effect of dietary plant stanol esters on hepatic lipids and mRNA levels

	Control diet	Stanol ester diet
Lipids (μg/mg protein)		
Cholesteryl ester	46.6±1.7	17.6±3.4* (38)
Free cholesterol	17.7±2.1	12.3±0.4* (69)
Triglycerides	106.8±4.3	66.2±10.7*(62)
MRNA levels(% of control diet)		
LDL receptor		
SB34	100±14	102±13
GAPDH	100±25	98±10
HMG-CoA synthase		
SB34	100±19	92±13
GAPDH	100±29	87±11
Cholesterol 7α-hydroxylase		
SB34	100±19	94±9
GAPDH	100±28	90±9
Sterol 27-hydroxylase		
SB34	100±7	104±10
GAPDH	100±12	102±8

Mice were treated with 1%(wt/wt) plant stanols or control diet (respective diet groups 5 and 1, table 1) for 8 weeks. Livers were isolated immediately after the VLDL-apolipoprotein B and VLDL-triglyceride production experiment. In liver homogenates, free cholesterol, cholesteryl ester and triglycerides were determined using HPTLC as described in Methods. *Values in parentheses* represent the percentage of the value obtained in mice on control diet. mRNA was isolated from liver homogenates as described in Methods. Statistically significant differences between control and treated mice are indicated by an asterisk * ($P<0.05$, Mann-Whitney U). Data are given as means±SD (N=3 pools of two mouse livers each).

In line with the mRNA measurements, no effect of 8 weeks plant stanol ester feeding on microsomal cholesterol 7α-hydroxylase activity was found (table 4). Furthermore, no effects of plant stanol ester treatment on bile flow and the biliary outputs of bile acids and phospholipids were found (table 4). In addition, control and plant stanol ester-treated mice had similar bile acid pool sizes and hepatic bile acid synthesis rates (table 4). However, compared with controls, dietary plant stanol esters significantly reduced biliary cholesterol outputs by more than 50%. As a consequence, the biliary cholesterol saturation index was significantly decreased by 55% (table 4).

TABLE 4. Effect of plant stanol ester feeding on flow and composition of bile and cholesterol 7α-hydroxylase enzyme activity

	Control diet	Stanol ester diet	
Cholesterol 7α-hydroxylase activity	10.7±0.7	11.7±4.0	(110)
Bile flow	7.2±1.1	7.1±1.0	(94)
Output			
Bile acid output	438.8±209.6	403.8±129.3	(92)
Cholesterol output	4.3±1.3	2.1±01*	(49)
Phospholipid output	70.1±7.5	74.3±9.9	(105)
Bile cholesterol saturation index	0.982±0.566	0.442±0.055*	(45)
Bile acid pool size	11.3±5.0	11.4±2.1	(101)
Hepatic bile acid synthesis	171±25	186±14	(109)

Cholesterol 7α-hydroxylase enzyme activity was determined in hepatic microsomes as described in Methods and expressed as nmol/hr/mg protein. Bile was collected for 15 minutes, immediately after creation of a gallbladder fistula and collecting gallbladder bile. Bile flow, expressed as μl/min/100g bodyweight, was determined gravimetrically. Output rates, expressed as nmol/min/100g body weight, were calculated by multiplying bile flow with concentration. Bile acid pool size, expressed as μmol/100g body weight, and hepatic bile acid synthesis, expressed as nmol/min/100g body weight, were determined as described in Methods. *Values in parentheses* represent the percentage of the value obtained in mice on control diet. An asterisk indicates statistically significant differences between control diet and stanol ester diets *($P<0.05$, Mann-Whitney U). Data represent means ± SD of six mice per group.

VLDL production

To assess whether the decreased hepatic cholesterol levels in plant stanol ester fed mice had an effect on secretion of cholesterol into the circulation, we measured VLDL-triglyceride and apolipoprotein B production and the lipid content of nascent VLDL (table 5). Plant stanol ester feeding had no effect on the secretion by the liver of VLDL-triglyceride. In addition, VLDL-apolipoprotein B production was also unchanged, indicating that the number of VLDL particles produced by the liver was not affected by plant stanol ester feeding. However, dietary plant stanol esters significantly reduced the absolute amounts of cholesteryl ester (-70%) and free cholesterol (-28%) per nascent VLDL particle, resulting in a net decreased hepatic cholesterol output (-58%). This is indicative of the secretion of a β-VLDL-like particle less enriched in cholesterol. In contrast, the average phospholipid and triglyceride contents of nascent VLDL were not increased, the latter in line with the absence of a change in serum triglyceride levels.

TABLE 5. Effect of plant stanol ester feeding on production of VLDL-triglyceride and VLDL-apolipoprotein B and on lipid composition of nascent VLDL

	Control diet	Stanol ester diet
VLDL-triglyceride production (μmol/hr/kg bodyweight)	80.8±13.1	98.7±19.5 (122)
VLDL-apolipoprotein B production (10^3 dpm/hr/kg bodyweight)	145±45	131±59 (90)
Nascent VLDL lipid composition (μmol/mg apolipoprotein B)		
cholesteryl ester	13.4±3.0	3.8±1.8* (28)
free cholesterol	6.3±1.3	4.4±1.2* (70)
triglycerides	17.4±2.0	20.5±4.9 (117)
phospholipids	9.6±1.0	8.0±2.1 (83)

Mice were treated with 1%(wt/wt) plant stanols or control diet (respective diet groups 5 and 1, table 1) for 8 weeks. After ^{35}S-methionine and Triton WR-1339 injection triglyceride and de novo apolipoprotein B production and lipid composition of VLDL were determined as described in Methods. *Values in parentheses* represent the percentage of the value obtained in mice on control diet. Statistically significant differences between control diet and stanol ester diet are indicated by an asterisk * ($P<0.05$, Mann-Whitney U). Data are given as means±SD of 6 mice per group.

Discussion

In this study with transgenic apoE*3-Leiden mice, plant stanol esters dose-dependently decreased serum cholesterol levels, mainly in the VLDL and IDL fractions, and to a lesser degree in LDL. No effect on serum triglycerides was found. Dietary plant stanol esters lowered liver lipid levels and reduced hepatic secretion of cholesterol via VLDL and bile. No adverse health effects upon plant stanol ester feeding were observed.

Similar to our findings, plant stanol esters lower serum cholesterol without affecting serum triglycerides in mildly hypercholesterolaemic non-diabetic humans, although the hypocholesterolaemic effect is mainly in the LDL fraction (26). In addition, in non-insulin dependent diabetic patients dietary plant stanol esters reduce serum cholesterol levels in VLDL and LDL without having an effect on serum triglycerides, which is even more comparable to our findings in mice than in non-diabetic humans (27). The sensitive hypocholesterolaemic response to plant stanol ester feeding in apoE*3-Leiden mice is explained by the dominance of the apoE*3-Leiden mutation, defecting the clearance of post-prandial lipoproteins (13). This leads to accumulation of these lipoproteins and results in a human-like lipoprotein profile under dietary challenge with fat and cholesterol (13,15). Due to this phenotype, any change in cholesterol input is reflected by changes in serum cholesterol levels.

Plant stanol ester feeding reduced serum plant sterol/cholesterol ratios, indicating inhibition of the intestinal absorption of plant sterols and cholesterol, which is in line with observations in humans (28) and other rodents (1,2) As a consequence of the decreased intestinal cholesterol absorption, leading to a reduced flux of esterified- and free-cholesterol from the intestines to the liver via chylomicrons, we found that stanol ester feeding resulted in a reduction in hepatic cholesteryl ester and free cholesterol content. Theoretically, a decrease in the hepatic pool of free cholesterol would lead to up regulation of genes involved in cholesterol synthesis and the LDL receptor gene. This process is mediated via sterol regulatory element binding proteins (SREBP's), that act as sensitive sensors of the putative regulatory pool of free cholesterol in the cells (29). We found that plant stanol ester feeding did not alter mRNA levels of HMG CoA synthase and the LDL receptor, indicating that the liver was not depleted of cholesterol in these cholesterol-fed mice. The livers of stanol ester-treated animals still contained considerable amounts of cholesterol as compared with chow-fed apoE*3-Leiden mice (cholesterol ester in the latter is 3-5 μg/mg protein). It has been shown that intravenous infusion

of a high dose of phytosterols can inhibit hepatic cholesterol synthesis (30) and that HMG CoA reductase mRNA is reduced in sitosterolemic livers (31). Although we cannot fully exclude the possibility that accumulation of plant stanols in the liver may explain the lack of effect on HMG CoA synthase and LDL receptor mRNAs, we think this is unlikely. In our experiment liver levels were not measured, but serum sterol levels were decreased and although serum plant stanol levels were increased, they remained markedly lower than the plant sterol concentrations. Furthermore, plant sterols and stanols are efficiently removed from the liver into bile by specific transport mechanisms (32). In addition, at least high tissue β-sitosterol concentrations do not inhibit HMG CoA reductase activity and mRNA levels (30).

In line with observations in humans (28) and other rodents (1,2), plant stanol ester treatment did increase whole-body cholesterol synthesis, which is reflected by an elevated serum ratio of lathosterol/cholesterol in apoE*3-Leiden mice. There may be several other reasons for this apparent discrepancy. The cholesterol synthesis, in which HMG CoA reductase plays a key role, is regulated, next to regulation of mRNA levels, at multiple levels, i.e. gene transcription, mRNA and protein stability, enzyme activity and availability of substrate (33). Furthermore, it should be noted that lathosterol is a marker of whole-body cholesterol synthesis. The liver and intestines are the major organs involved in cholesterol synthesis (34). However, it is conceivable that a reduced supply of lipoprotein cholesterol in stanol ester-treated mice may also lead to up-regulation of synthesis in other tissues. The compensatory up-regulation of whole-body cholesterol synthesis, in response to the reduced intestinal cholesterol absorption, is obviously not sufficient to overcome the hypocholesterolaemic effect of stanol ester treatment.

An additional reason why the LDL receptor is not regulated is that, in contrast to humans, in mice liver cholesterol homeostasis is mainly regulated via cholesterol synthesis rather than via LDL receptor mediated uptake of cholesterol (34). This finding also indicates that the hypocholesterolaemic effect of plant stanol esters cannot be explained by an increased LDL receptor-dependent clearance of apolipoprotein B-containing lipoproteins in apoE*3-Leiden transgenic mice.

Plant stanol ester feeding had no effect on hepatic mRNA levels and enzyme activity of bile acid synthetic enzymes nor on biliary bile acid output. These data indicate that the decreased hepatic cholesterol content had no effect on the neutral and acidic pathways in bile acid synthesis. This is in line with observations in mildly hypercholesterolaemic non-insulin dependent

diabetic men, in which no change was found in fecal bile acid content (28). In contrast, we found that dietary plant stanol esters decreased the biliary cholesterol saturation index as a result of a reduced biliary cholesterol output, while the bile flow and biliary outputs of bile acids and phospholipids were unaffected. In addition, the pool size and hepatic synthesis of bile acids were not influenced. These data show that in apoE*3-Leiden mice the reduced hepatic cholesterol content due to plant stanol ester feeding was rate-limiting for the biliary cholesterol output. This resulted in excretion of less saturated bile, which is prognostic for a reduced risk of cholesterol gallstone formation (35). In literature, there are no data on effects of dietary plant stanol esters on biliary cholesterol excretion in experimental animals. In human studies biliary cholesterol excretion was both increased (36) and unchanged (37) by plant stanol ester feeding. These data indicate that plant stanol ester feeding does not lead to a decreased lithogenicity of bile in humans, which is at variance with apoE*3-Leiden mice.

As an explanation for the decreased serum cholesterol levels, we found that the decreased liver cholesterol content led to a reduced hepatic output of cholesteryl ester and free cholesterol in nascent VLDL, without affecting the output-rates of VLDL-triglycerides and the amount of VLDL-particles (apolipoprotein B). Thus, plant stanol ester treatment resulted in the hepatic production of cholesterol poor and therefore less atherogenic βVLDL particles. Data from *in vivo* and *in vitro* studies in animals indicate that hepatic cholesterol, specifically the amount of cholesteryl ester regulates apolipoprotein B secretion and that the hepatic metabolic cholesterol pool is required for secretion and transport of triglycerides in VLDL and vice versa (38-40). In addition, in non-insulin dependent diabetic patients the LDL-apolipoprotein B production rate was lowered by dietary plant stanol esters (36). Our finding that there was no change in VLDL-apolipoprotein B production upon plant stanol ester treatment indicates that in mildly cholesterol-fed apoE*3-Leiden mice the size of the hepatic cholesteryl ester pool did not become rate-limiting for apolipoprotein B secretion.

In apoE*3-Leiden mice plant stanol ester feeding lowered the liver triglyceride content. The biochemical background of this finding awaits further investigation. The lowered liver triglyceride content implicates that during assembly of nascent VLDL the reduced cholesteryl ester incorporation was not compensated by an increased incorporation of triglycerides, as observed.

In conclusion, in apoE*3-Leiden mice the reduced hepatic cholesterol content upon plant stanol ester feeding was responsible for the serum cholesterol-

lowering effect via a decreased incorporation of cholesterol in nascent VLDL. In addition, the reduced hepatic cholesterol content led to a reduced biliary cholesterol saturation. Reduction of the lithogenicity of bile may be an additionally favorable feature of plant stanol ester consumption, which may only be applicable to these mice. We have recently shown that feeding these mice with plant stanol esters also dramatically reduced the extent and severity of atherosclerosis (41).

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Chapter 3

Dietary vegetable oil and wood derived plant stanol esters reduce atherosclerotic lesion size and severity in apoE*3-Leiden transgenic mice

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Abstract

The hypolipidaemic and anti-atherosclerotic effects of vegetable oil- and wood-based dietary plant stanol esters were compared in female apoE*3-Leiden transgenic mice at relevant plasma cholesterol levels. The plant stanol esters derived from vegetable oil (sitostanol 65.7%, campestanol 30.1%) had different contents of sitostanol and campestanol than the plant stanol esters derived from wood (sitostanol 87.6%, campestanol 9.5%) or from a mixture of vegetable oil and wood (sitostanol 73.0%, campestanol 24.7%). The mice (10 per group) received for 38 weeks a control diet or diets containing 1.0%(w/w) plant stanol esters derived from either vegetable oil, wood or a mixture of both. Vegetable oil (-46%), wood (-42%) and vegetable oil/wood (-51%) plant stanol esters decreased the plasma cholesterol levels ($P<0.0001$) by reducing the cholesterol content in plasma very low density-, intermediate density- and to a lesser extent in low density-lipoprotein. Plant stanol ester feeding did not change plasma triglyceride levels. Dietary plant stanol esters reduced the atherosclerotic lesion area by 91±13% (vegetable oil), 97±4% (wood) and 78±34% (vegetable oil / wood) ($P<0.0001$) and the severity from regular intimal fatty streaks/mild plaques (on average type 2-3 lesions) in controls to individual intimal foam cells (<type 1 lesions) in the treatment groups ($P<0.0001$). Plant stanol esters had no effect on adherence of monocytes to the vessel wall. Feeding of plant stanol esters dramatically reduced, independent of its sources, the extent and severity of atherosclerotic lesions, by decreasing VLDL-, IDL- and to a lesser extent LDL-cholesterol in apoE*3-Leiden transgenic mice.

Introduction

Plant sterols are structurally closely related to cholesterol. Plant sterols like β-sitosterol and campesterol are synthesized in plant cells where they are abundantly present. Saturated forms of plant sterols, or plant stanols, occur in nature only in trace amounts. However, by saturation of plant sterols, large amounts of plant stanols can be obtained. Simply, through trans-esterification with rapeseed oil fatty acids, plant stanols and plant sterols can be converted into more fat soluble plant stanol- and sterol-esters which makes them more suitable for addition to fat-containing foods such as margarine and spreads. Several studies performed in humans showed that both dietary plant stanol

esters and plant sterol esters lowered plasma cholesterol levels by reducing LDL-cholesterol without affecting HDL-cholesterol (1-4). In humans, dietary plant sterol esters reduce LDL-cholesterol levels equally potent as plant stanol esters (5,6). Plasma cholesterol reductions as a result of feeding different mixtures of un-esterified plant stanols and sterols have also been observed in hamsters, rabbits and rats (7,8). Dietary addition of un-esterified or esterified plant stanols and plant sterols lower plasma cholesterol by inhibiting the intestinal cholesterol absorption (9-11). Dietary addition of un-esterified plant stanols and sterols have been shown to attenuate atherosclerosis in the ascending aorta and coronary arteries of rabbits and in the aortic root of apoE deficient mice (12,13). In humans and in animal models no evidence for atherosclerosis attenuating properties of stanol esters is available. In addition, it is not known whether there are differences between campestanol- and sitostanol-esters.

The aim of our study was to compare vegetable oil and wood-derived plant stanol esters, having different sitostanol/campestanol ratios with respect to their hypolipidaemic and anti-atherosclerotic properties. In this study, we chose apoE*3-Leiden transgenic mice as a model for hyperlipidemia and for assessing atherosclerosis. Originally, these mice were developed as a model for human familial dysbetalipoproteinaemia (FD) (14). We chose these mice, because they have a lipoprotein profile with abundant VLDL/LDL sized lipoproteins (15,16) that is comparable to human patients with FD (17). In these mice, the accumulation of VLDL and VLDL-remnant lipoproteins (IDL and LDL) in plasma is primarily caused by a defective hepatic uptake of VLDL (18). These mice can easily be adjusted to desired plasma cholesterol levels relevant to the human situation and they respond well to dietary interventions (15,19). Furthermore, they are highly susceptible to diet-induced atherosclerosis (15,20) and the atherosclerotic lesions show close resemblance to human pathology (21,22).

We found that plant stanol esters, irrespective of their source and composition, reduced the size and severity of the atherosclerotic lesions in the aortic root. Lowered plasma cholesterol levels in the VLDL-, IDL- and LDL-sized fractions caused the reduced atherosclerosis.

Methods

Animals and diets

Three months old female apoE*3-Leiden transgenic mice of line #2, expressing the human apoE*3-Leiden gene (23) were used for the experiments. Transgenic mice were F15-F17 generation of breeding male apoE*3-Leiden transgenic mice with female C57BL/6Jico mice (Ifa-Credo Institute bv, Someren, The Netherlands). Identification of transgenity occurred with an ELISA for human apoE, as previously described (15). The animals were housed in wire-topped Macrolon cages with a layer of sawdust as bedding. Institutional guidelines for animal care were followed in all experiments.

All diets and water were given ad libitum to the animals. Before the start of the study, animals were kept on standard rat/mouse chow (SRM-A, Hope Farms, Woerden, The Netherlands). During the experimental run-in period of 4 weeks, animals were fed a cholesterol-containing semi-synthetic control diet (table 1).

Table 1. Composition of the diets

Diet components	Diet			
	Control	Vegetable oil stanol esters	Wood stanol esters	Vegetable oil / Wood stanol esters
	g /100g diet			
Western fat*	11	11	11	11
Sucrose	38.5	38.5	38.5	38.5
Corn starch	10	10	10	10
Casein	20	20	20	20
Cellulose	5.95	5.95	5.95	5.95
Cholesterol	0.25	0.25	0.25	0.25
Shortenings†				
Control	8	0	0	0
Experimental	0	8	8	8
Plant stanols	0.0	1.00	1.00	1.00
% of total plant stanols:				
Sitostanol	0.0	65.7	87.6	73
Campestanol	0.0	30.1	9.5	24.7
β-Sitosterol	0.0	2.1	1.1	1.1
Campesterol	0.0	1.1	0.8	0.85
Sitostane	0.0	0.3	0.5	0.25
Campestane	0.0	0.1	0.1	0.12

* Western fat consisted of 40.7 %(w/w) mono-unsaturated fatty acids, 17.4%(w/w) poly-unsaturated fatty acids and 41.9%(w/w) saturated fatty acids. Total fat provides 37.2 energy %, sucrose 35.3 energy %, cornstarch 9.4 energy % and casein 18.8 energy %.

† The control shortening contained no stanol esters. The experimental shortenings contained stanol esters with the indicated composition. In addition, all diets contained 1% choline chloride (50% w/v), 0.2% methionine, 4.85% mineral mixture and 0.25% vitamin mixture.

Study design

After the run-in period, animals were randomly separated into 4 dietary groups and received four different diets for 38 weeks. These groups were fed a control diet or diets enriched with plant stanol esters derived from vegetable oil-, wood- or vegetable oil/wood-sources (table 1). These dietary plant stanol esters obtained from different sources had different sitostanol/campestanol ratios (table 1). The dietary shortenings (table 1) were provided by Raisio Benecol Ltd, Finland. Food intake was determined periodically at group level by weighing the amount of food consumed and was expressed as gram/mouse/day.

Serum lipid and lipoprotein analysis

Blood samples were taken from the tail vein after 4 hours of fasting. Levels of plasma total cholesterol and triglycerides (without measuring free glycerol) were measured enzymatically using commercially available kits (236691, Boehringer Mannheim, Germany and 337-B, GPO-Trinder kit, Sigma Chemical Co. St Louis, USA). For determination of serum lipoprotein distribution, 400 μl of pooled serum was subjected to density gradient ultracentrifugation at 40.000 rpm in an SW41 rotor (Beckman Instruments, Inc., Geneva, Switzerland) for 18 hours at 4°C, followed by fractionating the volume into 500 μl fractions. In all fractions, density was measured using a DMA 602M densitometer (Paar, Germany), cholesterol and triglycerides were measured as described above using a commercially available kit (701904; GPO-PAP kit, Sigma Chemical Co. St Louis, USA).

Histological assessment of atherosclerosis

After 38 weeks of diet feeding, mice were anaesthetized using 1.6 ml/kg Dormicum (Roche) and 2.0 ml/kg Hypnorm (Janssen Pharmaceutica) and were then sacrificed. The entire hearts plus aortas down to the diaphragm were dissected and fixed (24 hours, 4°C) with Zinc Formal-Fixx^{tm} (Shandon/Lipshaw, Pittsburg, USA,1:4 diluted in de-ionized water). The hearts were then sectioned just below the atria, perpendicular to the axis of the aorta and the aortas were separated from the hearts just below the aortic arch. The tissues were dehydrated and paraffin embedded. Thereafter, the hearts were sectioned perpendicular to the axis of the aorta, starting with the heart and working in the direction of the aortic arch as described by Paigen et al. (24). Once the aortic root was identified by the appearance of the aortic valve leaflets, serial 5 μm sections were taken and mounted at 30 μm intervals on 3-AminoPropyl-3-EtoxySilane (APES) coated slides. Sections were air dried for 24 hours at 37 °C and were then stained with Hematoxylin-Phloxin-Saffron (HPS). Monocytes were immunostained with a rabbit anti-serum against murine macrophages (AIA31240, diluted 1/1500, Accurate Chemical and Scientific).

Quantification and characterization of atherosclerosis and monocyte adhesion

Per animal, full colour images of 4 sections of the aortic root were acquired at 30 μm intervals with a CCD camera (HV-C10, Hitachi, Japan) that was connected to a light microscope (Microphot-FXA, Nikon, Japan) equipped with a 4x objective. The CCD camera was connected to a PC running Qwin image analysis software (Leica imaging systems Ltd, England). The images were all captured under identical lighting and software settings and stored in tiff format.

Thereafter, atherosclerotic lesion areas in the valvular region were measured manually using a pen and drawing board III (Calcomp digitizer products division analyzer, CA, USA) that was connected to a PC and total lesion areas were determined using Qwin image analysis software. The same operator, who was blinded for the group allocation, performed all analyses.

For characterization of the severity of atherosclerosis, the arterial lesions were classified into five categories as described by van Vlijmen et al. (15) using the same cross-sections as used for lesion quantification. This classification is slightly different from the classification of the AHA (25) in which lesions are divided into 6 types. Type 1 lesions (early fatty streaks) comprise up to 10 foam cells, present in the intima. Type 2 lesions (regular fatty streaks) consist of more than 10 foam cells, present in the intima. Type 3 lesions (mild plaques) have a fibrous cap and foam cells also present in the media. Type 4 lesions (moderate plaque) are more progressed lesions with an affected media, but without loss of architecture of the media. Type 5 lesions (severe plaque) have a severely affected media, broken elastic fibers and cholesterol clefts, calcification and necrosis are frequently observed.

Monocyte adhesion was quantified by counting monocytes adhered to the endothelium of the aorta in the valve region in 4 serial HPS stained 5 μm paraffin cross-sections at 30 μm intervals and expressed as average number of monocytes per cross-section. For quantification of monocyte adhesion the above-described light microscope equipped with a 25x objective instead of a 4x objective was used.

Statistical analysis

All data are presented as mean±SD. For multiple group comparisons ANOVA with Tukey's correction was used. P-values less than 0.05 were considered as significant. All data were statistically analyzed using SPSS for Windows (release 8.0).

Results

Plasma cholesterol and triglyceride levels

Dietary plant stanol ester enrichment, independent of its source, had no influence on food intake and body weights. During the complete study the food intake was 2.4±0.5 (gram/mouse/day) in all groups. The body weights gradually increased from 20±1 grams at the beginning of the study to 27±2, 29±3, 29±4 and 27±4 grams at the endpoint, respectively.

Compared with controls, dietary vegetable oil-, wood- and vegetable oil/wood-derived plant stanol esters significantly decreased plasma cholesterol levels (figure 1). For all three treated groups, the maximal decreases in plasma cholesterol were already obtained after 3 weeks. There was no difference in cholesterol-lowering potencies between the different plant stanol ester

sources. During the complete study plant stanol ester feeding had no effect on the plasma triglyceride levels (not shown).

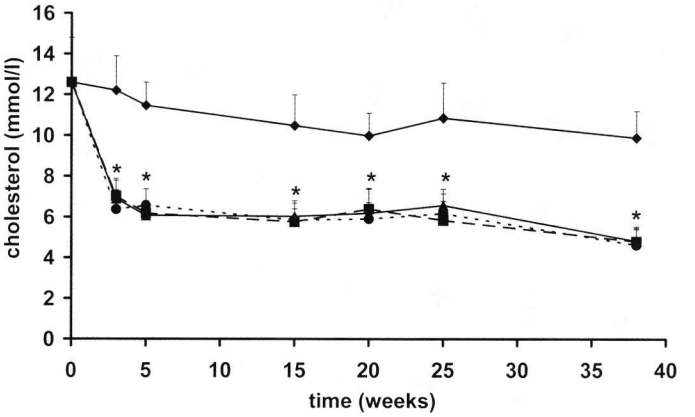

Figure 1. Effect of plant stanol ester feeding on plasma cholesterol levels during the study period of 38 weeks.

The different groups (N=10 per group) are indicated by the following marks: (◆) control diet, (■) or diets with plant stanol esters derived from vegetable oil, (●) wood, or (▲) vegetable oil/wood. Statistically significant differences between control diet and all three plant stanol ester diets are indicated by an asterisk * ($P<0.0001$).

To investigate which lipoproteins were responsible for the plasma cholesterol-lowering effect of plant stanol ester feeding, we made plasma lipoprotein profiles (figure 2), and determined the plasma cholesterol levels of the different lipoproteins (table 2). The ultracentrifugation profiles revealed that plant stanol ester feeding mainly lowered VLDL and IDL cholesterol whereas LDL cholesterol appeared to be less markedly lowered. The plasma lipoprotein cholesterol levels of the different treatment groups (table 2) support these findings by showing a larger decrease of plasma cholesterol in VLDL/IDL and VLDL/IDL/LDL than in LDL alone. However, compared with controls, the decreases in plasma cholesterol levels of LDL, VLDL/IDL/LDL and VLDL/IDL due to plant stanol ester feeding were all highly significant ($P<0.0001$). Furthermore, plant stanol ester feeding had no effect on HDL cholesterol levels (figure 2), neither on the triglyceride levels of all lipoproteins (not shown).

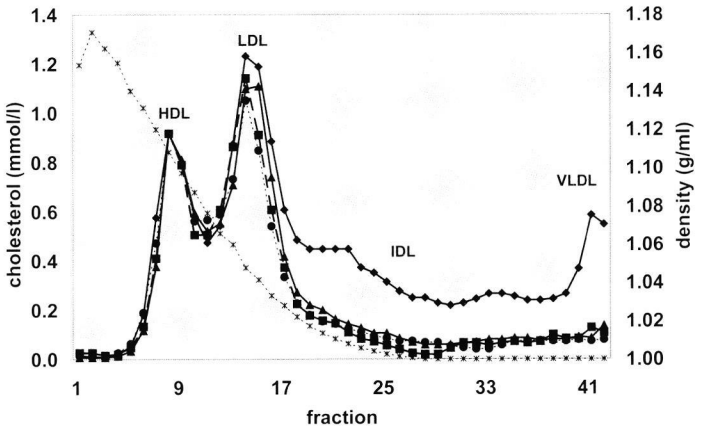

Figure 2. The effect of dietary plant stanol esters on lipoprotein profiles for cholesterol after 25 weeks of treatment.

The pooled ultracentrifugation profiles were made as described in "Methods". The different groups are indicated by the following marks: (◆) control diet, or (■) vegetable oil-, (●) wood- or (▲) vegetable oil/wood- containing diets. The (×) marks indicate the density gradient of the lipoprotein profile.

Table 2. Effect of dietary plant stanol esters on cholesterol levels in apoB-containing lipoproteins.

	Diet			
Cholesterol	Control	Vegetable oil stanol esters	Wood stanol esters	Vegetable oil / Wood stanol esters
	(mmol/l)*			
Plasma	11.7±1.1	6.4±0.8** (54)	6.8±0.7** (58)	5.8±0.7** (49)
VLDL/IDL/LDL	9.5±0.9	4.3±0.6** (46)	4.8±0.5** (50)	3.8±0.4** (40)
VLDL/IDL	5.7±0.5	1.4±0.2** (24)	1.7±0.2** (30)	1.3±0.2** (23)
LDL	3.8±0.4	3.0±0.4** (78)	3.1±0.3** (81)	2.5±0.3** (85)

*Cholesterol levels, expressed as (mmol/l) are average of all time-points measured between 3 and 38 weeks of the study period (see figure 1). Lipoprotein cholesterol levels of individual mice (N=10 per group) were extrapolated from plasma total cholesterol levels (figure 1) using the lipoprotein profiles for cholesterol presented in figure 2. In these profiles the ratios of the area under the curve (AUC) of individual lipoprotein cholesterol levels to the AUC of the total lipoprotein profiles were integrated. The AUC of LDL was calculated from fractions 12-18 and the AUC of IDL/VLDL was calculated from fractions 19-43. Values between parentheses represent the percentage of the value obtained in mice on control diet. Statistically highly significant differences between control diet and plant stanol ester diets are indicated by two asterisks ** ($P<0.0001$).

Atherosclerotic lesion area and severity

At the study endpoint the plasma cholesterol exposures (mmol/l x week) of the animals that received dietary plant stanol esters (vegetable oil 257±31;wood 274±29;vegetable oil/wood 240±25) were significantly lower ($P<0.0001$) than the cholesterol exposure of the control animals (460±45). Exposures between the three experimental groups were similar.

After 38 weeks of treatment, compared with the control diet plant stanol ester feeding significantly ($P<0.0001$) reduced atherosclerotic lesion area (figure 3). There were no significant differences in atherosclerotic lesion areas between the different plant stanol ester fed groups.

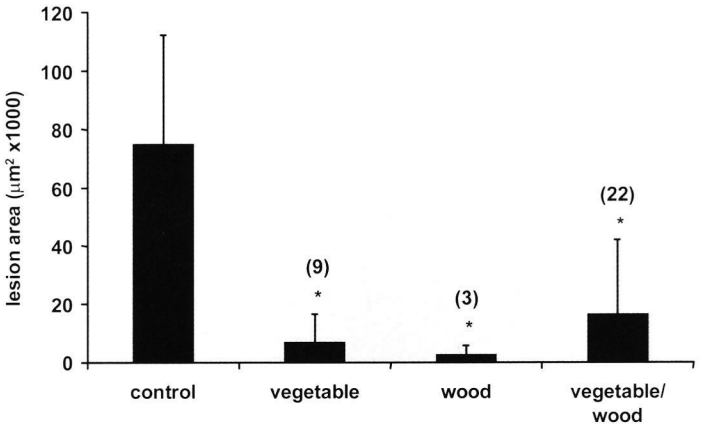

Figure 3. The effect of dietary plant stanol esters on the atherosclerotic lesion area in the aortic root.

Individual atherosclerotic lesion areas (N=10 per group) were determined in 4 consecutive cross-sections per animal, overlapping a 110 μm region as described in "Methods". *Values in parentheses* represent the percentage of the value obtained in mice on control diet. An asterisk indicates statistically significant differences between control diet and stanol ester diets * ($P<0.0001$).

Characterization of atherosclerosis severity showed that the control group had on average type 2-3 lesions, while the dietary plant stanol ester groups had on average lesions with less than 10 macrophages per lesion (type 1). In order to discriminate between early and established atherosclerotic lesions, the classified lesions were assigned into categories of type 0-2 and type 3-5, respectively (figure 4). In the controls, the percentages of type 0-2 lesions and type 3-5 lesions were 60 and 40, respectively. In contrast, in the different plant stanol ester-treated groups, this ratio significantly ($P<0.0001$) shifted to more than 90% of type 0-2 lesions and less than 10% of type 3-5 lesions. However, the increased percentage of type 0-2 lesions is mainly the result of the markedly decreased number of type 3-5 lesions and is not the result of an increased absolute number of type 0-2 lesions (not shown). This is clearly reflected by the pronouncedly decreased lesion size as shown in figure 3, which is similar to the decreased percentage of type 3-5 lesions as seen in figure 4.

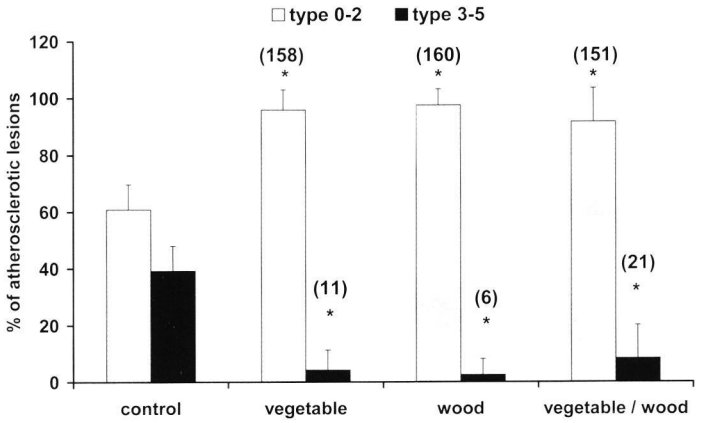

Figure 4. Effect of dietary plant stanol esters on the distribution of early and established atherosclerotic lesions.

Data (N=10 per group) are expressed as the percentage of atherosclerotic lesions that were classified type 0-2 (early) or type 3-5 (established) and the sum of early and established lesions is 100%. Classification of lesion severity was done as described in "Methods". Statistically significant differences between control diet and plant stanol ester diets are indicated by an asterisk * ($P<0.0001$). *Values in parentheses* represent the percentage of the value obtained in mice on control diet. Data were obtained from the same 4 cross-sections per animal of which atherosclerotic lesion area was quantified.

Photomicrographs, representative for the atherosclerotic lesions found in the different treatment groups, visualize that plant stanol ester feeding decreased lesion area and severity (figure 5).

Since adherence of monocytes to the activated endothelium is one of the first steps in the atherosclerotic process, we also quantified the number of monocytes attached to the vessel wall. There was no difference in monocyte adherence between the control group and the groups that received plant stanol esters ($P=0.168$). The number of monocytes adhered per cross-section were 8±3 (controls), 5±3 (vegetable oil-), 6±2 (wood-) and 6±3 (vegetable oil/wood-derived stanol esters), respectively. A photomicrograph of monocytes adhered to the aortic vessel wall is depicted in figure 6.

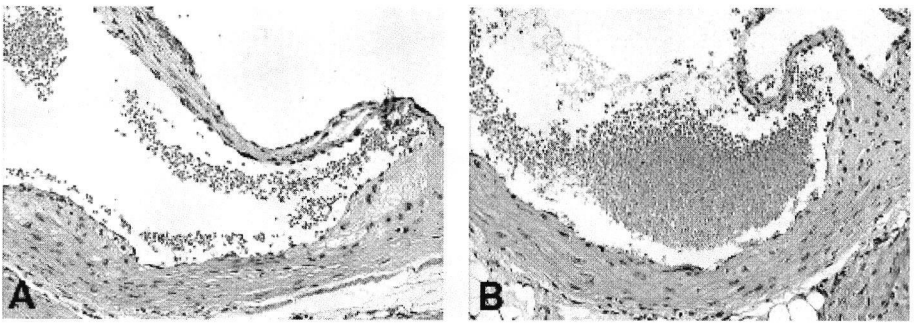

Figure 5. Photomicrographs (magnification 10x) of the aortic root.
The atherosclerotic type 2 lesions in (A) are representative for the control lesions while the type 1 lesion on the valve cusp attachment in (B) is representative for lesions present in the aortic root of plant stanol ester-treated mice. The 5 μm paraffin cross-sections were stained with hematoxylin-phloxin-saffron.

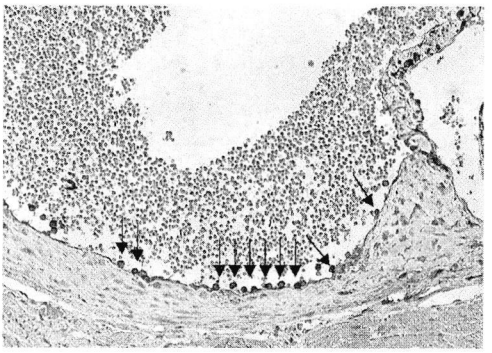

Figure 6. Representative photomicrograph (magnification 10x) of aortic vesselwall with white and red blood cells present at the luminal side of the aorta.
Monocytes and macrophages were stained immuno-histochemically with an AIA31240 antibody. The monocytes adhered to the endothelium are marked with black arrows.

Discussion

We found that in apoE*3-Leiden transgenic mice plant stanol esters derived from vegetable oil, wood and vegetable oil/wood sources, irrespective of their sitostanol/campestanol content, dramatically reduced atherosclerotic lesion

area and severity, mainly as a consequence of reduced VLDL- and IDL-cholesterol levels. Furthermore, plant stanol ester feeding decreased the atherosclerotic lesion severity from regular intimal fatty streaks/mild plaques (type 2-3 lesions) in controls to lesions consisting of individual foam cells (type 1 lesions) in the plant stanol ester-treated groups.

We found that the vegetable oil- and wood-derived dietary plant stanol esters, having different sitostanol/campestanol ratios, similarly reduced plasma cholesterol levels and atherosclerotic lesion area. Our data indicate that there are no large differences in plasma cholesterol- and atherosclerosis-reducing potencies between sitostanol ester and campestanol ester. Furthermore, these data are in line with findings in normolipidemic humans, where no differences in serum cholesterol-lowering potencies between vegetable oil and wood based plant stanol ester mixtures were found (26). In addition, we determined plasma cholesterol exposures, because in apoE*3-Leiden transgenic mice there is a strong relationship between plasma cholesterol exposure and the amount of atherosclerosis in the aortic root (20). When the three dietary plant stanol ester groups in our study are considered as one group, plant stanol ester feeding was responsible for a 44% lowered plasma cholesterol exposure, which led to an 89% reduction of atherosclerotic lesion area. Reduced cholesterol levels in all apoB-containing lipoproteins, VLDL, IDL and LDL, were responsible for the lowered plasma cholesterol exposures. However, in these mice the cholesterol-lowering effect of plant stanol esters was more pronounced in VLDL/IDL fractions (70-77% reduction, $P<0.0001$) than in LDL (15-22% reduction, $P<0.0001$). This implicates that reduced plasma levels of the atherogenic lipoproteins VLDL and IDL were the primary cause of the reduced atherosclerotic lesion area and severity. Our data are on reduction of lipoprotein cholesterol at variance with observations in mildly hypercholesterolaemic humans, where the cholesterol-lowering effect of plant stanols and sterols is mainly confined to LDL (27). However, although the hypocholesterolaemic effect of stanol esters on LDL was less extensive as the effect on VLDL/IDL, in these mice this effect was still of the same magnitude as in humans (LDL 10-15% reduced) (27). In addition, the cholesterol-lowering effect of plant stanols cannot always entirely be subscribed to LDL in humans. For example, in hypercholesterolaemic patients with non-insulin dependent diabetes mellitus, plant stanol esters have also been observed to significantly lower VLDL-cholesterol levels (28).

Atherosclerosis reducing properties of un-esterified plant stanol and plant sterol feeding have been shown in rabbits and apoE-deficient mice

respectively (12,13). Compared with these studies, we have chosen for a setup of our study that is more comparable to the human situation. We used plant stanol mixtures that were trans-esterified with rapeseed oil, which is similar to the plant stanol ester margarines that are available for human consumption. In contrast, un-esterified plant stanols/sterols were used in the above-described rabbit and apoE-deficient mice studies. Furthermore, we used diets with a fat content and fatty acid composition that is typical for Western diets and that is comparable to the diets given to humans in the study of Miettinen et al. (1). In the rabbits (12) and apoE-deficient mice (13) the plasma cholesterol levels were far more extreme than the plasma cholesterol levels of the apoE*3-Leiden transgenic mice. In addition, in this study apoE*3-Leiden mice had a mildly atherogenic plasma lipoprotein distribution with elevated levels of apoB-containing lipoproteins. Due to the moderate plasma cholesterol levels (controls, average 12 mmol/l), the apoE*3-Leiden mice had slowly developed atherosclerosis with a moderate severity after 38 weeks of feeding. In contrast, the extreme cholesterol levels in rabbits and apoE-deficient mice resulted in severe atherosclerosis after 10 to 18 weeks (12,13).

In our study, we found that the amount of monocytes adhered to the endothelium of the aortic root was low, even after 38 weeks of feeding in the control situation at plasma cholesterol levels of 12 mmol/l. This indicates that in the observed stage of the atherosclerotic process activation of the endothelium was still low. We found that reduction of plasma cholesterol levels as a result of plant stanol ester feeding had no effect on monocyte adherence, which indicates that the reduction of atherosclerosis was caused by a reduction of the plasma cholesterol exposure alone.

In conclusion, dietary plant stanol esters, independent of their source or sitostanol/campestanol ratios, dramatically reduced the size and severity of atherosclerotic lesions in the aortic root. This was caused by a lowering of plasma cholesterol confined to the VLDL-, IDL- and to lesser extent LDL-sized fractions.

Acknowledgements

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Chapter 4

HOE 402 lowers serum cholesterol levels by reducing VLDL lipid production, and not by inducing the LDL receptor, and reduces atherosclerosis in wild-type and LDL receptor-deficient mice

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submitted

Abstract

Previous rodent studies suggest that the potent hypolipidaemic agent 4-amino-2-(4,4-dimethyl-2-oxo-1-imidazolidinyl)pyrimidine-5-N-(trifluoromethyl-phenyl) carboxamide monohydrochloride (HOE 402) is an inducer of the LDL receptor (LDLR). Using wild-type and heterozygous and homozygous LDLR knock-out (LDLR +/0 and LDLR 0/0) mice, fed a low or high cholesterol diet, we investigated whether HOE 402 specifically induces the LDLR and whether other pathways are affected. Upon treatment with 0.05% w/w HOE 402, the serum cholesterol levels of wild-type, LDLR +/0 and LDLR 0/0 mice, were maximally reduced by 53, 56, and 73%, respectively ($P<0.05$), by reducing levels in very low density (VLDL), intermediate density (IDL), and low density lipoprotein (LDL) cholesterol, whereas high density lipoprotein cholesterol levels were increased. The observations that HOE 402 exhibited no effect on *in vivo* clearance of 125I-labelled LDL in wild-type mice, and clearly reduced serum cholesterol levels in LDLR 0/0 mice, indicate that the LDLR is not the main target for the compound. In wild-type mice, production of VLDL-TG and cholesterol were reduced by more than 50% by HOE 402 ($P<0.05$), whereas VLDL apolipoprotein B (ApoB) secretion was unaffected, indicating that HOE 402 treatment changes the size, rather than the number of the secreted VLDL particles. The reduced VLDL production was accompanied by a 22% decreased hepatic cholesteryl ester concentration ($P<0.05$). Additionally, HOE 402 treatment strongly reduced the aortic content of atherosclerotic lesions by 90% and 72% in LDLR +/0 and LDLR 0/0 mice, respectively ($P<0.01$). In conclusion, HOE 402 is a potent cholesterol-lowering compound, which inhibits VLDL production, and consequently attenuates atherosclerosis development.

Introduction

A high LDL cholesterol level is an independent risk factor for the development and progression of atherosclerosis in humans, but the risk can be substantially reduced by different cholesterol-lowering therapeutic modalities. Nowadays, treatment is mainly focused on: (i) inhibition of endogenous HMG-CoA reductase by statins; (ii) the reduction of VLDL secretion by nicotinic acid and derivatives; (iii) inhibition of intestinal uptake of bile acids and cholesterol; and (iv) activation of the nuclear peroxisome proliferator-activated receptor by fibric

acid derivatives (1-3), the latter leading to lowered serum TG and cholesterol levels, and increased HDL cholesterol levels.

HOE 402 (4-amino-2-(4, 4-dimethyl-2-oxo-1-imidazolidinyl)pyrimidine-5-N-(trifluoromethyl-phenyl) carboxamide monohydrochloride), is a lipid-lowering compound with a mechanism that is potentially different from those described above. HOE 402 strongly reduced serum LDL-cholesterol levels in hamsters and Watanabe heritable hyperlipidaemic rabbits (4,5). The effect of HOE 402 could not be attributed to interference with intestinal cholesterol absorption or hepatic sterol synthesis (5). LDL-clearance rate, however, was doubled in HOE 402-treated hamsters and rabbits. Moreover, LDL-binding sites and LDLR-mRNA in cultured HepG2 cells incubated with HOE 402 were three to four-fold increased compared with control, suggesting that HOE 402 induced the upregulation of LDLR activity (4). However, the *in vivo* specificity of HOE 402 as a LDLR inducer remained unclear. To investigate whether HOE 402 acts via induction of the LDLR, the present study was performed in wild-type, heterozygous and homozygous LDLR deficient mice. On a high fat/cholesterol diet LDLR deficient mice exhibit elevated levels of LDL and VLDL, caused by an impaired LDLR mediated clearance (6,7). We argued that if HOE 402 acts directly on the LDLR, it should be ineffective in LDLR 0/0 mice.

We found that HOE 402 is a potent cholesterol-lowering and anti-atherosclerotic compound that acts independently of the LDLR.

Methods

Animals, diets and study design

Three-to five-month-old female wild-type, LDLR +/0 and LDLR 0/0 mice, crossed back on a C57Bl6/j background (IFA-Gedo and Jackson Labs) were used. All animals were housed in wire-topped cages with sawdust as bedding, and all diets and water were given ad libitum. Food consumption was assessed weekly at group level.

Three semi-synthetic pelleted diets (8) (Hope Farms), were used in this study. Diet J contained, all weight by weight, 50% sucrose, 20% acid casein, and 12% corn starch. The W and N diets contained 40% sucrose, 15% cocoa butter, and 10% corn starch, whereas diet W contained 0.25% cholesterol and diet N 1% cholesterol and 0.5% cholate. Added to these diets was 0% (control diets) or 0.05% HOE 402 (Hoechst AG). At the start of a 2-week run-in period, diets were changed from chow to one of the semi-synthetic diets described above. Thereafter, animals were randomized in age-matched groups (N=6-9), and intervention periods of 4 weeks (LDL clearance and VLDL production) or 21 weeks (atherosclerosis) started, using diets enriched with 0.05% HOE 402 (treated) or without this compound (controls).

Blood samples were drawn from the tail vein after 4 hr of fasting. At the study endpoint, fasted (4 hr) mice were anaesthetized with 2.5 mL/kg Dormicum and 2.5 mL/kg Hypnorm (Janssen Pharmaceutica), blood was collected by orbital puncture, and mice were sacrificed via cervical dislocation.

Serum and liver lipids

Serum cholesterol and TG levels were determined individually using commercially available enzymatic kits, and ultracentrifugation lipoprotein profiles were made of pooled sera as previously described (9). For determination of liver lipids, livers were mechanically homogenized in phosphate buffered saline (pH 7.4), protein contents were measured (10), cholesterol acetate was added as an internal standard followed by lipid extraction (11), and separation by thin-layer chromatography and quantification of the bands (12).

LDL clearance and VLDL production

Human LDL, isolated by ultracentrifugation, was radio-iodinated using [^{125}I]-monochloride (13), to a specific activity of 100 to 300 cpm/ng of protein, and extensively dialysed against PBS/10 mM EDTA at 4°C. Wild-type mice on diet J were injected in the tail vein with 200 µL NaCl (0.9%) containing freshly isolated [^{125}I]-labelled human LDL (10 µg protein). Heparinized blood samples were drawn from the tail vein 5 min (100% reference value), 40 min, and 2, 5, 10 and 24 hr after LDL injection. Radioactivity was determined in these samples; after 24 hr, 90% of the radioactivity was still associated with the ApoB fraction as determined by the method described in the VLDL-production study.

After 4 hr of fasting, wild-type mice on diet J were anaesthetized as described above. *In vivo* hepatic VLDL ApoB synthesis and VLDL-TG production were determined after intravenous [^{35}S] methionine and Triton WR-1339 injections (9). Fifty mL blood samples were drawn from the tail vein at 0.5, 10, 20, 30 and 60 min after injections. Blood, collected 60 min after injections, was used for quantification of newly synthesized ApoB in nascent VLDL. Therefore, nascent VLDL was isolated after ultracentrifugation, ApoB was precipitated with isopropanol, as described previously (9), and radioactivity was determined in ApoB48 and ApoB100 after 4-20% SDS-PAGE gradient gel electrophoresis under reducing conditions (14).

Assessment of atherosclerosis

LDLR +/0 and 0/0 mice were sacrificed after a 5-month intervention period on diet N and W, respectively, with and without 0.05% (w/w) HOE 402. Aortas from the aortic origin to the iliac bifurcation were put in 3.7% formaldehyde at 4°C until use. After adventitial fat removal, the aortas were cut open longitudinally via the outer curvature and pinned en face (15) on a silicone basement using insect pins. Then fat-containing lesions were specifically stained with Oil Red O (Aldrich Chemical Co.), and lesion areas were measured quantitatively by using a drawing tube connected to a light microscope (MOP-videoplan, Kontron). Lesion areas and total aortic areas were selected by hand, using a digitalised tablet, and a computer (Videoplan evaluation software for image analysis) calculated the size of the selected areas. The same operator blindly scored all thoracic aortas, from origin to the diaphragm.

Statistical analysis

Data are expressed as mean ± SD. For comparisons of two groups, data were evaluated by the nonparametric Mann-Whitney test for unpaired data. Differences between control and HOE 402-treated groups were considered as statistically significant when $p < 0.05$.

Results

Effect of HOE 402 on serum lipid levels in wild-type, LDLR +/0 and LDLR 0/0 mice

The cholesterol and TG lowering potencies of HOE 402 were evaluated in wild-type, LDLR +/0, and in LDLR 0/0 mice. For this purpose, these mice were put on the following diets, with or without HOE 402 added: The wild-type and LDLR +/0 mice received for four weeks a lipogenic sucrose-containing diet J and a high fat/cholesterol-cholate containing diet N. In addition, LDLR +/0 and LDLR 0/0 mice received for 21 weeks, high fat/cholesterol containing diets N and W, respectively. LDLR 0/0 mice did not receive diet N, but received diet W, which contains no sodium cholate and less cholesterol than diet N. This was done because serum cholesterol levels of LDLR 0/0 mice on diet N would become too high, and these mice would develop a severe hypercholesterolaemia with additional pathologies such as cutaneous xanthomatosis (16).

In all mouse strains used, the applied dietary HOE 402 dose (0.05% w/w) had no effects on body weight, weight gain, food intake and on serum enzyme activities of the liver damage parameter ALAT (data not shown). Furthermore, HOE 402 had no effect on serum TG levels in all mice strains on the different diets (data not shown).

The serum cholesterol levels of the wild-type, LDLR +/0 and LDLR 0/0 mice are given in table 1. Compared with controls, HOE 402 reduced serum cholesterol levels in wild-type and LDLR +/0 mice fed diet J, but this effect was more pronounced on diet N. HOE402 potently reduced serum cholesterol levels in LDLR +/0 and LDLR 0/0 mice after 21 weeks of treatment. These cholesterol-lowering effects of HOE 402 were more pronounced than after 4 weeks in LDLR +/0 mice, and after 11 weeks in LDLR 0/0 mice.

Table 1. Effect of HOE 402 on serum cholesterol levels in wild-type, LDLR +/0 and LDLR 0/0 mice on diets J, W and N

	wild-type		LDLR +/0		LDLR 0/0	
	control	HOE 402	control	HOE 402	control	HOE 402
Diet	serum cholesterol mmol/L					
J						
week 4	2.5±0.3	1.8±0.4* (72)	6.8±0.9	5.3±0.9* (78)		
W						
week 11					50.4±10.2	33.6±13.3 (67)
week 21					49.2±13	13.1±2.4** (27)
N						
week 4	7.3±0.7	3.4±0.3** (47)	18.4±4.1	12.4±3.1* (67)		
week 21			29.4±4.3	12.9±3.1** (44)		

Serum cholesterol concentrations were measured in wild-type and LDLR +/0 mice on diets J and N after 4, and 21 weeks (LDLR +/0 mice) of feeding, and in LDLR 0/0 mice on diet W after 11 and 21 weeks of feeding with or without 0.05% (w/w) HOE 402 (N=6-8 per group). At baseline, serum cholesterol levels of control and HOE 402-treated mice were the same (data not shown). *Values in parentheses* represent the percentage of the value obtained in mice on the control diet. HOE 402 had no effect on serum TG levels. Statistically significant differences between control and treated mice are indicated by *$p<0.05$, **$p<0.005$.

In wild-type (figure 1A), LDLR +/0 (figure 1B), and in LDLR 0/0 mice (figure 1C), HOE 402 reduced serum cholesterol levels of VLDL/IDL/LDL by 86, 57 and 63%, respectively, whereas the respective HDL-cholesterol levels were increased by 21, 54 and 47%.

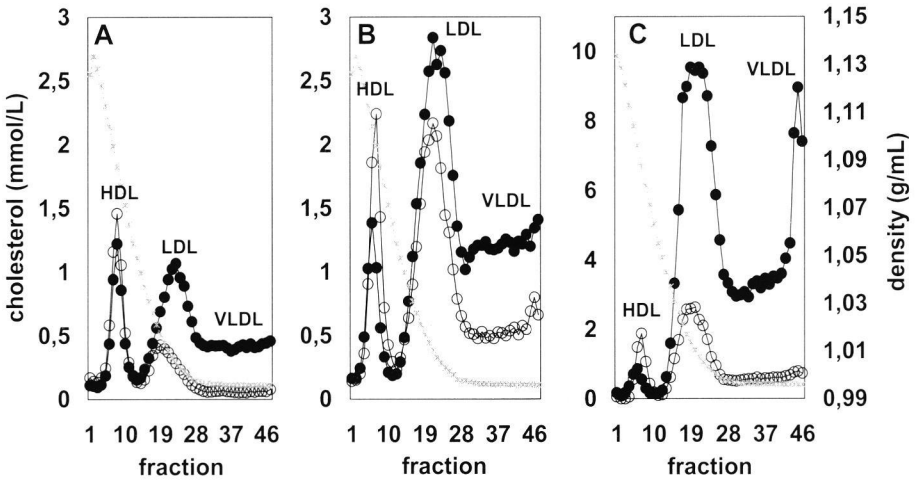

Figure 1. Effect of HOE 402 on lipoprotein profiles in wild-type (A), LDLR +/0 (B) and LDLR 0/0 (C) mice.

Ultracentrifugation profiles of pooled serum of wild-type (N=6 per group) and LDLR +/0 mice (N=8 per group), fed diet N, were made after 4 weeks of treatment. The profiles of LDLR 0/0 mice, fed diet W (N=8), were made after 21 weeks of treatment. Control (●) and HOE 402 treatment (○); x indicates the density (g/mL) gradient. The lipoproteins are classified by their density, being VLDL: d < 1.006, IDL: 1.006-1.019, LDL: 1.019-1.063, and HDL: d > 1.063 g/mL.

Effects of HOE 402 on LDL clearance and VLDL production

The finding that LDLR deficiency did not influence the serum cholesterol-lowering potency of HOE 402, suggests that HOE 402 reduced serum cholesterol levels via another mechanism than by inducing the LDLR. To study the mechanism of action, we investigated whether HOE 402 modulated the LDLR, and whether HOE 402 could alter VLDL production in wild-type mice on the lipogenic diet J.

By measuring the serum clearance of injected human iodinated LDL in mice that had received diets without or with HOE 402 (figure 2), no effect of HOE 402 on the clearance of LDL was found. For this clearance study human LDL was used, because in contrast to mouse LDL, it contains no apoE and can therefore be more specifically cleared by the LDLR than mouse LDL which can also be cleared by the apoE requiring LDLR-related protein (LRP).

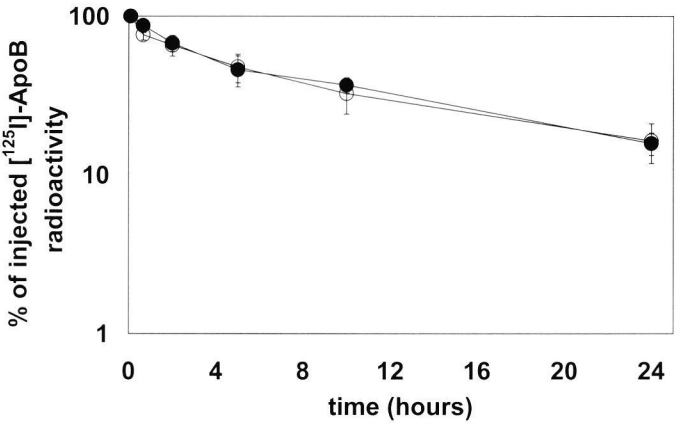

Figure 2. Clearance of human LDL-ApoB in wild-type mice. Mice were fed the sucrose rich diet J for 4 weeks.

Fasted mice were injected with 10 μg of [^{125}I]-labelled human LDL protein. Blood was collected at 40 min, and 2, 5, 10, and 24 hr, and [^{125}I]-ApoB was measured. Control (●) (N=6), and HOE 402 treated (○) (N=6). Statistically significant differences between control and treated mice were not present.

Subsequently, the effect of HOE 402 on different aspects of VLDL production after Triton WR 1339 injection, was studied by measuring the production rates of VLDL-TG and VLDL ApoB (figure 3), and the cholesterol content of nascent VLDL. Compared with controls, the serum TG production rate was reduced by more than 50% in HOE 402-treated mice. In contrast, the synthesis rate of VLDL-ApoB was not changed. Furthermore, the cholesterol content of nascent VLDL was also diminished by HOE 402 (112 ± 2 vs 65 ± 7 μg/mL, $p < 0.05$). These findings indicate that HOE 402 had no effect on the number of VLDL particles produced, but resulted in the release of TG- and cholesterol-poor VLDL in the circulation.

If in these wild-type mice, HOE 402 had a serum cholesterol-lowering effect via the induction of the LDLR, an increased flux of cholesterol from the circulation to the liver could lead to an increased hepatic cholesterol storage. However, we found that HOE 402 reduced the liver cholesterol ester content by 22% in these mice (6.5 ± 0.6 vs 5.1 ± 0.7 μg/mg protein, $p<0.05$). Additionally, no changes in the hepatic mRNA levels of HMG-CoA synthase were found (data not shown).

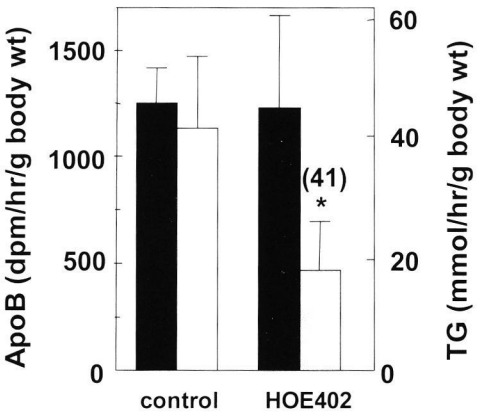

Figure 3. VLDL-ApoB and -TG production in wild-type mice.
After 4 weeks of feeding diet J, fasted control (N=7) and HOE 402-treated (N=7) mice were anaesthetized, and intravenously injected with [^{35}S]-methionine and Triton WR-1339. Thereafter, [^{35}S]-ApoB ■ and TG □ production rates were determined as described in methods. The value between parenthesis represents the percentage of the value obtained in mice on control diet. Statistically significant differences between control and treated mice are indicated by an asterisk * $p < 0.05$.

The effect of HOE 402 in atherosclerotic lesion size in aortas of LDLR +/0 and LDLR 0/0 mice

LDLR +/0 and LDLR 0/0 mice were fed the atherogenic diets N and W, for 21 weeks. In these mice HOE 402 significantly ($P<0.005$) reduced the serum cholesterol exposures (weeks x mmol/L) by 47% in LDLR +/0 mice (controls 462±53; HOE 402 246±53), and by 41% in LDLR 0/0 mice (controls 979±143; HOE 402 574±221). These reductions were accompanied by a strong decrease in the atherosclerotic lesion area in the HOE 402-treated groups (figure 4).

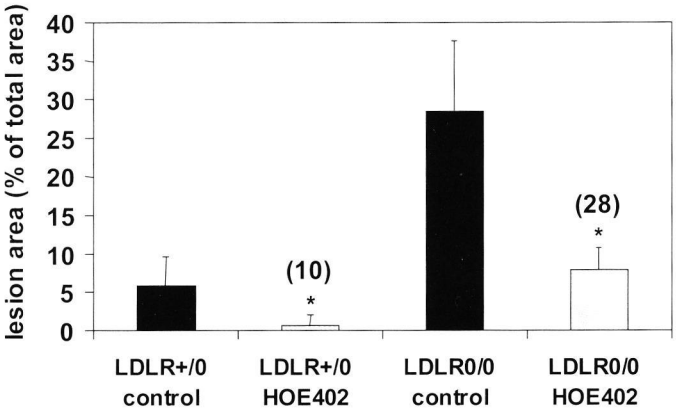

Figure 4. Effect of HOE 402 on atherosclerotic lesion area in aortas of LDLR +/0 and LDLR 0/0 mice.

Aortic lesion areas were expressed as a percentage of total aortic area from its origin to the renal artery branch points. Lesions were stained with Oil Red O in aortas of LDLR +/0 mice fed diet N and LDLR +/0 mice fed diet W for 21 weeks with and without HOE 402. Filled bars ■ controls (N=8 per group); open bars □ HOE 402 (N=8 per group). Values in parentheses represent the percentage of the value obtained in mice on the control diet. Statistically significant differences between control and treated mice are indicated by * $p < 0.01$.

In figure 5 representative photographs of Oil Red O stained thoracic aortas are shown. As can be seen in these photographs, the atherosclerotic lesions were mostly present in the aortic arch. More than 90% of the lesions were located in this area.

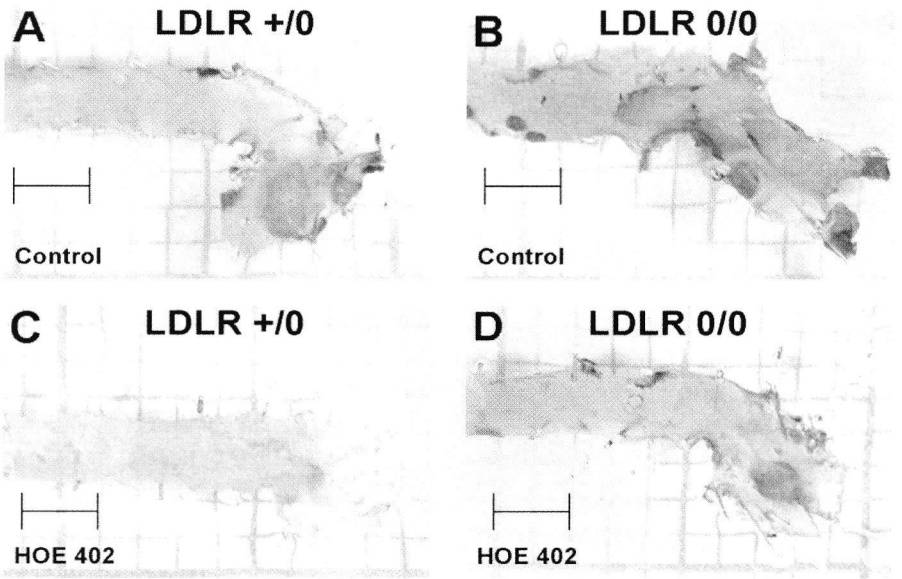

Figure 5. Representative en face photographs of Oil Red O stained lesions in the aortic arches of LDLR +/0 (A,C) and LDLR 0/0 mice (B,D) fed a control diet (A,B) or a diet containing 0.05% w/w HOE 402 (C,D).

Scale bars represent 2 mm.

Discussion

The present study demonstrated the hypolipidaemic activity of the compound HOE 402 in wild-type, LDLR +/0 and LDLR 0/0 mice, showing strong reductions in serum levels of the ApoB-containing lipoproteins VLDL, IDL and LDL. Treatment with HOE 402 had no effect on LDL-clearance, but reduced VLDL-TG and cholesterol production without affecting ApoB production. These findings indicate that HOE 402 inhibits VLDL lipid production rather than inducing the LDLR, as suggested previously (4,5).

A similar reduction in serum cholesterol levels by HOE 402 as we found in mice was observed in hyperlipidaemic Watanabe rabbits, where mainly LDL-cholesterol was lowered (4). In cholesterol-fed hamsters cholesterol levels were similarly lowered in all lipoprotein fractions (5). In addition, HOE 402 had no effect on serum lipids in homozygous LDLR-deficient rabbits (4). Based on

the latter data and on the fact that HOE 402 treatment upregulated LDLR mRNA levels in HepG2 cells (4), it was concluded that HOE 402 is an LDLR inducer. However, the present study shows that in mice the LDLR is not the primary target of HOE 402, since HOE 402 treatment reduced serum cholesterol levels in LDLR 0/0 mice, and the compound had no effect on the clearance of human LDL in wild-type mice. In addition, we found that in wild-type mice treatment with HOE 402 resulted in the production of nascent VLDL particles that are relatively poor in TG and cholesterol. Thus, this reduced VLDL lipid secretion may explain the marked serum VLDL/IDL/LDL-cholesterol-lowering properties of HOE402 in these mice. Concomitantly, we found that HOE 402 treatment reduced hepatic cholesteryl ester contents, which may form an explanation for the reduced VLDL-cholesterol secretion.

The precise molecular mechanism of action of HOE 402 is presently unknown, but there are some similarities with other hypolipidaemic agents. Several animal studies have shown that plant stanol esters, ACAT inhibitors, and HMG-CoA reductase inhibitors reduce serum levels of cholesterol and ApoB-containing lipoproteins by a decrease in VLDL lipid secretion, and that the latter strongly depends on the hepatic cholesterol ester content (9,17-19). The primary mechanisms of action of these treatments are inhibition of intestinal cholesterol absorption; of endogenous cholesterol esterification, thereby also inhibiting intestinal cholesterol absorption (20); and of endogenous cholesterol synthesis, respectively. However, it is unlikely that HOE 402 inhibited the intestinal cholesterol absorption, since no effect on intestinal cholesterol absorption was observed in hamsters (5), and intestinal cholesterol absorption inhibitors are effective in both hamsters and mice (9,21). In addition, there are strong indications that inhibition of ACAT or of HMG-CoA reductase are not the primary target of HOE 402, since HOE 402 did not inhibit ACAT activity in HepG2 cells (data not shown), and HOE 402 did not inhibit endogenous cholesterol synthesis in hamsters (5) and in HepG2 cells (4). Moreover, if HOE 402 had suppressed endogenous cholesterol synthesis in these mice, an upregulation of mRNA levels of HMG CoA synthase, an enzyme in the cholesterol synthetic pathway, could have been expected (22). However, we found no effect of HOE 402 treatment on hepatic HMG-CoA synthase mRNA levels in wild-type, LDLR +/0 and LDLR 0/0 mice (data not shown). Thus, although inhibition of intestinal cholesterol absorption, and inhibition of endogenous esterification and synthesis of cholesterol show similar hypocholesterolaemic effects as HOE 402, the biochemical background

of the cholesterol-lowering properties of HOE 402 is different, and needs to be further investigated.

In this study, we found that HOE 402 potently reduced serum levels of ApoB-containing lipoproteins in LDLR 0/0, LDLR +/0 and wild-type mice, but also led to moderately increased levels of HDL in LDLR 0/0 and LDLR +/0 mice. Both changes are beneficial with respect to the development of atherosclerosis and can explain the strong reduction in aortic atherosclerosis found in mice treated with HOE 402.

In conclusion, in mice the hypocholesterolaemic effect of HOE 402 was not caused by induction of LDLR activity as found in other animal models, but by reducing the VLDL-TG and VLDL-cholesterol secretion. The hypocholesterolaemic effect of HOE 402 resulted in a strong reduction of the atherosclerotic lesion area of the aorta in these mice.

Acknowledgements

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Chapter 5

Long-term dietary vitamin E reduces atherosclerotic lesion size and increases plaque stability in heterozygous LDL receptor-deficient mice

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submitted

Abstract

Background - The effect of natural vitamin E on the progression of atherosclerosis and composition of the atherosclerotic plaque was studied in mice at a serum cholesterol level and lipoprotein profile that is relevant to the human situation.

Methods and results - Female heterozygous low density lipoprotein (LDL) receptor-deficient mice received for 58 weeks a control diet with a basal vitamin E content of 0.0035% (w/w) (N=8) or the same diet supplemented with 0.07% (w/w) vitamin E (N=10). Serum cholesterol levels were on the average 11.9±4.4 mmol/l (controls) and 11.7±2.9 mmol/l (vitamin E) and the LDL/HDL ratio was 6.5 in both groups. A vitamin E enriched diet caused a 30% decrease ($P<0.05$) of the atherosclerotic lesion size in the aortic root. Lesions of the vitamin E-treated mice showed a 16% increase ($P<0.05$) in the collagen content, a 541% increase ($P<0.01$) in the amount of smooth muscle cells and a non-significant 45% decrease ($P=0.146$) in the macrophage content of the atherosclerotic plaques.

Conclusion - This study demonstrates that a long-term vitamin E diet reduces the progression of atherosclerotic lesions and increases the collagen content of the lesions and the amount of smooth muscle cells in the fibrous cap and shoulder region of the lesions, without having any effect on serum lipid levels. Therefore, vitamin E treatment results in a more stable plaque phenotype in heterozygous LDL receptor-deficient mice, at serum lipid, lipoprotein and vitamin E levels relevant to the human situation.

Introduction

Vitamin E is a lipid soluble antioxidant that is mainly transported by LDL in blood. Oxidative modification of lipoproteins is generally believed to play a central role in the development of atherosclerosis (1), and vitamin E has been found to inhibit oxidative modification of LDL *ex vivo* (2) and to lower urinary isoprostane levels (3-5), *in vivo* measures of oxidative stress (6). However, with respect to protective effects against atherosclerosis, contradictory effects of vitamin E have been reported in humans and in animal models (7). Two out of the five major clinical intervention trials did not find that vitamin E produced any protective effect against cardiovascular events (8,9). The other three trials found reduced risks of developing angina pectoris (10), the mildest and earliest

form of cardiovascular event, and of non-fatal myocardial infarction (11), and composite cardiovascular disease endpoints and myocardial infarction (12). Several rodent studies reported that vitamin E did not protect against atherosclerosis (13-15) whereas other studies in severely hypercholesterolaemic rodents found an atherosclerosis-attenuating effect of vitamin E (6,16-21). However, in some of these latter studies where vitamin E attenuated atherosclerosis, vitamin E treatment also lowered serum cholesterol levels (17,18,20,21). Therefore, from these studies it was not possible to conclude whether atherosclerosis was attenuated due to the hypocholesterolaemic effect or the antioxidant or other properties of vitamin E. Furthermore, in none of the reported studies was the effect of vitamin E on plaque composition studied in detail.

The aim of this study was to assess the effects of mild vitamin E supplementation on the progression of atherosclerosis and plaque composition at moderate serum cholesterol levels that are relevant to the human situation. For this purpose, we used heterozygous LDL receptor-deficient mice. When given a semi-synthetic cholesterol-containing diet, these mice develop atherosclerosis at moderate serum cholesterol levels with lipoprotein profiles that resemble those in humans.

We found that dietary vitamin E enrichment caused a 30% reduction in atherosclerotic lesion size and a significant increase in the characteristics of plaque stabilization.

Methods

Animals and diets

Three-month-old ♀ heterozygous LDL receptor-deficient mice (22), F5 backcrossed on C57BL6, were used and housed as previously described (23). Before the start of the study, mice received standard chow. During the experimental period of 58 weeks, the mice (8 control, 10 vitamin E-treated) were kept on semi-synthetic diets (23,24) that contained (all percentages weight by weight) 40.5% sucrose, 16% fat comprising 13, 18 and 3 energy% of saturated, mono-unsaturated and poly-unsaturated fatty acids, respectively, 1% cholesterol and 0.25% cholate, and 0.0035% vitamin E (control) or 0.07% vitamin E (D-α-tocopherol, Sigma, T3634).

Serum and plasma parameters

Blood samples were taken at 0, 2, 6, 11, 17, 21, 27, 32, 40 and 58 weeks for measurement of the serum cholesterol and triglyceride levels. Furthermore, serum cholesterol exposures were determined and cholesterol ultracentrifugation profiles were made, as previously described (23). Cholesterol levels that were present in the respective VLDL/IDL *(17-23)*, LDL *(7-22)* and

HDL *(1-6)* fractions were determined by calculating the areas under the curves of the ultracentrifugation profiles. Pooled serum vitamin E concentrations were determined using HPLC (4). Pooled serum levels of serum amyloid A (SAA, Perbio) and soluble intracellular adhesion molecule-1 (sICAM-1) were determined by ELISA (Biosource International). Von Willebrand factor (vWf) was determined in pooled EDTA plasma by ELISA, using antisera from Dakopatts (Glostrup), basically as described by Ingerslev (25), using rat plasma for calibration. Concentrations of 8, 12-iso-iPF2α-VI were determined on pooled plasma samples taken at 40 and 42 weeks using a GC-MS assay, according to Pratico *et al.* (6).

Tissue preparation, histology and quantification of atherosclerosis

At the study endpoint, fasted mice (4 hours) were anaesthetized (2.5 ml/kg Dormicum and 2.5 ml/kg Hypnorm, Janssen Pharmaceutica) and then sacrificed. Dissection, fixation, dehydration and paraffin-embedding of the hearts/aortas were done as previously described (23). Serial (5 μm) cross-sections were made at the region of the aortic valve leaflets, as described by Paigen (26), and mounted on slides at 35 μm intervals.

Cross-sections were stained with haematoxylin-phloxin-saffron (HPS), Sirius red or von Kossa with light green counter-staining for quantification of atherosclerosis and visualization of collagen and calcification, respectively. Macrophages were detected after incubation with a primary anti-macrophage antibody (AIA31240, dilution 1:1500, Accurate Chemicals and Scientific), followed by a secondary biotinylated donkey anti-rabbit antibody (RPN 1004, dilution 1:3200, Amersham) and avidin-biotin-horseradish peroxidase (K-0355, Dako). Smooth muscle cells were detected with a primary mouse monoclonal anti-muscle α-actin antibody (clone 1A4, dilution 1:750, Sigma) and incubated with a secondary peroxidase-conjugated rabbit anti-mouse antibody (P-0260, dilution 1:300, Dako). Peroxidase activity of macrophages and smooth muscle cells was visualized with a Nova Red substrate kit (SK-4800, Vector Labs) and cross-sections were counter-stained with hematoxylin.

Per animal, full colour images of five consecutive cross-sections of the aortic root were acquired and captured as previously described (23), using a light microscope equipped with a 4x objective (lesion area, collagen, macrophages and smooth muscle cells) or a 10x objective (calcification). Quantification of atherosclerotic lesion areas, cholesterol clefts/necrosis and media thickness occurred manually and lesion contents of collagen, calcification, macrophages and smooth muscle cells were automatically quantified by setting a threshold colour for this specific staining in a pre-selected area, using Qwin image analysis software (Leica Imaging Systems Ltd).

The severity of atherosclerosis was classified into five types (23,27) using the classification system of the AHA. To discriminate between early and established lesions, the five lesion types were further divided into type 1-3 lesions (early fatty streaks, regular fatty streaks and mild plaques) and type 4-5 lesions (moderate and severe plaques) (23). The same operator who was blinded for the group allocation performed all analyses.

Statistical analysis

All data are presented as mean±SD. Differences between groups were evaluated using the non-parametric independent samples Mann-Whitney U-test (SPSS for Windows). P-values less than 0.05 were considered significant.

Results

Serum lipids

Body and total liver weights and food intake were not affected by the vitamin E-enriched diet (data not shown). During the study, average serum vitamin E concentrations were doubled upon the addition of vitamin E (controls 21.6±3.3 μmol/l, vitamin E-treated 44.1±3.3 μmol/l, $P<0.05$), whereas vitamin E had no effects on serum cholesterol (figure 1A) and triglyceride (not shown) levels. Mean serum cholesterol levels (week 4 to 58) in the control and the vitamin E - treated group were 11.9±4.4 and 11.7±2.9 mmol/l, respectively. Mean serum triglyceride levels (week 4 to 58) were 0.07±0.04 mmol/l (controls) and 0.07±0.03 mmol/l (vitamin E group). Furthermore, vitamin E had no effect on the distribution of cholesterol over the different lipoproteins (figure 1B). In control and treatment groups 25% of cholesterol was present in VLDL and IDL, 65% in LDL and 10% in HDL, giving an LDL/HDL ratio of 6.5.

Oxidative stress and inflammation

Oxidative modification of LDL plays an important role in the development of atherosclerosis (1). Therefore we determined the plasma levels of the F2-isoprostane 8,12-iso-iPF2α-VI, a marker of oxidative stress *in vivo*. Vitamin E reduced the plasma 8,12-iso-iPF2α-VI concentrations by 21% on average (controls 1.32±0.20 ng/ml; vitamin E 1.04±0.05 ng/ml), indicating that vitamin E reduced oxidative stress *in vivo*.

Since inflammation plays an important role in the pathogenesis of atherosclerosis (28), and in man some markers of inflammation can predict the risk of cardiovascular events, we measured the levels of SAA and sICAM-1 in serum, and of vWf in plasma. Vitamin E had no effects on the levels of sICAM-1 and vWf (data not shown), indicating that vitamin E had no effect on the activation state of the endothelium. However, after 21 and 48 weeks of treatment vitamin E potently reduced the serum SAA level (μg/ml) by 36% (control 14.6±6.1; vitamin E 6.1±2.8) and 76% (control 95.8±24.6; vitamin E

23.0±3.6), respectively. This finding indicates that vitamin E had anti-inflammatory properties.

Atherosclerotic lesion size and composition

Average serum cholesterol exposures (mM*week) were 621±177 for controls and 599±79 for vitamin E-treated mice (P=0.73), indicating that the observed effects of vitamin E are independent of serum cholesterol levels.

Representative photomicrographs of the aortic root show that lesions of vitamin E-treated mice had a moderately reduced size and a thicker fibrous cap (figure 2).

Vitamin E treatment reduced the atherosclerotic lesion area in the aortic root by 30% (P=0.016) as compared with the control group (figure 3A). Qualitative assessment of the progression of atherosclerosis revealed that vitamin E tended to cause a shift from (type 4-5) severe lesions to (type 1-3) fatty streaks. However, the change did not reach significance: 22±14% of control lesions was type 1-3 and 78±14% was type 4-5, whereas lesions of vitamin E-treated mice were for 33±17% type 1-3 and 67±17% type 4-5 (P=0.146). The majority of the lesions were exarcerbated by the presence of cholesterol clefts and necrosis (type 4-5). Some of these lesions had a loss of architecture of the media (type 5).

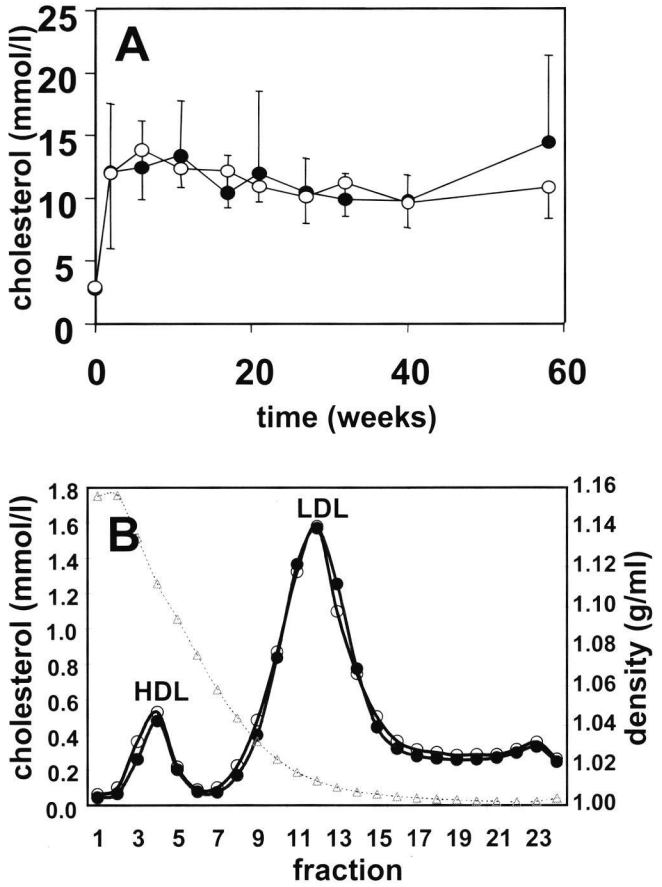

Figure 1. (A) Serum cholesterol levels during the study period of 58 weeks; (B) distribution of cholesterol over lipoprotein fractions.

Filled circles • controls; open circles ○ vitamin E-treated mice. In (B) + indicates the density gradient. No differences between control and treatment values were found.

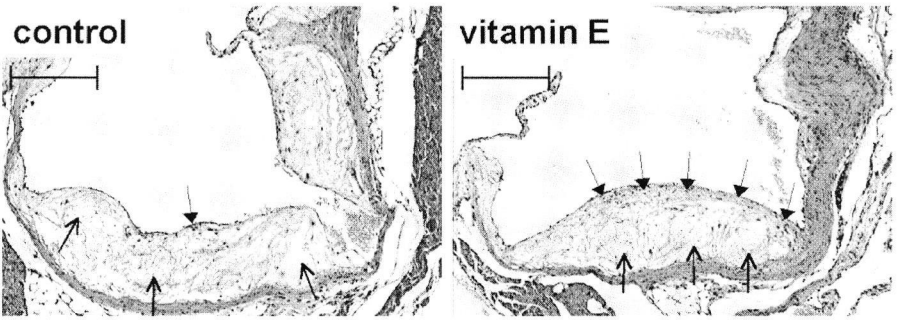

Figure 2. Representative photomicrographs of cross-sections of the aortic root from control or vitamin E-treated mice.

The fibrous caps of the lesions are indicated with filled arrows. Note a much thicker fibrous cap after vitamin E treatment. Open arrows indicate cholesterol clefts and necrosis. The cross sections were stained with haematoxylin, phloxin and saffron. Scale bars represent 250 μm.

To assess whether dietary vitamin E also had an effect on the composition of the lesions, we quantified in histological stainings the amount of collagen, calcification and cholesterol clefts/necrosis and in immunohistochemical stainings the amounts of macrophages and smooth muscle cells. Vitamin E treatment increased the collagen content of the atherosclerotic plaques by 16%, $P<0.05$ (figure 3B). Both in control and vitamin E-treated mice, collagen covered the major part of total lesion area, with the exception of the necrotic areas and the regions containing cholesterol clefts (figure 2 and 4). Additionally, the vitamin E-enriched diet led to a more than 5-fold increase ($P<0.01$) in the number of smooth muscle cells of the atherosclerotic lesions (figure 3E).

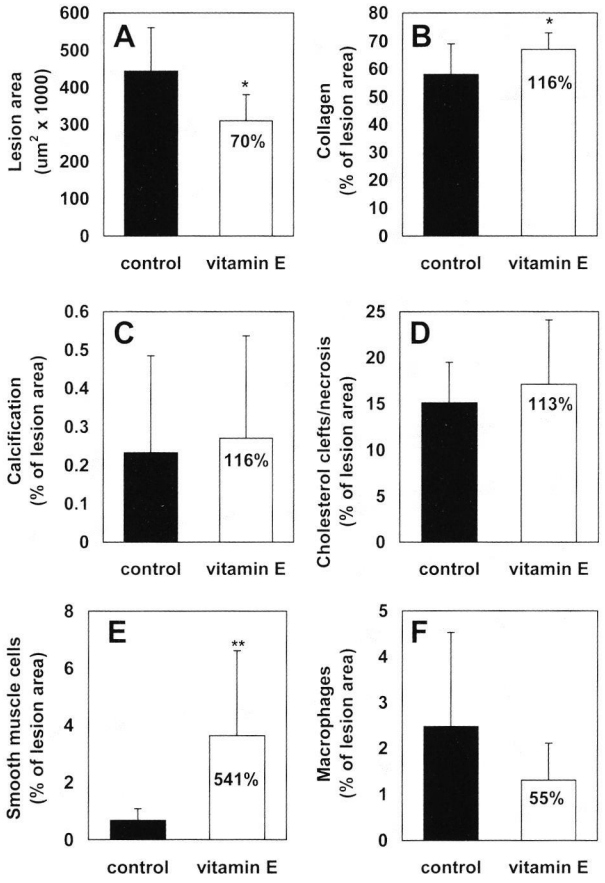

Figure 3. Effect of vitamin E on area (A) and lesion contents of collagen (B), calcification (C), cholesterol clefts/necrosis (D), smooth muscle cells (E) and macrophages (F).
Solid bars, mice on control diet; open bars, vitamin E-treated mice. Areas are means of 5 cross-sections at 30 μm intervals, overlapping a region of 145 μm of the aortic root. Statistically significant differences between control and treated mice are indicated by * $p < 0.05$; ** $p < 0.01$.

Representative pictures (figure 4) show that the lesions of vitamin E-treated mice contained more collagen at the luminal side (A,B), and had a smooth muscle cell-rich fibrous cap and shoulder region (H), while significantly less smooth muscle cells were present at the luminal side of the control lesions (G). No difference in media thickness was observed (controls 22.6±4.6 μm; vitamin E 19.7±4.8 μm, P=0.35). The lesion content of macrophages tended to

be lowered in the vitamin E-treated mice, but this was not significant (P=0.146) (figure 3F). Furthermore, the macrophages were mainly present at the luminal side and shoulder regions of the lesions (figure 4 E,F). The lesion contents of calcium deposits (figure 3C, P=0.83) and cholesterol clefts + necrosis (figure 3D, P=0.70) were also not different in mice fed the control or the vitamin E-enriched diet. Representative pictures show that the lesions of control and vitamin E-treated mice were poor in calcium deposits, present in the core of the lesions (figure 4 C,D). The absolute lesion areas of macrophages, clefts and necrotic tissue were reduced in parallel with the total lesion area in the vitamin E-treated mice.

Altogether, these results demonstrate that treatment with vitamin E led to the development of smaller atherosclerotic lesions that contained more collagen and smooth muscle cells. Although no effect on the amount of macrophages and cholesterol clefts/necrosis was found, the observed changes in structure indicate that vitamin E treatment resulted in the formation of a more stable plaque that is less prone to rupture.

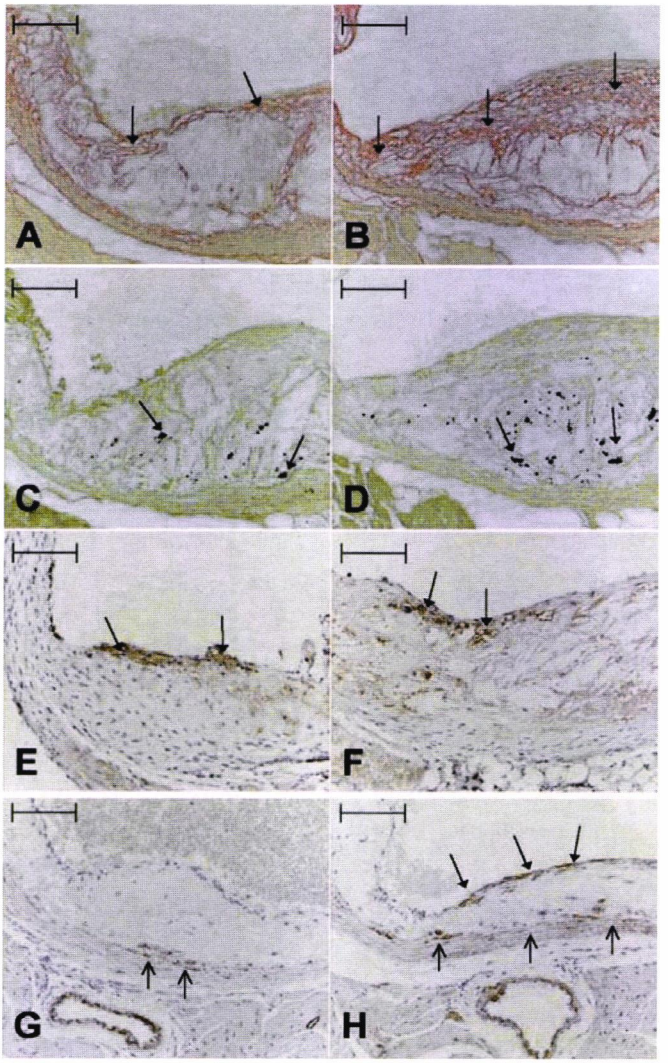

Figure 4. Representative photomicrographs of serial cross-sections of the aortic root of control (A,C,E,G) or vitamin E-treated (B,D,F,H) mice.

(A,B) Sirius red staining for collagen positive areas, indicated with arrows (red staining). (C,D) von Kossa staining for detection of calcification (arrow, black staining). Macrophages (E,F) and smooth muscle cells (G,H) were detected using monoclonal antibodies (filled arrows, red/brown staining). The smooth muscle cells in the media, indicated with open arrows (G,H), were not included in the measurements. Scale bars represent 125μm.

Discussion

This study was primarily focused on the anti-atherogenic effect of vitamin E supplementation on cholesterol levels and a lipoprotein profile relevant to the human situation. We used a vitamin E dose that, compared with controls, doubled serum vitamin E levels without affecting serum lipid levels. A similar increase is found in healthy humans given 400 IU/day of vitamin E orally (3). We found that natural vitamin E reduced the atherosclerotic lesion area in the aortic root by 30% and increased the collagen (+16%) and smooth muscle cell (+541%) contents of the atherosclerotic plaques, indicative of the formation of a more stable plaque.

Two studies conducted in mice showed no beneficial effect of vitamin E on the development of atherosclerosis (14,15). In one study, however, adverse effects on serum HDL were found. In C57BL6 mice, a very high dosage of dietary vitamin E (2% wt/wt) significantly increased the serum total antioxidant status, but reduced serum total and HDL-cholesterol levels and had no effect on atherosclerosis (14). In our study the much lower dietary vitamin E dose (0.07% wt/wt) caused no change in serum total and HDL-cholesterol levels.

We showed that a two-fold increase in serum vitamin E levels, from 22 to 44 μmol/l, due to a moderate dose of vitamin E (0.07% wt/wt), was sufficient in attenuating the progression of atherosclerosis. In a recent study with apoE-deficient mice, having about two-fold higher lipid levels, a dietary vitamin E dose two-fold higher (0.15% wt/wt) than used in our study caused a four-fold increase in serum vitamin E levels to 136 μmol/l, and reduced atherosclerotic lesion size by more than 60% (6). The much higher serum vitamin E levels in the latter study can be explained by the higher vitamin E dose and the higher plasma cholesterol levels both predominantly contained in β-VLDL, the most abundant lipoprotein in apoE-deficient mice. Additionally, VLDL is a better acceptor for vitamin E than LDL (29), the dominant lipoprotein in the heterozygous LDL receptor-deficient mice used in our study. In contrast, in another study with apoE-deficient mice, a moderate dosage of vitamin E (0.05% wt/wt D,L-α-tocopherol) comparable to the present study, in combination with β-carotene (0.05% wt/wt) led to a more than five-fold increase in serum vitamin E levels to only 15 μmol/l and had no effect on atherosclerosis (15). These opposing findings may be explained by differences in the serum vitamin E/cholesterol ratio. Reviewing the above-mentioned data and the data from our study, we conclude that in mice serum vitamin E levels below ~2 μmol/mmol cholesterol are ineffective in inhibiting atherosclerosis

development and that doubling to about 4 μmol/mmol cholesterol is sufficient to inhibit atherosclerosis. The finding that comparable dietary vitamin E contents led to different plasma vitamin E levels can possibly be explained by the differences in the composition of the diets (amounts of fat) and the different α-tocopherol enantiomers used in the different studies. We used the natural, physiologically most active D-α-tocopherol form.

Oxidative stress is an important factor in the pathogenesis of atherosclerosis (1). Therefore, we determined the level of the isoprostane iPF2α-VI in plasma, being an *in vivo* marker of oxidative stress. We found that after 40-42 weeks of treatment, vitamin E reduced the level of the isoprostane iPF2α-VI in plasma, indicating that vitamin E reduced oxidative stress *in vivo* in these mice. In line with our present findings, vitamin E attenuated atherosclerosis and diminished plasma and urinary isoprostane levels in mice (6) and rabbits (16), respectively. These studies, including the present study, indicate that in rodents the antioxidant properties of vitamin E may be responsible for the attenuation of atherogenesis.

Inflammation and endothelial activation play a central role in the pathogenesis of atherosclerosis (28). Clinical trials have provided evidence that C-reactive protein is an independent risk factor for cardiovascular disease (30), while SAA levels are strongly correlated (31,32). In addition, there are indications from prospective studies that elevated serum sICAM-1 levels are associated with an increased risk of cardiovascular events (33-35), which also holds true for the vascular adhesion molecule-1 (VCAM-1) (34). Furthermore, vWf, known as a marker of endothelial activation, has recently been shown to be a risk factor for recurrent myocardial infarction (36). We therefore measured the levels of SAA, sICAM and vWF in a situation with marked atherosclerosis. The finding that vitamin E potently reduced the levels of serum SAA indicates that vitamin E had anti-inflammatory properties. In addition, this finding suggests that in LDLR +/0 mice anti-inflammatory properties of vitamin E could have been responsible for its anti-atherosclerotic effects. However, the finding that the levels of sICAM-1 and vWf had not been reduced by vitamin E indicate that vitamin E did not inhibit endothelial activation. Nevertheless, these findings do not exclude the possibility that the anti-inflammatory properties of vitamin E could have interfered with the development of atherosclerosis, because in mice there are no clear data on the link between the systemic levels of these markers and their local upregulation in the atherosclerotic plaque.

Former animal studies only assessed whether vitamin E had an effect on the aortic lesion size and did not evaluate plaque composition. There is clear evidence, especially with respect to acute coronary events, that the composition of the plaque and its susceptibility to rupture are more important than its size alone (37,38). Plaques vulnerable to rupture have a thin fibrous cap with a reduced smooth muscle cell content, a reduced collagen content and a large soft lipid core, whereas stable plaques have a thick fibrous cap that protects the blood from contact with thrombogenic material in the lipid rich core of the atheroma (37,38). We found to our knowledge for the first time that vitamin E treatment increased the amount of smooth muscle cells in the fibrous cap and shoulder region of the lesions and the collagen content of the lesions. Additionally, vitamin E had no effect on media thickness, which indicates that vitamin E does not increase smooth muscle cell migration from the media towards the fibrous cap and shoulders region. Furthermore, a non-significant trend towards a decreased amount of macrophages was observed in lesions of vitamin E-treated mice. Together, these data are strongly indicative of the lesion-stabilizing properties of vitamin E.

Our finding that vitamin E increased the smooth muscle cell content in the fibrous cap and shoulder region appears to be at variance with *in vitro* studies where vitamin E reduces smooth muscle cell proliferation by inhibiting protein kinase C (39), and an *in vivo* study where vitamin E reduces the smooth muscle cell density in early lesions of hypercholesterolaemic rabbits (40). However, it is possible that vitamin E reduces smooth muscle cell proliferation in the early formation of atherosclerosis, whereas it may protect smooth muscle cells from differentiation into macrophages or cell death in more advanced stages of atherosclerosis.

Smooth muscle cells are known to synthesize collagen (38), potentially explaining the increased amount of collagen in the lesions of vitamin E-treated mice. On the other hand, the increased collagen content can also result from a decreased enzymatic activity of matrix metalloproteinases (MMPs) which are primarily synthesized by macrophages (41). We suggest that vitamin E alters the balance between an increased collagen biosynthesis and a reduced enzymatic degradation of collagen. However, the exact role of vitamin E in the regulation of matrix metabolism has yet to be further investigated.

Four clinical intervention studies investigating the effect of vitamin E on cardiovascular disease, namely ATBC (11), CHAOS (10), GISSI (8), HOPE (9), and SPACE (12), have so far been published. The GISSI (300 mg D,L-α-tocopherol per day) and HOPE (400 IU/day) trials showed that intervention

with vitamin E for 3.5 and 4.5 years, respectively, had no beneficial effect on cardiovascular outcomes such as cardiovascular death, non-fatal myocardial infarction and stroke. The CHAOS trial showed that 1.4 years of treatment with vitamin E (400 or 800 IU/day) had a protective effect on the incidence of non-fatal myocardial infarctions, whereas the SPACE study reported that 1.4 years of (D-α-tocopherol, 800 IU/day) treatment reduced composite cardiovascular endpoints and myocardial infarctions. In the ATBC trial it was found that during a follow-up period of 4.7 years 50 mg/day of vitamin E resulted in a significant 9% decrease in the risk of developing angina pectoris, the mildest and often the first sign of coronary heart disease. A plausible explanation for the absence of a common protective effect against cardiovascular disease in the clinical intervention trials described above might be that elderly high-risk patients were investigated, in which the earliest events in atherosclerosis already had been triggered. Thus, vitamin E is most probably ineffective in reducing the progression of atherosclerosis or in regression, but may only be effective in inhibiting atherosclerosis when treatment starts at the earliest onset of atherosclerosis. This hypothesis is implicitly supported by several animal studies, including our study, in which vitamin E treatment that started at the earliest onset of atherosclerosis protected against the development of atherosclerosis (6,16,19,21) On the other hand, the CHAOS and SPACE trials show primarily reductions of acute events such as fatal and non-fatal myocardial infarctions, which points strongly to the stabilization of existing atherosclerotic plaques. In conclusion, this study demonstrates that long-term vitamin E treatment reduces the progression of atherosclerosis and increases the stability of the plaque in heterozygous LDL receptor-deficient mice. Both the antioxidant and anti-inflammatory properties of vitamin E may have been responsible for these beneficial effects. In addition, these effects were found to be relevant for the human situation at serum lipid, lipoprotein and vitamin E levels.

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Chapter 6

Vitamin E reduces the macrophage rich area of atherosclerotic lesions at moderate cholesterol levels in apoE*3-Leiden transgenic mice

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submitted

Abstract

We investigated in apoE*3-Leiden mice, the anti-atherosclerotic effect of vitamin E at a high cholesterol level or at a moderate cholesterol level due to cholesterol-lowering treatment with atorvastatin. Methods - After 4 weeks on a semi-synthetic diet (0.0035% w/w vitamin E), mice were randomized into 4 groups that received for 25 weeks: this diet with (N=14) or without 0.07% (w/w) vitamin E (N=14) enrichment (high cholesterol level), or: this diet enriched with 0.01% (w/w) atorvastatin (moderate cholesterol level) with or without 0.07% (w/w) vitamin E. Results - Vitamin E lowered plasma cholesterol levels at a high cholesterol level by 18% ($P<0.0001$) by reducing the cholesterol level of circulating apoB-containing lipoproteins by 20% ($P<0.0001$). At a moderate cholesterol level, vitamin E had no effect on plasma cholesterol levels. Vitamin E treatment resulted in 1.6-fold increases in the level of plasma vitamin E at a high cholesterol level ($P<0.05$) and at a moderate cholesterol level ($P<0.05$). At both a high and moderate cholesterol level, vitamin E had no effects on the circulating levels of the inflammation markers serum amyloïd A (SAA), soluble intracellular adhesion molecule-1 (sICAM-1), and von Willebrand factor (vWf), and on the urinary excretion of the in vivo oxidative stress marker 8-iso-PGF2α. At a high cholesterol level, vitamin E had no effect on the urinary nitrate excretion. However, at a moderate cholesterol level vitamin E increased the urinary nitrate excretion ($P<0.05$) by 1.2-fold and 1.9-fold after 11 and 23 weeks of treatment, respectively. Vitamin E treatment had no effects on the size, the severity, and the collagen content of the atherosclerotic lesions. In addition, at a high cholesterol level, vitamin E had no effect on the macrophage area of the atherosclerotic lesions. However, at a moderate cholesterol level vitamin E reduced the macrophage area of the atherosclerotic plaques by 58% ($P<0.01$). Conclusions - Vitamin E did not attenuate atherosclerosis at a high cholesterol level, whereas at a moderate cholesterol level vitamin E had a beneficial effect on atherosclerosis, as reflected by the diminished macrophage area of the atherosclerotic plaques. Additionally, at a moderate cholesterol level, vitamin E can possibly improve vessel function, as indicated by an increased urinary nitrate excretion.

Introduction

The beneficial effects of vitamin E on the development of atherosclerosis are not clear. Five major clinical intervention trials on the anti-atherosclerotic effects of vitamin E, named ATBC (1), CHAOS (2), GISSI (3), HOPE (4) and SPACE (5) showed divergent effects. Vitamin E had no effect in two studies (3,4) and demonstrated beneficial effects on cardiovascular disease in three other studies: a small reduction in angina pectoris (1), and a reduced number of non-fatal myocardial infarctions (2,5). Several rodent studies showed no effects of vitamin E on atherosclerosis (6-8), whereas in other studies vitamin E reduced the progression of atherosclerosis, independently of the modulation of plasma lipid levels (9-11). The anti-atherosclerotic mechanism of vitamin E is not fully understood, but may be related to the inhibition of LDL oxidation, which is thought to be an important process in the early development of atherosclerosis (12). Studies in humans and animals have found that vitamin E lowers the urinary levels of F2-isoprostanes (10,11,13,14), an *in vivo* measure of oxidative stress. These data may indicate that the antioxidant activity of vitamin E determines its anti-atherosclerotic properties. However, other potential atherosclerosis-attenuating properties of vitamin E have also been reported (15). These are independent of its antioxidant activity and related to the post-transcriptional inhibition of protein kinase C activity, thereby improving endothelial function, inhibiting smooth muscle cell proliferation, and limiting platelet aggregation (16). In addition, vitamin E has been found to inhibit monocyte-endothelial cell adhesion *in vitro*, possibly by inhibiting the activation of the transcription factor nuclear factor-kappaB (NF-kappaB) (15).

In this study we investigated whether vitamin E treatment reduced the progression of atherosclerosis at a high cholesterol level and at a moderate cholesterol level in an hyperlipidaemic mouse model. The hypothesis to be tested was that vitamin E can only have anti-atherosclerotic effects at moderate cholesterol levels. We used aggressive lipid-lowering therapy with atorvastatin in order to reduce plasma cholesterol to a moderate level without changing the composition of the diets. In addition, aggressive lipid-lowering therapy reflects the trend in clinical practice to treat increased cholesterol levels in patients. As animal model, we used apoE*3-Leiden transgenic mice, because these mice show a human-like lipoprotein profile with abundant VLDL/LDL-sized lipoproteins (17,18), caused by a defective clearance and lipolysis of remnant lipoproteins (19). Furthermore, when given semi-synthetic sucrose-based diets, serum lipid levels of these mice can easily be varied by

changing the amount of cholesterol and fat supplied via the diet (17). In addition, these mice develop diet-induced atherosclerosis, closely resembling the human pathology (20-23). Furthermore, apoE*3-Leiden mice have shown to be highly responsive to atorvastatin by reducing the levels of atherogenic lipoproteins and the development of atherosclerosis (22).
This study demonstrates that on the one hand treatment with vitamin E did not reduce the progression of atherosclerosis at a high cholesterol level. On the other hand, at a moderate cholesterol level, vitamin E reduced the macrophage area in the atherosclerotic lesions of apoE*-Leiden mice.

Methods

Animals and diets

Three-to four-month old female apoE*3-Leiden transgenic mice of the F18-F19 generation cross-bred on C57BL/6Jico line (The Broekman Institute bv, Someren, The Netherlands), were used. Identification of transgenity was carried out with an ELISA for human apoE. The animals were housed in wire-topped Macrolon cages with a layer of sawdust as bedding and all diets and water were given ad libitum.
Before the start of the study, animals were fed on standard rat/mouse chow. During the experimental run-in period of 4 weeks, animals were fed a semi-synthetic diet (22,23), containing (all percentages weight by weight) 41% sucrose, 16% fat (9% saturated, 6% mono-unsaturated, and 1% poly-unsaturated), 0.75% cholesterol, 0.02% cholate and 0.0035% vitamin E. After the run-in period, animals were randomized into four groups for plasma cholesterol levels and age. For the remaining 25 weeks, the control group continued to receive the run-in diet, whereas the three treatment groups received the same diet, but enriched with either vitamin E (vitamin E group) to 0.07% (D-α-tocopherol, Sigma, T3634), or with 0.01% atorvastatin (Lipitor, Pfizer; atorvastatin group), or with both 0.07% vitamin E and 0.01% atorvastatin (co-treatment group). Food intake was determined periodically at group level by weighing the amount of food consumed and was expressed as gram/mouse/day. The experiments were approved by the TNO Committee on Animal Welfare.

Plasma and urine parameters

Blood samples were taken from the tail vein after 4 hours of fasting. Levels of plasma cholesterol and triglycerides were measured enzymatically, and cholesterol ultracentrifugation profiles were made as previously described (23). Plasma pools of 3-4 mice were used to determine apoB levels according to Mancini (24). Pooled plasma vitamin E (α-tocopherol) concentrations were quantified using HPLC (25). After 20 weeks of treatment, lag time (min) and propagation rates (nmol dienes/mg/min) of copper-induced VLDL/LDL oxidation were determined in triplicate (26).

Serum levels of serum amyloid A (SAA, Perbio) and soluble intracellular adhesion molecule-1 (sICAM-1) were determined by ELISA (Biosource International). Von Willebrand factor (vWf) was determined in EDTA plasma by ELISA, using antisera from Dakopatts (Glostrup), basically as described by Ingerslev (27), using rat plasma for calibration. Pools of urines of 3-4 mice were collected during 17h periods after 7, 11 and 23 weeks of treatment. Urinary isoprostane and nitrate levels were quantified using GC-MS (28,29), and were corrected by urinary creatinine concentration to limit variability due to changes in renal excretory function. Urinary creatinine was determined spectrophotometrically by the alkaline picric acid method in an automated analyser (Beckman).

Tissue preparation, histology and quantification of atherosclerosis

After 25 weeks of treatment, mice were anaesthetized using 1.6 ml/kg Dormicum (Roche) and 2.0 ml/kg Hypnorm (Janssen Pharmaceutica) and were then sacrificed. After dissection, the hearts were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS) (48 hours, 4 °C, pH 7.4), and were then dehydrated and paraffin embedded. Thereafter, the hearts were sectioned perpendicularly to the axis of the aorta, starting with the heart and working in the direction of the aortic arch as described by Paigen *et al.* (30). Once the aortic root was identified by the appearance of the aortic valve leaflets, serial 5 mm sections were taken and mounted at 50 mm intervals on 3-AminoPropyl-3-EthoxySilane (APES)-coated slides. Sections were air-dried (24 hours, 37 °C). Cross-sections were stained with haematoxylin-phloxin-saffron (HPS) or Sirius red for the quantification of atherosclerosis and the visualization of collagen, respectively. Macrophages were detected after incubation with a primary anti-macrophage antibody (AIA31240, dilution 1:1500, Accurate Chemicals and Scientific), followed by a secondary biotinylated donkey anti-rabbit antibody (RPN 1004, dilution 1:3200, Amersham) and avidin-biotin-horseradish peroxidase (K-0355, Dako). Smooth muscle cells were detected using a primary mouse monoclonal anti-muscle α-actin antibody (clone 1A4, dilution 1:750, Sigma) and followed by a secondary peroxidase-conjugated rabbit anti-mouse antibody (P-0260, dilution 1:300, Dako). Peroxidase activity was visualized using a Nova Red substrate kit (SK-4800, Vector Labs) and cross-sections were counter-stained with haematoxylin.

Per animal, full colour images of 4 consecutive cross-sections of the aortic root were acquired and captured as previously described (23), using a light microscope equipped with a 4x objective. Lesion contents of collagen, macrophages and smooth muscle cells were automatically quantified by setting a threshold value for the intensity of the specific staining in a pre-selected area, using Qwin image analysis software (Leica Imaging Systems Ltd).

The severity of atherosclerosis was classified into five types (23), using the classification system of the AHA. To discriminate between early and established lesions, the five lesion types were further divided into type 1-3 lesions (early fatty streaks, regular fatty streaks and mild plaques) and type 4-5 lesions (moderate and severe plaques). One operator (who was blinded to the group allocation) performed all the analyses.

Statistics

All data are presented as mean±SD. Overall differences between groups were evaluated, using the non-parametric Kruskal-Wallis test. If this test reached significance, multiple comparisons between the two groups were done using the Mann-Whitney U-test for unpaired samples. Multivariate linear regression analysis was used to determine which parameters associated with atherosclerotic lesion size. P values less than 0.05 were considered significant. All statistics were performed using SPSS for Windows (version 10).

Results

Plasma levels of lipids, lipoproteins and vitamin E

Neither vitamin E nor atorvastatin treatment had an effect on food intake and body and liver weight, indicating that treatments did not induce unfavourable health effects.

Over the treatment period, plasma cholesterol levels were on average 21.0 ± 2.4 mM in the control group (figure 1A, table 1). At a high cholesterol level, vitamin E treatment decreased plasma cholesterol levels on the average by 18% ($P<0.0001$), by lowering the cholesterol levels of the apoB-containing lipoproteins IDL and LDL (figure 1, table 1). Furthermore, vitamin E reduced the plasma cholesterol exposure by 12% ($P<0.05$) (table 1). However, after 25 weeks of treatment, the cholesterol-lowering effect of vitamin E was not apparent, and plasma apoB levels were similar to the control group (table 1). At a moderate cholesterol level, vitamin E had no effects on the plasma cholesterol levels and on the composition of the circulating lipoproteins (table 1, figure 1). Atorvastatin reduced plasma total cholesterol levels by 50%($P<0.0001$), by decreasing the amounts of the apoB-containing lipoproteins (figure 1A, table 1). All treatments had no effect on plasma triglyceride levels.

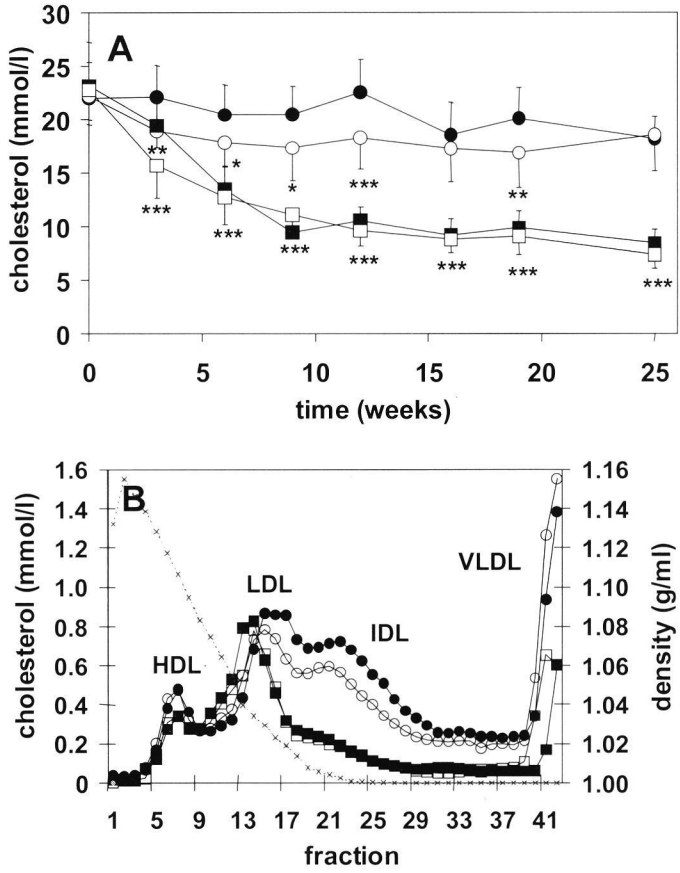

Figure 1. (A) Plasma cholesterol levels during the study period of 25 weeks; (B) Distribution of cholesterol over lipoprotein fractions after 9 weeks of treatment, using ultracentrifugation. ●, controls; ○, vitamin E; ■, atorvastatin; □, atorvastatin + vitamin E. In (A) statistically significant differences between control and treatment groups are indicated by $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. In (B), X indicates the density gradient. For the lipoprotein profiles pooled plasma was used. The fractions 4-9 correspond to HDL, 10-18 to LDL, 19-39 to IDL, and 40-42 to VLDL. The number of mice per group is indicated in table 1.

Plasma vitamin E levels are presented in table 1. At a high and moderate cholesterol level, vitamin E treatment resulted in 1.6-fold increases in plasma vitamin E levels, respectively ($P<0.05$). Furthermore, atorvastatin treatment non-significantly decreased ($P=0.20$) plasma vitamin E levels, as compared with the control group.

Table 1. Plasma levels of total, VLDL/IDL/LDL and HDL cholesterol, total apoB, and α-tocopherol

group size	Control N=14	Vitamin E N=13	Atorvastatin N=13	Vitamin E + Atorvastatin N=13
	High cholesterol level		Moderate cholesterol level	
		mmol/l		
Total cholesterol	21.0±2.4	17.1±2.7** (82)	10.5±1.3**	10.2±1.4** (97)
VLDL/IDL/LDL-cholesterol	19.2±2.2	15.4±2.4** (80)	9.0±1.1**	8.8±1.2** (98)
HDL-cholesterol	1.8±0.2	1.7±0.3 (95)	1.5±0.2**	1.4±0.2** (93)
Triglycerides	1.3±0.2	1.3±0.3 (100)	1.3±0.2	1.3±0.3 (100)
		mmol/l*week		
Cholesterol exposure	501±75	443±57* (88)	300±37**	298±33 (99)
		(μg/mL)		
ApoB	368±59	346±25 (94)	220±18* ^{x}	196±22* ^{x} (89)
		(μmol/l)		
Vitamin E	38.1±1.3	59.1±5.0* (155)	27.0±8.1	43.8±4.0^{+} (162)

Cholesterol and triglyceride concentrations are averages of six time-points between 6 and 25 weeks, as indicated in figure 1. Lipoprotein cholesterol levels of individual mice were calculated from plasma total cholesterol levels, using lipoprotein profiles determined at 6, 9 and 25 weeks. The areas under the curves (AUCs) of VLDL/IDL/LDL and HDL were calculated from lipoprotein fractions 10-42 and 4-9 respectively. Plasma apoB was measured at t=25 weeks. Plasma vitamin E (α-tocopherol) levels are averages of pooled plasma, taken at 3, 9, 19 and 25 weeks. The areas under the curves (AUCs) of VLDL/IDL/LDL and HDL were calculated from lipoprotein fractions 10-42 and 4-9 respectively. Plasma apoB was measured at t=25 weeks. Plasma α-tocopherol levels are averages of pooled plasmas, taken at 3, 9, 19 and 25 weeks. Values between parentheses represent the percentage of the value obtained from controls at a high cholesterol level, whereas at a moderate cholesterol level the percentage of the value obtained from atorvastatin treatment is represented. Statistically significant differences between control and treatment groups: * $p< 0.05$; ** $p< 0.0001$; between atorvastatin and vitamin E/atorvastatin treatment: $^{+}$ $p< 0.05$; and between vitamin E and treatment groups: ^{x} $p< 0.05$.

Serum levels of SAA and sICAM-1 and plasma levels of vWf

Inflammation plays an important role in the pathogenesis of atherosclerosis (31), and in man some markers of inflammation can predict the risk of cardiovascular events. For this reason, at the endpoint we measured the levels of SAA and sICAM-1 in serum, and of vWf in plasma (table 2).

At both a high and moderate cholesterol level, treatment with vitamin E had no effect on the circulating levels of SAA, sICAM-1 and vWf. In addition, atorvastatin had no effects on the levels of sICAM-1 and vWf, respectively. However, as compared with the control group and treatment with vitamin E, atorvastatin reduced SAA levels by 71% ($P<0.0001$).

Oxidative stress and endogenous nitrate production

Oxidative modification of LDL plays an important role in the development of atherosclerosis (12). Therefore we measured the levels of urinary isoprostane (8-iso-PGF2α) excretion as a marker for *in vivo* oxidative stress (table 2), and the lag time and propagation rate of copper-induced VLDL/LDL oxidation, as measure for the susceptibility of atherogenic lipoproteins to oxidative modification. In addition, we determined the urinary excretion rates of nitrate, a measure for whole-body nitric oxide production (table 2).

Treatments with vitamin E and atorvastatin had no effect on urinary isoprostane excretion, indicating that these treatments did not attenuate *in vivo* oxidative stress under the conditions applied. However, Vitamin E increased the lag time of VLDL/LDL oxidation by 1.2-fold at a high cholesterol level (controls 248±23 min, vitamin E 301±22 min) and by 1.5-fold at a moderate cholesterol level (atorvastatin 231±1 min, vitamin E/atorvastatin 350±22 min). In addition, vitamin E reduced the propagation rate of VLDL/LDL oxidation (nmol/mg protein/min) by 15% at a high cholesterol level (controls 1.04±0.11, vitamin E 0.89±0.04) and by 18% at a moderate cholesterol level (atorvastatin 0.88±0.03, vitamin E/atorvastatin 0.72±0.08). Furthermore, atorvastatin treatment had no effect on the lag time of VLDL/LDL oxidation, whereas atorvastatin reduced the propagation rate by 15%.

Vitamin E had no effect on the urinary nitrate excretion at a high cholesterol level, whereas at a moderate cholesterol level, after 11 and 23 weeks of vitamin E treatment, the nitrate excretion rate had gradually increased by 1.7-fold and 2.4-fold, respectively ($P<0.05$). This finding indicates that vitamin E led to a gradual increase of whole-body NO production at a moderate cholesterol level. Furthermore, atorvastatin had no effect on urinary nitrate excretion.

Table 2. Effects on serum SAA, sICAM-1 and plasma vWf levels, and on urinary excretions of isoprostane and nitrate

	Control	Vitamin E		Atorvastatin	Vitamin E + Atorvastatin	
	High cholesterol level			Moderate cholesterol level		
	μg/ml					
SAA	5.1±2.3	5.5±1.6	(107)	1.5±1.8 ** ^{xx}	1.4±0.5 ** ^{xx}	(93)
sICAM-1	53.8±8.3	53.6±5.4	(99)	56.8±6.5	53.0±5.2	(93)
	% of reference plasma					
vWf	206±108	256±71	(124)	310±76	212±107	(68)
	Urinary 8-iso-PGF2α (nmol/mol creatinine)					
7 weeks	480±254	390±141	(81)	139±76	278±271	(200)
11 weeks	226±80	178±128	(79)	156±65	153±118	(98)
23 weeks	229±206	119±94	(52)	62±29	117±51	(189)
	Urinary nitrate (μmol/mmol creatine)					
7 weeks	337±26	313±76	(93)	319±114	360±114	(113)
11 weeks	284±32	329±62	(116)	377±61	464±88*	(123)
23 weeks	247±73	285±37	(115)	261±76	527±167* $^{+x}$	(194)

Levels of serum amyloid A (SAA), serum soluble intracellular adhesion molecule-1 (sICAM-1), and plasma von Willebrand Factor (vWF) were measured individually (N=13-14) at the study endpoint (T=25 weeks). Urinary isoprostane (8-iso-PGF2α) and nitrate levels (N=4-5 pools of N=3-4 mice) were corrected for urinary creatinine, and were measured after 7, 11 and 23 weeks of treatment. At the same time-point, values between parentheses represent the percentage of the value obtained from controls at a high cholesterol level, whereas at a moderate cholesterol level the percentage of the value obtained from atorvastatin treatment is represented. Statistically significant differences between control and treatment groups: * $p< 0.05$; ** $p< 0.0001$; between atorvastatin and vitamin E/atorvastatin treatment: $^{+}p< 0.05$; and between vitamin E and treatment groups: $^{x}p< 0.05$; $^{xx}p< 0.0001$.

Size, severity and composition of atherosclerotic plaques

The size, severity and the relative areas of collagen, smooth muscle cells, and macrophages of the atherosclerotic plaques (table 3), were quantified to assess the effects of the treatments on the development and stability of the atherosclerotic plaques.

Vitamin E had no effects on the size, severity and the collagen contents of the atherosclerotic lesions, at a high and moderate cholesterol level, respectively. See also the representative photomicrographs of the size (figure 2 A-D) and the collagen contents (figure 2 E-H) of the lesions. In addition, at a high cholesterol level the macrophage area of the atherosclerotic lesions had not been influenced by vitamin E. However, at a moderate cholesterol level, vitamin E treatment led to a 58% reduction of the macrophage areas of the atherosclerotic lesions ($P<0.0001$). See also the representative photomicrographs (figure 2 I-K).

Table 3. Atherosclerotic lesion size, severity, and composition

	Control	Vitamin E		Atorvastatin	Vitamin E + Atorvastatin	
Group size	N=14	N=13		N=13	N=13	
	High cholesterol level			Moderate cholesterol level		
	($\mu m^2 \times 10^3$)					
Lesion size	226±48	186±54	(82)	54±32***	39±17***	(72)
	% of total lesions					
Lesion severity						
early lesions	43±17	46±23	(106)	65±17**	68±15**	(105)
established lesions	57±17	54±23	(96)	35±17**	32±15**	(91)
	% of lesion area					
Collagen	66±9	70±6	(107)	78±5**	79±7 ***	(101)
Macrophages	8.0±2.7	6.5±4.1	(81)	8.6±4.1	3.6±2.2*** ^{xx}	(42)
Smooth muscle cells	0.26±0.18	0.26±0.19	(100)	0.24±0.21	0.28±0.32	(117)

The size, severity and composition of atherosclerotic lesions were determined as described in Methods. The atherosclerotic plaque contents of collagen and macrophages are presented as a percentage of the size of early (type 1-3) and established (type 4+5) lesions, whereas contents of smooth muscle cells are presented as a percentage of total (type 1-5) lesion size. Areas are means of 4 cross-sections per mouse at 50 μm intervals, overlapping a region of 170 μm of the aortic root. Values between parentheses represent the percentage of the value obtained from controls at a high cholesterol level, whereas at a moderate cholesterol level the percentage of the value obtained from atorvastatin treatment is represented. Statistically significant differences between control and treatment groups are indicated by * $p< 0.05$, ** $p < 0.001$, *** $p < 0.0001$; and between atorvastatin and vitamin E/atorvastatin are indicated by $^{x}p < 0.05$, $^{xx}p<0.01$, $^{xxx}p < 0.005$.

These photomicrographs of the collagen (figure 2 E-H) and the macrophage positive areas (figure 2 I-L) also show that the largest portion of the atherosclerotic plaques consisted of collagen, followed by macrophages, whereas a small part of the lesions comprised smooth muscle cells (not shown). In contrast with vitamin E, atorvastatin potently diminished the size ($P<0.0001$) and the severity ($P<0.001$) of the atherosclerotic lesions. In addition, treatment with atorvastatin increased the lesional collagen content by 1.2-fold ($P<0.001$). However, atorvastatin had no effect on the macrophage area of the atherosclerotic lesions. Treatment with vitamin E or with atorvastatin had no effect on the smooth muscle cell content of atherosclerotic plaques (table 3).

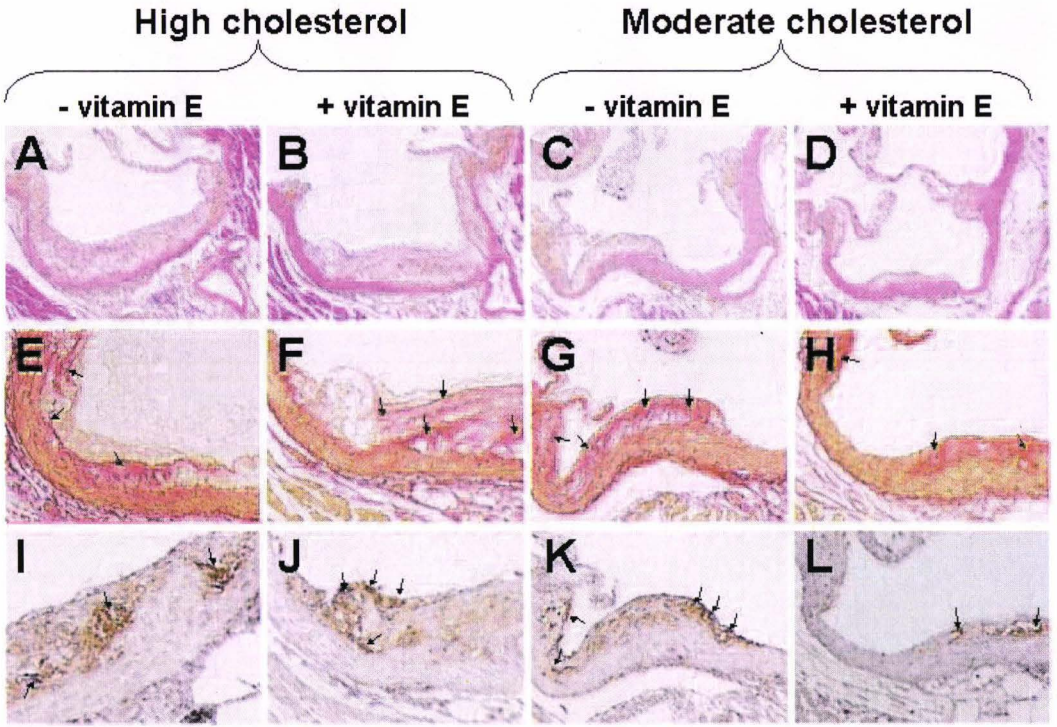

Figure 2. Representative photomicrographs of serial cross-sections of the aortic root of control (A, E, I), vitamin E-treated (B, F, J), atorvastatin-treated (C, G, K) and vitamin E/atorvastatin-co-treated (D, H, L) mice. HPS staining (A-D, magnification 4x) for quantification lesion size; Sirius red staining (E-H, magnification 8x) for collagen positive areas, indicated with arrows. Macrophages (I-L, magnification 8x) were detected using monoclonal antibodies, and present as brown staining, as indicated by filled arrows.

Discussion

In this study, we investigated in apoE*3-Leiden mice the effect of vitamin E on the progression of atherosclerotic lesions at a high and moderate cholesterol level, respectively. Vitamin E had no anti-atherosclerotic effects at a high cholesterol level, whereas at a moderate cholesterol level vitamin E diminished the macrophage areas of the atherosclerotic lesions. This effect had been found in addition to the potent anti-atherosclerotic effect of aggressive cholesterol-lowering treatment with atorvastatin.

There is clear evidence from clinical practice, especially with respect to coronary events in cardiovascular disease patients, that the composition of the plaque and its susceptibility to rupture, are more important factors than its size alone (60,61). Clinical studies revealed that plaques vulnerable to rupture have a large soft lipid pool mainly consisting of macrophages and a thin collagen-poor fibrous cap. The finding that vitamin E reduced the lesional macrophage area at a moderate cholesterol level is favourable, because in lesions macrophages can destabilize plaques by stimulating the degradation of matrix components of the lesion (32). However, the mechanism behind this reduced macrophage content of atherosclerotic plaques remains to be investigated. One possible mechanism might be a diminished attachment of monocytes to the endothelium. Indeed, several *in vitro* studies have shown that vitamin E reduced monocyte adhesion to the endothelial cells, and could inhibit oxLDL-associated upregulation of ICAM-1 and VCAM-1. However, *in vivo* evidence is lacking (33). In addition, it is possible that vitamin E diminished foam cell formation through inhibition of atherogenic lipoprotein oxidation, or through direct inhibition of pkC-mediated macrophage proliferation. For instance, in apoE-deficient mice increased titres of auto-antibodies to oxLDL paralleled the development of atherosclerotic lesions (34), mainly consisting of macrophages (35), whereas treatment with vitamin E attenuated atherosclerosis in these mice, and resulted in reduced oxLDL antibody titers and aortic and urinary F2-isoprostane levels, markers of *in vivo* oxidative stress (36). However, it seems to be unlikely that this proposed mechanism is responsible for reducing the macrophage content in atherosclerotic plaques of apoE*3-Leiden mice, because in these mice we found no inhibitory effect of vitamin E on oxidative stress *in vivo*, as reflected by the unchanged urinary F2-isoprostane excretion. In addition, vitamin E can inhibit protein kinase C activation *in vivo*,

independently of it's antioxidant properties (37), and *in vitro* studies have shown that oxLDL stimulates the proliferation of macrophages through the activation of pkC (38). However, for the lesional macrophages, the *in vivo* relevance of these findings has not been proven.

Atorvastatin treatment reduced plasma cholesterol levels by 50%, mainly by decreasing the amount of circulating apoB-containing lipoproteins. In apoE*3-Leiden mice, a potent inhibitory effect on the production of the amount of nascent VLDL particles is responsible for the cholesterol-lowering effect of atorvastatin (unpublished data). The level of cholesterol reduction aimed at was to be comparable to the aggressive lipid-lowering therapy in hypercholesterolaemic patients receiving 80 mg/day atorvastatin (39). Cholesterol-lowering treatment with atorvastatin resulted in potent anti-atherosclerotic effects, being large reductions in the size and severity, and an increased collagen content of the atherosclerotic lesions. These findings indicate that atorvastatin has plaque-stabilizing properties in apoE*3-Leiden mice, which is in line with clinical evidence on the effectiveness of statins in reducing cardiovascular disease (62). In line with our data, lipid-lowering by statin therapy or by diet also increased collagen contents of atherosclerotic plaques in rabbits (63). In the latter studies, collagen accumulation was accompanied by a reduced activity of matrix metalloproteinases (MMPs). MMPs are mainly produced by macrophages, but also by smooth muscle cells (64). Thus, a similar inhibitory effect of atorvastatin on MMP activity may have been responsible for the increased collagen content in atherosclerotic plaques of apoE*3-Leiden mice.

Atorvastatin treatment tended to decrease plasma vitamin E levels, in line with clinical studies, which showed significantly reduced vitamin E levels (40). This reduction is explained by the hypocholesterolaemic properties of statins, because the apoB-containing lipoproteins are the main carriers of vitamin E in plasma (26,41).

In apoE*3-Leiden mice, vitamin E treatment increased vitamin E levels by 1.6-fold at high and moderate cholesterol levels, respectively, which is comparable with the increase in humans given 200 IU/day of vitamin E given orally (26).

We have previously shown that at a comparable cholesterol level, a similar dose of vitamin E had more effective anti-atherosclerotic properties in heterozygous LDL receptor-deficient (LDLR +/0) mice (42) than in the presently used apoE*3-Leiden mice. In the LDLR +/0 mice vitamin E led to an attenuation of the size of the atherosclerotic lesions and increased the areas of

collagen and of smooth muscle cells in the fibrous cap and shoulder regions of the atherosclerotic lesions. These differences in effectiveness of vitamin E might be explained by the larger increase in plasma vitamin E levels in the LDLR +/0 mice (2.0-fold) than in the present study with apoE*3-Leiden mice (1.6-fold). In addition, in the present study the potent anti-atherosclerotic effects of aggressive cholesterol-lowering with atorvastatin may also have overshadowed the additional anti-atherosclerotic effects of vitamin E in apoE*3-Leiden mice.

In these mice, we found that this 1.6-fold increase in the plasma vitamin E level failed to attenuate atherosclerosis at a plasma cholesterol level of 21 mmol/l, whereas it reduced the amount of macrophages in atherosclerotic lesions at the lower plasma cholesterol level of 10 mmol/l. This difference in effectiveness can be explained by the level of cholesterol. In the present study the ratio of the apoB-containing liporoteins VLDL/IDL/LDL to HDL is greater at a high cholesterol level (vitamin E-treated 9/1) than at a moderate cholesterol level (vitamin E-treated 6.2/1). Furthermore, a higher cholesterol level allows atherosclerotic plaques to develop more rapidly in apoE*3-Leiden mice (17). These findings indicate that vitamin E is ineffective in attenuating atherosclerosis at a high cholesterol levels, because the atherosclerotic plaques progress too rapidly. In contrast, a previous study with apoE-deficient mice showed that vitamin E potently reduced the atherosclerotic lesion size at a high plasma cholesterol level of 19 mmol/l on average (10). However, in this study vitamin E treatment led to an almost 5-fold increase in the level of plasma vitamin E, whereas in the present study with apoE*3-Leiden mice the plasma vitamin E level had been increased by 1.6-fold. The above-mentioned studies indicate that for the anti-atherosclerotic effectiveness of vitamin E treatment, at a higher cholesterol level the plasma vitamin E level must be increased to a greater extent. More specifically, vitamin E treatment is only effective in attenuating atherosclerosis when the plasma ratio of the level of vitamin E to cholesterol has been elevated to a level of at least 4 μmol/mmol cholesterol, as is the case in our studies with apoE*3-Leiden and LDLR +/0 mice, and in the study of Pratico *et al.* (10) with apoE-deficient mice, respectively.

Inflammation and endothelial activation play an important role in the pathogenesis of atherosclerosis (31). There is growing evidence from clinical trials that C-reactive protein is an independent risk factor for cardiovascular disease (29), while SAA levels are strongly correlated (43,44). Concerning sICAM-1, there are indications from prospective studies that there is a link

between elevation of it's serum levels and an increased risk of cardiovascular events (45-47), which also holds true for the vascular adhesion molecule-1 (VCAM-1) (46). Furthermore, vWf, known as a marker of endothelial activation, has recently been shown to be a risk factor for recurrent myocardial infarction (48). We therefore measured the levels of SAA, sICAM and vWF in a situation with marked atherosclerosis. The finding that vitamin E and atorvastatin had no effects on the levels of sICAM-1 and vWf indicate that these lipid-lowering and antioxidant treatments had no protective effect on the activation state of the endothelium. In addition, vitamin E had no effect on the levels of SAA. In contrast, atorvastatin reduced the SAA levels by more than 70%. This finding suggested that anti-inflammatory properties of atorvastatin could have been responsible for its anti-atherosclerotic effects. Therefore, we used multiple regression analysis, but we found that the SAA level was less predictive of atherosclerotic lesion size ($P=0.051$) than serum cholesterol exposure ($P<0.0001$). This indicates that in apoE*3-Leiden mice atorvastatin mainly attenuated atherosclerosis by reducing circulating levels of cholesterol, rather than by suppressing inflammation.

Since oxidative modification of LDL plays a central role in the initiation of atherosclerosis (12), we measured *ex vivo* copper-induced VLDL/LDL oxidation, a measure for the susceptibility of atherogenic lipoproteins to undergo oxidative modification. Moreover, we quantified the urinary excretion of the isoprostane 8-iso-PGF2α, a marker of oxidative stress *in vivo*. Although atorvastatin and vitamin E synergistically increased the lag time and reduced the propagation rate of VLDL/LDL oxidation, both compounds had no effect on the urinary excretion of 8-iso-PGF2α, indicating that these treatments had no effect on oxidative stress *in vivo*. The lack of effect of vitamin E on isoprostane excretion is at variance with other studies that showed a lowering effect of vitamin E on urinary isoprostane excretion (10,11,13,14).

We found that after cholesterol-lowering treatment with atorvastatin, vitamin E increased the urinary nitrate excretion, an indicator of whole-body NO production. Together with citrulline, NO is the product of oxidation of L-arginine by NO-synthase, of which there are two species, being endothelial NO-synthase (eNOS) and inducible NO-synthase (iNOS). NO produced by eNOS has potentially anti-atherosclerotic properties, including improvement of endothelium-dependent vasodilation (49), and in rodents an increased NO production due to dietary enrichment with L-arginine leads to the attenuation of atherosclerosis (11,50). However, the role of NO produced by iNOS in the development of atherosclerosis is not established (51-54). There is no direct

evidence that vitamin E can modulate eNOS or iNOS activity *in vivo*, although vitamin E can stimulate eNOS acticity in platelets *in vitro*, via protein kinase C (PKC)-dependent phosphorylation of eNOS (55). In addition, vitamin E has been found to improve *in vivo* endothelium-dependent vasodilation in humans (56). However, in theory vitamin E can also preserve eNOS activity in an indirect fashion, by the inhibition of superoxide anion (O_2 -) formation, thereby diminishing peroxynitrite generation. Peroxynitrite is a highly reactive radical that damages the endothelium and inhibits eNOS activity (57,58). However, the finding that vitamin E had no effect on *in vivo* oxidative stress in apoE*3-Leiden mice, as reflected by an unchanged urinary isoprostane excretion, makes it unlikely that vitamin E induced NO production by inhibiting peroxynitrite formation. Furthermore, the increased urinary nitrate excretion could also have been the result of an interaction between atorvastatin and vitamin E, because there is evidence that atorvastatin can promote NO production via the activation of eNOS by decreasing endothelial caveolin-1 abundance (59). However, further investigations will be needed to elucidate the effects and interactions of vitamin E and atorvastatin on NO production *in vivo*.

In conclusion, this study shows that in apoE*3-Leiden transgenic mice, vitamin E is ineffective in attenuating atherosclerosis at a high cholesterol level. However, the finding that vitamin E diminished the macrophage area of the atherosclerotic lesions at a moderate cholesterol level, indicates that vitamin E can have a plaque stabilizing property in these mice. Furthermore, the increased urinary nitrate excretion due to treatment with vitamin E indicates that vitamin E may have a beneficial effect on vascular function at a moderate cholesterol level in these mice. In addition, we have indications that in apoE*3-Leiden mice, the anti-atherosclerotic effects of atorvastatin are mainly caused by its cholesterol-lowering properties.

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Chapter 7

General discussion and future perspectives

General discussion

The studies that we presented in this thesis provide evidence that (i) several lipid-lowering compounds, including plant stanol esters (chapters 2 and 3), HOE 402 (chapter 4), atorvastatin (chapter 5), and (ii) the natural antioxidant vitamin E, either alone or in combination with atorvastatin (chapters 5 and 6, respectively) can effectively attenuate the development of atherosclerosis in genetically modified hyperlipidaemic mice. Furthermore, these studies have produced new insights into the anti-atherosclerotic mechanism of these compounds.

Plant stanol esters

In our studies on the effects of plant stanol esters on plasma lipid levels and atherosclerosis development, we used apoE*3-Leiden mice, because their serum cholesterol levels can be titrated to any desired level by adjustment of the fat, cholesterol and cholate contents in semi-synthetic diets (1). ApoE*3-Leiden mice respond well to compounds that affect lipid metabolism (2-6), and they have a lipoprotein profile resembling the human situation, especially of patients with FD. Additionally, these mice are very useful for the assessment of atherosclerosis, because the morphology of their atherosclerotic lesions also resembles that of their human counterparts (1,7-9).

Using apoE*3-Leiden mice as a model, we found in chapter 2 that the diminished intestinal cholesterol absorption due to plant stanol ester treatment, as reflected by a reduced serum ratio of non-cholesterol sterols to cholesterol, resulted in a reduced hepatic cholesterol content. In addition, we found that this reduced hepatic cholesterol content resulted in a compensatory up-regulation of endogenous cholesterol synthesis, as reflected by an increased serum lathosterol/cholesterol ratio. However, it is obvious that the up-regulated cholesterol synthesis was not sufficient to overcome the hypocholesterolaemic effect of plant stanol esters. Moreover, we found that the reduced hepatic cholesterol content was rate-limiting for the cholesterol supplies to newly produced VLDL and bile. This led to the production of cholesterol-poor VLDL particles, but had no effect on the amount of newly produced VLDL particles, as reflected by an unchanged apoB production. Subsequently, the reduced VLDL-cholesterol production led to reductions in the plasma levels of the apoB-containing lipoproteins VLDL/IDL/LDL.

In humans, there are also indications that plant stanol ester treatment leads to a decreased VLDL production (10), and a compensatory upregulation of endogenous cholesterol synthesis (11). However, the finding that in apoE*3-Leiden mice the reduced hepatic cholesterol content was not rate-limiting for VLDL-apoB production, is in contrast to other *in vivo* and *in vitro* animal studies (12-14). There are two possible explanations for this discrepancy. First, the hepatic cholesterol content that we measured could not be representative of the metabolic cholesterol pool that regulates apoB production. Second, in these mice plant stanol ester treatment did not reduce the hepatic cholesterol content to an extent that was rate-limiting for apoB synthesis.

The findings that in apoE*3-Leiden mice plant stanol ester treatment reduced the VLDL cholesterol production and had no effect on the hepatic mRNA levels of the LDL receptor, indicate that treatment mainly lowers cholesterol levels by inhibiting VLDL production, and not by increasing the LDL receptor-mediated lipoprotein clearance. However, in order to prove this assumption, one should assess in these mice, the effect of plant stanol ester treatment on the LDL receptor-mediated clearance of LDL.

Furthermore, we found in these mice, that the reduced biliary cholesterol output due to plant stanol ester treatment resulted in a decreased cholesterol saturation index of bile, which is prognostic of a reduced risk of developing cholesterol gallstones (15). This effect of plant stanol esters on bile composition has not been observed in humans (16), a result which is at variance with our results in mice. A possible explanation for this difference could be that in humans, compensation mechanisms exist for maintaining hepatic cholesterol homeostasis, i.e. the upregulation of the LDL receptor-mediated lipoprotein clearance and cholesterol synthesis, which are more tightly controlled than in mice. In view of this, treatment would not lead to a reduced hepatic cholesterol pool that regulates the cholesterol output in bile in humans.

In apoE*3-Leiden mice, plant stanol ester treatment more potently reduced serum VLDL/IDL levels than LDL, which is at variance with findings in mildly hypercholesterolaemic humans, where plant stanol esters mainly reduce circulating levels of LDL. However, our findings are more comparable to those in patients having NIDDM, where stanol ester treatment also leads to reduced VLDL cholesterol levels (17). As compared with normolipidaemic humans, NIDDM patients have an increased VLDL production and a reduced clearance of apoB-containing lipoproteins (18,19). In addition, in patients with NIDDM the hypocholesterolaemic effect of plant stanol esters did not result in an

increased fractional catabolic rate of LDL apoB (17), indicative of an unchanged LDL receptor-mediated lipoprotein clearance. Thus, it is possible that in NIDDM patients the cholesterol-lowering effect of plant stanol esters depends more on reducing the VLDL cholesterol production than on an increasing the clearance of circulating LDL particles. Therefore, the hypolipidaemic effects of plant stanol esters in apoE*3-Leiden mice may be more comparable to those in NIDDM patients than to non-diabetic humans.

We found that plant stanol esters, irrespective of their source and composition, had equally potent cholesterol-lowering properties (chapter 3). These data are in line with findings in normolipidaemic humans, where the source and composition of stanol esters also had no effect on their cholesterol-lowering potential (20). In addition, we found in these mice that the equal cholesterol-reductions that were obtained with the stanols derived from the different sources, resulted in comparable attenuations in the size and severity of the atherosclerotic lesions in the aortic root. Obviously, one could conclude from these findings that it is as well possible to use vegetable oil as plant stanol source, instead of pine wood, which is the currently used source for plant stanol esters for incorporation into foods.

Plant stanol and sterols that were not esterified to fatty acids have also been shown to attenuate atherosclerosis due to their cholesterol-lowering properties in hypercholesterolaemic rabbits and apoE-deficient mice, respectively. Compared with those studies, our studies have been performed in conditions that were more comparable to the human situation, i.e. (i) we used plant stanol mixtures that were trans-esterified to rapeseed oil, as used in the plant stanol ester enriched margarines; (ii) the diets had a fat content and fatty acid composition comparable to human diet; and (iii) the plasma cholesterol levels were far less extreme, causing a more realistic rate of development of atherosclerosis. Moreover, if these data may be extrapolated to the human situation, these findings obviously suggest that plant stanol esters can effectively attenuate atherosclerosis development in humans with elevated plasma cholesterol levels.

HOE402

HOE 402 has been shown to be a potent lipid-lowering compound in hamsters and rabbits (21,22). In combination with data concerning the HepG2 cell, these *in vivo* rodent studies indicated that the cholesterol-lowering effect of HOE 402

occurs through the induction of LDL receptor activity. However, in chapter 3 we found no indications that in mice HOE 402 exerts its cholesterol-lowering effects by the modulation of LDL receptor-mediated lipoprotein clearance. Firstly, HOE 402 also effectively lowered serum cholesterol levels in the absence of the LDL receptor, in LDL receptor-deficient mice, by lowering levels of the apoB-containing lipoproteins VLDL/IDL/LDL. Secondly, in wild-type mice HOE 402 treatment resulted in a potent hypocholesterolaemic effect without affecting the clearance of human LDL. In order to assess the LDL receptor-mediated lipoprotein clearance, we used human LDL instead of mice LDL because the first is devoid of apoE.

We found in these mice that HOE 402 treatment reduced the hepatic cholesterol content, which led to a reduced hepatic VLDL-lipid production, without affecting the amount of newly produced VLDL particles. This finding indicates that HOE 402 treatment resulted in the production of lipid-depleted VLDL particles. Furthermore, we found that HOE 402 had no effect on the hepatic mRNA levels of HMG-CoA synthase, an enzyme involved in the endogenous cholesterol synthesis. The latter finding indicates that inhibition of the endogenous cholesterol synthesis cannot explain the hepatic cholesterol reduction. However, in order to exclude the effects of HOE 402 on cholesterol synthesis, one should assess the effects of HOE 402 on measures of endogenous cholesterol synthesis, such as the serum ratio of a cholesterol precursor (lathosterol, squalene, or lanosterol) to cholesterol. However, other mechanisms, such as the inhibition of endogenous ACAT, could also be responsible for the reduced hepatic cholesterol content, although no effect of HOE 402 on ACAT activity has been found in HepG2 cells.
Thus, in contrast to hamsters and rabbits, a reduced hepatic cholesterol content upon HOE 402 treatment, and the consequently reduced hepatic VLDL-lipid production, is most probably the underlying mechanism of the cholesterol-lowering potential of HOE 402 in mice. Furthermore, we found that HOE 402, as a consequence of its cholesterol-lowering properties, reduced the development of atherosclerosis in the thoracic aortas of LDLR-deficient mice.

Vitamin E

The study that we presented in chapter 5, was primarily focused on the anti-atherogenic effect of vitamin E supplementation on cholesterol levels and a lipoprotein profile relevant to the human situation. For this purpose, we used

heterozygous LDL receptor-deficient mice. In addition, in chapter 6 we assessed in apoE*3-Leiden mice whether vitamin E had anti-atherosclerotic effects at a high cholesterol level of approximately 20 mM, or at a moderate cholesterol level of 10 mmol/l on average, after lipid-lowering with atorvastatin. This hypothesis was also tested from a clinical perspective, in order to assess whether patients on statin therapy would benefit from additional vitamin E supplementation. There is considerable evidence that inflammation plays a key role in the development of atherosclerosis (23). The circulating levels of several inflammation markers, including SAA (24,25), vWf (26), and sICAM-1 (27-29) have been associated with an increased risk of cardiovascular disease. In addition, levels of vWf and sICAM-1 are commonly used as markers for endothelial activation, which is considered an important event in the initiation of atherosclerosis. Therefore, we also assessed in chapters 5 and 6 whether vitamin E had any effect on these markers.

In chapter 5, we found that vitamin E reduced the serum levels of SAA, which indicates that the anti-atherosclerotic properties of vitamin E may be explained by an anti-inflammatory effect. At variance with these findings, the SAA levels had not been reduced by vitamin E in apoE*3-Leiden mice (chapter 6), at a comparably moderate cholesterol level. However, in these mice vitamin E had been given in combination with atorvastatin, and atorvastatin reduced the SAA levels by 71%. Therefore, in this study the potential reduction of the level of SAA by vitamin E may have been masked by atorvastatin.

However, the finding that vitamin E had no effect on the circulating levels of vWf and sICAM-1 in both LDLR +/0 (chapter 5) and apoE*3-Leiden mice (chapter 6), indicates that vitamin E had no protective effect on the activation state of the endothelium. Nevertheless, these findings do not exclude the possibility that the anti-inflammatory properties of vitamin E could have interfered with the development of atherosclerosis, because in mice there are no clear data on the link between the systemic levels of these markers and their local upregulation in the atherosclerotic plaque.

Oxidative modification of LDL is an important factor in the initiation and progression of atherosclerosis (30). Therefore, we measured in LDLR +/0 mice (chapter 5) and in apoE*3-Leiden mice (chapter 6) the levels of the F2-isoprostane 8-iso-PGF2α in urine and 8,12-iso-iPF2α-VI in plasma, respectively, as indicators of oxidative stress *in vivo*. Vitamin E reduced the plasma F2-isoprostane level by 21% in LDLR +/0 mice. This finding indicates that vitamin E reduced oxidative stress. In contrast, vitamin E had no effects on the urinary excretion of 8-iso-PGF2α in apoE*3-Leiden mice, which

indicates that *in vivo* oxidative stress had not been inhibited by vitamin E. However, together with the data of Pratico *et al.* (31) that vitamin E effectively attenuated atherosclerotic lesion size and reduced F2-isoprostane levels in urine, plasma and vascular tissues of apoE-deficient mice, one can conclude that the antioxidant properties of vitamin E play an important role in the attenuation of atherosclerosis in mice.

Together with citrulline, NO is the product of oxidation of L-arginine by endothelial and inducible NO-synthase, eNOS and iNOS respectively. NO has potentially anti-atherosclerotic properties, including improvement of endothelium-dependent vasodilation (32), and in rodents an increased NO production due to dietary enrichment with L-arginine leads to the attenuation of atherosclerosis (33,34). In addition, vitamin E has been found to improve *in vivo* endothelium-dependent vasodilation in humans (35). We found that vitamin E increased whole-body nitric oxide (NO)-production in the apoE*3-Leiden mice (chapter 6), as reflected by an increased urinary excretion of nitrate. The increased NO-production may have been caused by an improved eNOS activity in these mice. Indeed, vitamin E has been found to stimulate NO production in platelets *in vitro*, via protein kinase C (PKC)-dependent phosphorylation of eNOS (36). However, there is no direct evidence that vitamin E can induce eNOS activity *in vivo*. In addition, vitamin E may indirectly preserve eNOS activity, by the inhibition of superoxide anion (O_2-) formation, thereby diminishing peroxynitrite generation. Peroxynitrite is a highly reactive radical molecule that damages the endothelium and inhibits eNOS activity (37,38). Furthermore, the increased NO-production may have been caused by an interaction between vitamin E and atorvastatin, because there is evidence that atorvastatin can enhance NO production via the activation of eNOS by decreasing endothelial caveolin-1 expression (39). Thus, the biochemical background of the effects of vitamin E on NO production and the possible interactions between vitamin E and atorvastatin need to be further assessed.

There is clear evidence from the clinic that the composition of the plaque and its susceptibility to rupture are more important factors than its size alone, especially with respect to coronary events (40,41). Clinical studies revealed that plaques vulnerable to rupture have a large soft lipid pool mainly consisting of macrophages and a thin collagen-poor fibrous cap. Since we found in LDLR +/0 mice that vitamin E increased the collagen contents and enlarged the fibrous cap of the atherosclerotic lesions (chapter 5), these data are strongly indicative of the lesion-stabilizing properties of vitamin E. In addition, the finding that vitamin E potently reduced the macrophage area of

the atherosclerotic lesions of apoE*3-Leiden mice (chapter 6) is indicative of a lesion-stabilizing property, because macrophages can enhance the further progression and weakening of atherosclerotic lesions, by releasing pro-inflammatory cytokines and MMPs (42).

In these studies (chapters 5 and 6) the beneficial effects of vitamin E had been found at the same dose of vitamin E and at a moderately elevated cholesterol level (10-12 mmol/l). However, vitamin E had greater anti-atherosclerotic and plaque-stabilizing properties in LDR+/0 mice (chapter 5) than in apoE*3-Leiden mice (chapter 6). One factor possibly explaining this difference is that the plasma vitamin E level had been increased to a greater extent in LDLR +/0 mice (2.0-fold) than in apoE*3-Leiden mice (1.6-fold). Furthermore, the aggressive cholesterol-lowering treatment with atorvastatin, which resulted in a potent attenuation of atherosclerosis could have masked further anti-atherosclerotic effects of vitamin E.

In contrast with our findings in apoE*3-Leiden mice (chapter 6) where the development of atherosclerosis had not been attenuated by vitamin E at a high cholesterol level, vitamin E effectively attenuated atherosclerosis in apoE-deficient mice at a plasma cholesterol level of 19 mmol/l on average (31). In this study, treatment with a 2-fold higher vitamin E dose than we used, resulted in a 5-fold elevation of plasma vitamin E to a level that was 2-fold higher than in the apoE*3-Leiden mice. These data suggest that for the anti-atherosclerotic efficacy of vitamin E, the ratios of plasma vitamin E to cholesterol must be sufficiently elevated. However, in order to prove the link between the extent of the plasma vitamin E-elevation and the anti-atherosclerotic efficacy of vitamin E, another atherosclerosis study will be required in which different dosages of vitamin E are being used.

Five clinical intervention studies investigating the effect of vitamin E on cardiovascular disease, have been published (43-47). The GISSI (43) and HOPE (44) trials found no beneficial effects of vitamin E. However, the CHAOS (46) and SPACE (45) trials both showed that vitamin E protected against the incidence of non-fatal myocardial infarctions. In the ATBC trial (47), it was found that during a follow-up period, vitamin E decreased the risk of developing angina pectoris. Possible explanations for the absence of a comparable protective effect of vitamin E against cardiovascular disease in these clinical trials, are: (i) the inclusion of elderly high-risk patients, in whom the initiation of atherosclerosis had already been triggered; and (ii) the different dosages and intervention periods that were applied in these studies, if protective effects of vitamin E depend on its dose and length of treatment.

Concerning the dose of vitamin E that needs to be applied for sufficient antioxidant protection, 600 mg/day of vitamin E has shown to inhibit oxidative stress *in vivo*, as reflected by a reduced formation of F2-isoprostanes (48,49). Results from animal studies suggest that vitamin E treatment only protects against the development of atherosclerosis when started at the earliest onset of atherogenesis, and when plasma vitamin E levels were sufficiently elevated (31,50-52). To summarize, one could conclude from both clinical and animal studies that if treatment with vitamin E is started in younger people at an earlier stage of atherosclerosis, and if these are given a proper dose ($\geq$ 600 mg/day), one could expect more evident effects of vitamin E on cardiovascular disease.

Atorvastatin

In chapter 6, we reported the treatment of mice with atorvastatin alone, as a control for the combination treatment of vitamin E and atorvastatin. Atorvastatin treatment reduced serum cholesterol levels by 50%, by lowering serum apoB-containing lipoproteins, and potently reduced atherosclerotic lesion size (by 77%) and severity, which is in line with a previously reported study in apoE*3-Leiden mice (5). In addition, we found that atorvastatin treatment led to an increased collagen content of the atherosclerotic lesions, which is indicative of an increased plaque stability (42). This finding is in line with all major clinical statin trials, where treatment has been proven to reduce cardiovascular disease mortality (53). This is strongly indicative of the plaque-stabilizing properties of statins. The increased plaque collagen content might be secondary to the lipid-lowering properties of statins, because in rabbits it was found that lipid-lowering resulted in increased collagen content of atherosclerotic lesions (54). However, there is growing evidence that statins also have anti-atherosclerotic properties that are independent of their lipid-lowering properties (55), such as antioxidant effects (56), NO-mediated improvement of endothelial function (57), and anti-inflammatory properties (58). However, it is unlikely that in these mice atorvastatin had antioxidant properties or improved NO-mediated endothelial function, because we found no effect of atorvastatin on urinary isoprostane and nitrate excretions, markers of *in vivo* oxidative stress and whole-body NO-production, respectively. However, the finding that atorvastatin treatment led to a pronounced reduction of the levels of serum SAA, indicates that anti-inflammatory properties of atorvastatin could have been responsible for its anti-atherosclerotic efficacy.

Moreover, multiple regression analysis excluded this possibility, because plasma cholesterol exposure better predicted the size of the atherosclerotic lesions than the SAA level. Furthermore, in these mice SAA levels correlated with serum cholesterol levels. Thus, in apoE*3-Leiden mice we have no indications that atorvastatin has anti-atherosclerotic effects that are independent of its lipid-lowering properties. However, whether the non-lipid-related effects of statins are relevant for the development of atherosclerosis in humans, remains to be elucidated.

Future perspectives

An elevated LDL cholesterol level is a prominent risk factor for cardiovascular disease. In Western society, cardiovascular disease still is the most important cause of mortality. During the mid-1980s in the USA, and at the end of the 1980s in the Netherlands, the use of statins as LDL cholesterol-lowering drugs, became increasingly popular, especially, after large clinical intervention trials showed that statins effectively reduce cardiovascular disease mortality (53). Consumption of margarines enriched with plant stanol esters and plant sterol esters have also been shown to reduce cholesterol levels in mildly hypercholesterolaemic humans, by 10% on average, due to 12-15% reductions of LDL cholesterol. In addition, when these enriched margarines are given to hypercholesterolaemic patients on statin therapy, their additional serum cholesterol-lowering effect is equally as potent as treatment with plant stanol and sterol esters alone (10,59,60). This finding shows that the daily intake of plant stanol esters or sterol esters could be useful for further lowering plasma cholesterol levels in for instance poorly responding FH patients who receive statins, but still have high plasma cholesterol levels.

The anti-atherosclerotic effectiveness of lowering serum cholesterol levels using plant stanols and sterols, respectively, has not been proven in humans, because margarines and other foods enriched with plant stanol esters and sterol esters have only recently become available for human consumption. Their consumption does not lead to unfavourable side-effects, although it has been found that plasma levels of the carotenoids α- and β-carotene, and/or lycopene are slightly, but significantly decreased by consumption of plant stanol esters and plant sterol esters (20,59,61-63). Since these effects could interfere with the anti-atherosclerotic effectiveness of reducing serum cholesterol levels, plant stanol esters and plant sterol ester-

containing margarines have been fortified with carotenoids. On the basis of the results of rodent studies by others (64-66) and by us (chapter 3), that showed clear anti-atherosclerotic effects of reducing cholesterol levels using plant stanols and sterols, one can assume that treatment will also be effective in humans. According to the 2:1 rule of thumb, a 1% lowering of cholesterol would result in about 2% lowering of risk of cardiovascular disease mortality, or the inverse of the observational ratio of a 2% to 3% increase in risk for each 1% rise in cholesterol (67,68). If this rule is applied to the 10% cholesterol-lowering by plant stanol ester treatment, the risk of cardiovascular disease mortality is reduced by approximately 20%. If these foods prove to be as effective in reducing cardiovascular disease mortality and if they are used by a large proportion of the population, cardiovascular disease will indeed be substantially reduced. This would be a breakthrough for the popularity of functional foods in general. Functional foods are defined as manufactured foods for which scientifically valid health claims can be made. Caloric restriction due to moderate reductions of dietary fat intake leads to cholesterol reductions that are comparable to those obtained with plant stanol esters (69,70). Therefore, dietary fat reduction can also lead to a substantial lowering of the risk of cardiovascular disease at population level. However, in practice it is not feasible to persuade a complete population to enforce a long-term dietary fat restriction.

In addition, a group of potent pharmacological inhibitors of intestinal cholesterol absorption have been developed, of which the drug ezetimibe, has passed clinical phase II/III studies (71). This group of compounds inhibits the intestinal cholesterol absorption more specifically than plant stanol and sterol esters, via a yet to be identified integral membrane protein of 145 kDa, that is situated in the brush border membrane of intestinal enterocytes (72). In contrast with plant stanol and sterol esters that are meant for consumption by a large part of the population, ezetimibe is a drug that is developed for clinical applications. As compared with plant stanol and sterol esters, this drug more specifically inhibits the intestinal cholesterol absorption, and its use will therefore result in greater serum LDL-cholesterol reductions of probably 20% on average, and will not lead to reduced serum carotenoid levels. In the near future, phase III trials in patients with elevated cholesterol levels must confirm whether the co-administration of ezetimibe with statins or fibrates is safe and whether these drugs synergistically reduce plasma lipid levels and cardiovascular disease mortality.

Another potentially useful target for reducing serum cholesterol levels in a clinical setting, is the pharmacological induction of the LDL receptor-mediated clearance of atherogenic lipoproteins. HOE 402 is a cholesterol-lowering compound, which has been suggested to be a specific LDL receptor inducer. However, we found that HOE402 did not lower serum cholesterol levels by inducing the LDL receptor, but by reducing hepatic VLDL-production. The mechanism by which HOE 402 reduces VLDL production has not been elucidated. Nevertheless, the study with HOE 402 (chapter 3) shows that the inhibition of VLDL production is also a good approach for reducing cholesterol levels and for inhibiting atherosclerosis development. At present, no specific LDL receptor inducers are available, although statin treatment indirectly leads to the induction of the LDL receptor to compensate for a reduced hepatic cholesterol content due to the inhibition of endogenous cholesterol synthesis (73). If a specific LDL receptor inducer existed, it would be of great importance for assessing which hypercholesterolaemic patients would benefit from treatment. However, the LDL receptor remains an interesting target for reducing the levels of atherogenic lipoproteins.

We have shown that vitamin E can attenuate atherosclerosis in mildly hyperlipidaemic mice, when treatment leads to a sufficient increase in the serum ratio of vitamin E to cholesterol. However, it has been suggested that vitamin E is a more effective antioxidant and could therefore have more effective anti-atherosclerotic properties, when combined with another more hydrophilic antioxidant such as vitamin C (74) or coenzyme Q(10) (CoQ(10)) (75). These suggestions are based on the hypothesis that co-antioxidants neutralize the vitamin E radical molecules that appear when vitamin E scavenges radical molecules in the LDL particle (76). However, there is not much *in vivo* evidence that lipophilic co-antioxidants improve the anti-atherosclerotic effectiveness of vitamin E. One study has reported that CoQ(10) amplified the anti-atherosclerotic potential of vitamin E in apoE-deficient mice (75). Therefore, it would be of interest to assess in apoE*3-Leiden mice whether this combination has a synergistic effect on atherosclerosis, at mildly elevated plasma lipid levels and lipoprotein distribution that are more comparable to the human situation than in apoE-deficient mice. If this synergism is indeed present in apoE*3-Leiden mice, it would be interesting to test the effects of this combination on parameters of oxidative stress and inflammation in humans.

We found in apoE*3-Leiden mice that treatment with vitamin E at a moderate cholesterol level reduced the macrophage content of atherosclerotic

lesions, and increased urinary nitrate excretion. The biochemical background of these effects needs to be further assessed. In addition, the latter finding poses the question of whether vitamin E is beneficial for endothelial function, because NO plays a major role in the modulation of vascular function (32).

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Summary

Lipid-lowering therapies with statins have been shown to effectively reduce cardiovascular disease mortality. However, at present cardiovascular disease is still the number one cause of mortality in Western society. Therefore, the search for additional ways of reducing cardiovascular disease is of great interest. The primary aim of this thesis was to study the beneficial effects of antioxidant and lipid-lowering therapies on atherogenesis in hyperlipidaemic mice. As primary models of atherosclerosis, we used apoE*3-Leiden transgenic mice and LDL receptor-deficient (heterozygous and homozygous) mice. We used these mice, because they develop atherosclerosis and their plasma cholesterol levels can be titrated to a level that is relevant to the human situation.

In humans and hyperlipidaemic rodents, plant stanol esters reduce serum cholesterol levels by inhibiting intestinal cholesterol absorption. We investigated the consequences of this effect on serum lipid levels and hepatic lipid metabolism (chapter 2), and on the development of atherosclerosis (chapter 3), in apoE*3-Leiden transgenic mice. In addition, because plant stanol esters derived from vegetable oil and wood have different ratios of campestanol to sitostanol, we compared the hypolipidaemic and anti-atherosclerotic efficacy of vegetable oil and wood-based plant stanol esters in these mice. We found that plant stanol ester treatment dose-dependently reduced serum cholesterol levels, by lowering circulating levels of VLDL, IDL and to a lesser extent LDL, whereas no effects on serum levels of HDL and triglycerides were found (chapters 2 and 3). Treatment with plant stanol esters derived from different sources led to equally potent reductions in plasma cholesterol levels and in the size and severity of atherosclerotic lesions (chapter 3). The observation that the hypocholesterolaemic effectiveness of the different stanol ester sources was similar, indicates that campestanol inhibits the intestinal cholesterol absorption equally as effectively as sitostanol. Furthermore, stanol ester treatment led to potent decreases of liver cholesteryl ester, free cholesterol and triglyceride contents, but did not lead to changes in the hepatic production rates of VLDL-triglyceride and VLDL-apolipoprotein B. However, a plant stanol ester-enriched diet significantly decreased the amounts of cholesteryl esters and free cholesterol incorporated into nascent VLDL, resulting in the hepatic output of cholesterol-poor VLDL particles. Liver mRNA levels of LDL receptor, HMG-CoA synthase, cholesterol 7α-hydroxylase

and sterol 27-hydroxylase were not changed by a plant stanol ester diet. In line with observations in humans and other rodents, plant stanol esters increased whole-body cholesterol synthesis, as reflected by an increased serum lathosterol/cholesterol ratio. Plant stanol esters also significantly decreased the cholesterol saturation index in bile, which indicates a reduced lithogenicity of the bile in these mice, a finding which is at variance with observations of humans. We conclude that in apoE*3-Leiden transgenic mice, plant stanol esters lowered serum cholesterol levels, as a result of a reduced VLDL cholesterol secretion, and diminished the cholesterol saturation of the bile. These effects are the consequence of a decreased hepatic cholesterol content. In addition, plant stanol esters, irrespective of their source, potently reduced the extent and severity of atherosclerotic lesions, by decreasing circulating levels of VLDL-, IDL- and to a lesser extent LDL cholesterol, in apoE*3-Leiden transgenic mice.

Previous *in vitro* studies and *in vivo* studies with rodents suggested that the potent hypolipidaemic agent HOE 402 is an inducer of the LDL receptor (LDLR). We investigated whether this compound is also an LDL receptor inducer in mice (chapter 4). We found that treatment of wild-type and LDL receptor-deficient mice with HOE 402, reduced VLDL, IDL and LDL cholesterol levels, whereas HDL cholesterol levels were increased. The observations that HOE 402 exhibited no effect on *in vivo* clearance of [^{125}I]-labelled LDL in wild-type mice, and clearly reduced serum cholesterol levels in LDLR 0/0 mice, indicate that the LDLR is not the main target for the compound. In wild-type mice, production of VLDL-TG and VLDL-cholesterol were potently reduced by HOE 402, whereas VLDL-apolipoprotein B secretion was unaffected, indicating that HOE 402 treatment changes the size, rather than the number of the secreted VLDL particles. The reduced VLDL production was accompanied by a decreased hepatic cholesteryl ester concentration. Additionally, HOE 402 treatment strongly reduced the aortic content of atherosclerotic lesions in LDLR +/0 and LDLR 0/0 mice, respectively. We conclude that in mice HOE 402 lowers serum cholesterol levels by inhibiting the VLDL production, and, consequently, attenuates atherosclerosis development.

We investigated in heterozygous LDL receptor-deficient mice, whether treatment with natural vitamin E had beneficial effects on the size and composition of atherosclerotic plaques, at serum cholesterol levels and lipoprotein profiles that are relevant to the human situation (chapter 5). Vitamin E treatment led to a 2-fold increase in serum vitamin E levels, but had no effect on the levels of serum cholesterol, and on the LDL/HDL ratios. The levels of

plasma 8-iso-PGF2α and of serum SAA, being markers of oxidative stress and inflammation, had both been reduced by vitamin E.

Vitamin E led to a reduction of the atherosclerotic lesion size, and led to increases in the lesion content of collagen, and smooth muscle cells in the fibrous cap and shoulder regions of the lesions. Additionally, vitamin E had no effect on media thickness, which indicates that vitamin E did not increase smooth muscle cell migration from the media towards the fibrous cap and shoulders region of the lesions. Furthermore, vitamin E led to a non-significant decrease in the macrophage content of the atherosclerotic plaques. Together, these data are strongly indicative of lesion stabilizing properties of vitamin E. We conclude that long-term vitamin E treatment reduces the progression of atherosclerosis and increases the stability of the plaque. Both the antioxidant and anti-inflammatory properties of vitamin E may have been responsible for these beneficial effects.

Furthermore, we investigated in apoE*3-Leiden transgenic mice whether the natural antioxidant vitamin E attenuates atherosclerosis at a high cholesterol level, or at a moderate cholesterol level after treatment with the cholesterol-lowering drug atorvastatin (chapter 6). Treatment with vitamin E lowered plasma cholesterol levels at a high cholesterol level by reducing the cholesterol level of the apoB-containing lipoproteins. At a moderate cholesterol level, vitamin E had no effect on the plasma cholesterol levels. Vitamin E treatment resulted in 1.6-fold increases in the level of plasma vitamin E at a high and moderate cholesterol level. Since inflammation plays an important role in the pathogenesis of atherosclerosis, we measured the circulating levels of the inflammation markers serum amyloïd A (SAA), soluble intracellular adhesion molecule-1 (sICAM-1), and von Willebrand factor (vWf). The levels of these inflammation markers had not been reduced by vitamin E, indicating that vitamin E had no anti-inflammatory effects. In addition, vitamin E did not suppress the urinary excretion of 8-iso-PGF2α, indicating that vitamin E did not suppress oxidative stress *in vivo*. Furthermore, vitamin E had no effect on the urinary nitrate excretion at a high cholesterol level. However, at a moderate cholesterol level vitamin E increased the urinary nitrate excretion, indicative of an increased whole-body NO production. Since an increased NO production due to activation of eNOS has been associated with an improved vasodilation, vitamin E treatment may also result in an improved vessel function. Vitamin E had no effects on the size, the severity, and the collagen content of the atherosclerotic lesions. In addition, at a high cholesterol level, vitamin E had no effect on the macrophage area of the atherosclerotic lesions. However, at a

moderate cholesterol level vitamin E strongly reduced the macrophage area of the atherosclerotic plaques. Thus, in apoE*3-Leiden transgenic mice vitamin E did not attenuate atherosclerosis at a high cholesterol level, whereas at a moderate cholesterol level vitamin E had a plaque stabilizing property, notably a strong reduction of the macrophage content of the atherosclerotic plaques. Vitamin E less potently attenuated atherosclerosis in apoE*3-Leiden mice than in LDLR +/0 mice (chapter 5). Vitamin E was less effective in apoE*3-Leiden mice, because in these mice vitamin E treatment led to a smaller elevation of the plasma vitamin E level, and the anti-atherosclerotic effects of aggressive cholesterol-lowering with atorvastatin may have overshadowed the anti-atherosclerotic effects of vitamin E.

Furthermore, we have indications that in apoE*3-Leiden mice the cholesterol-lowering properties of atorvastatin are mainly responsible for the anti-atherosclerotic effects of atorvastatin.

Samenvatting

Hoewel lipidenverlagende therapieën met statines de door hart en vaatziekten veroorzaakte mortaliteit verlaagd hebben, zijn in de Westerse wereld hart en vaatziekten nog altijd de belangrijkste doodsoorzaak. Mede hierdoor staan andere mogelijkheden voor de behandeling van hart en vaatziekten nog steeds in de belangstelling.

Het primaire doel van dit proefschrift was om in muizen met verhoogde plasma lipidniveau's te bestuderen op wat voor een manier voedselcomponenten en lipidenverlagende medicijnen het lipoproteïnen metabolisme en de ontwikkeling van atherosclerose beïnvloeden. We hebben hiervoor apoE*3-Leiden transgene muizen en LDL receptor deficiënte muizen (heterozygoten en homozygoten) gebruikt, omdat deze atherosclerose ontwikkelen met een plasma cholesterolniveau dat relevant is voor de humane situatie.

Plantaardige stanol esters werken in mensen en in knaagdiermodellen cholesterolverlagend, door middel van remming van de cholesterolabsorptie in de darmen. Wij hebben in apoE*3-Leiden muizen bestudeerd welke gevolgen dit effect heeft voor de circulerende lipiden niveau's en de stofwisseling van de lever (Hoofdstuk 2), en voor de ontwikkeling van atherosclerose (Hoofdstuk 3). Ook hebben we in deze muizen de lipidenverlagende en anti-atherosclerotische eigenschappen van stanol esters die gezuiverd waren uit plantaardige olie en uit hout met elkaar vergeleken, omdat deze een verschillende verhouding van campestanol tot sitostanol bevatten (Hoofdstuk 3).

We vonden dat stanol esters op een dosis-afhankelijke manier serum cholesterolverlagend werkten, door circulerende niveau's van de atherogene lipoproteïnen VLDL, IDL, en in mindere mate LDL, te verlagen. Er werden geen effecten op de niveau's van HDL en triglyceriden gevonden. Stanol esters die afkomstig waren van hout en plantaardige olie, lieten vergelijkbare verlagingen in cholesterolniveau's en in de grootte en ernst van de atherosclerotische laesies zien. De eerste bevinding geeft aan dat campestanol en sitostanol de cholesterolabsorptie in de darm even effectief remmen. Behandeling met plantaardige stanol esters leidde tot verlaagde cholesterol en triglyceriden niveau's in de lever. Dit effect leidde niet tot verminderde lever productiesnelheden van triglyceriden en apoB in VLDL, maar leidde wel tot de productie van cholesterolarme nascente VLDL deeltjes.

De mRNA niveau's van de LDL receptor, HMGCoA synthase, cholesterol 7a-hydroxylase en sterol 27-hydroxylase in de lever, waren niet veranderd door behandeling met plantaardige stanol esters. Stanol ester behandeling leidde echter wel tot een verhoogde endogene cholesterolsynthese, ter compensatie van een verlaagde cholesterolabsorptie. In tegenstelling tot observaties bij de mens, vonden we dat plantaardige stanol esters de cholesterolexcretie van de lever in gal remden, wat leidde tot een verlaagde cholesterol verzadigingsindex, een indicatie voor een verminderd risico op de vorming van cholesterol-rijke galstenen.

Dus, in apoE*3-Leiden muizen veroorzaken plantaardige stanol esters (i) een cholesterolverlaging in VLDL, IDL en LDL, die wordt veroorzaakt door de productie van cholesterolarme nascente VLDL deeltjes, en (ii) een verlaagde cholesterolverzadiging van gal. Deze effecten zijn het gevolg van een verminderde hoeveelheid cholesterol in de lever. Tevens remmen stanol esters, onafhankelijk van hun bron, de ontwikkeling van atherosclerose zeer effectief, door de niveau's van de atherogene lipoproteïnen VLDL, IDL en LDL te verlagen (Hoofdstuk 3).

In eerdere *in vitro* studies en *in vivo* knaagdier studies werd geconcludeerd dat de sterk cholesterolverlagende stof HOE 402 een LDL receptor activator is. Wij onderzochten of HOE 402 ook in muizen cholesterolverlagend werkt door activering van de LDL receptor (Hoofdstuk 4). We vonden dat HOE 402 in zowel wild-type als in LDL receptor deficiënte muizen de cholesterolgehaltes van VLDL, IDL en LDL verlaagde, terwijl HDL cholesterolwaarden verhoogd werden. De bevindingen dat HOE 402 in wild-type muizen geen effecten had op de *in vivo* klaring van humaan [125I]-LDL, en in homozygote LDL receptor deficiënte muizen duidelijk cholesterol verlagend werkte, geven aan dat HOE 402 niet primair via inductie van de LDL receptor werkt. HOE 402 verlaagde in wild-type muizen de productie van cholesterol en triglyceriden in nascent VLDL, terwijl de VLDL-apoB productie onveranderd bleef. Dit geeft aan dat behandeling resulteerde in de productie van lipid arme nascente VLDL deeltjes. Deze verlaagde VLDL productie ging gepaard met een verminderde hoeveelheid veresterd cholesterol in de lever. Tevens vonden we dat HOE 402 in LDL receptor deficiënte muizen de ontwikkeling van atherosclerose in de aorta tegenging. Dus, in de muis werkt HOE 402 cholesterol verlagend door remming van de VLDL productie, wat leidt tot een geremde ontwikkeling van atherosclerose.

We hebben in heterozygote LDL receptor deficiënte muizen, met een serum cholesterolniveau en lipoproteïnenprofiel die relevant zijn voor de

humane situatie, onderzocht of behandeling met vitamine E de ontwikkeling van atherosclerotische laesies beïnvloedt (Hoofdstuk 5). Bevindingen uit de kliniek geven aan dat niet zozeer de grootte, maar vooral de samenstelling van de laesies en daaraan gekoppeld de kans op ruptuur van deze laesies bepalend zijn voor het risico op klinische gebeurtenissen die zijn gerelateerd aan hart- en vaatziekten. Behandeling met vitamine E leidde tot een tweevoudige verhoging van plasma vitamine E niveau's, maar had geen effect op serum cholesterolniveau's en op de verhouding LDL/HDL. Behandeling met vitamine E verlaagde de niveau's van plasma 8-iso-PGF2α en serum SAA, respectievelijk markers van oxidatieve stress en ontsteking.

De atherosclerotische laesies van de vitamine E behandelde muizen waren kleiner, hadden een grotere hoeveelheid collageen, en een grotere hoeveelheid gladde spiercellen in de fibreuze laag en schouder regio's van de laesies. Vitamine E had ook geen effect op de dikte van de media, wat aangeeft dat vitamine E behandeling de migratie van gladde spiercellen vanuit de media naar de fibreuze kap niet heeft gestimuleerd. Tevens was er een trend dat vitamine E de hoeveelheid macrofagen in de laesie verkleinde. In conclusie, vermindert vitamine E de ontwikkeling van atherosclerose, onafhankelijk van plasma lipid niveau's, en is de veranderde samenstelling van de atherosclerotische laesies een indicatie voor laesie stabiliserende eigenschappen van vitamine E.

Ook hebben we in apoE*3-Leiden transgene muizen bestudeerd of vitamine E de ontwikkeling van atherosclerose kan tegengaan bij een sterk verhoogd of bij een matig verhoogd cholesterolniveau na behandeling met een cholesterol verlagend medicijn atorvastatine (Hoofdstuk 6). Bij een sterk verhoogd cholesterolniveau had vitamine E met name in de apoB-bevattende lipoproteïnen een cholesterolverlagende werking. Deze cholesterolverlaging was niet aanwezig bij een matig verhoogd cholesterolniveau. Onafhankelijk van het cholesterolniveau resulteerde vitamine E behandeling in 1.6-maal verhoogde serum vitamine E niveau's. De niveau's van de onstekingsindicatoren SAA, sICAM-1 en vWf werden in de circulatie gemeten omdat ontstekingsprocessen een belangrijke rol in de ontwikkeling van atherosclerose spelen. Vitamine E behandeling had geen effect op de niveau's van SAA en sICAM-1 in serum en vWf in plasma, wat aangeeft dat vitamin E geen ontstekingsremmende werking heeft. Vitamine E had ook geen effect op de excretie van 8-iso-PGF2α in urine, wat aangeeft dat vitamine E oxidative stress *in vivo* niet tegengaat. Vitamine E behandeling leidde bij een matig verhoogd cholesterolniveau wel tot een sterk verhoogde excretie van nitraat in

urine, terwijl vitamine E bij een sterk verhoogd cholesterolniveau geen effect op de nitraatexcretie had. Deze bevinding zou erop kunnen duiden dat vitamine E bij een matig cholesterolniveau de vaatwandfunctie verbetert, omdat een verhoogde NO productie door activatie van eNOS tot een verbeterde vaatverwijding leidt. Vitamine E had bij beide cholesterolniveau's geen effect op de laesie grootte en ernst, en op de hoeveelheid collageen in de atherosclerotische laesies. Bij een sterk verhoogd cholesterolniveau had vitamine E ook geen invloed op de hoeveelheid macrofagen in de atherosclerotische laesies. Vitamine E leidde bij een matig verhoogd cholesterolniveau echter tot een sterke vermindering van de hoeveelheid macrofagen in de laesies. Ter conclusie, vermindert vitamine E in apoE*3-Leiden muizen bij een sterk verhoogd cholesterolniveau de ontwikkeling van atherosclerose niet. Vitamine E heeft bij een matig verhoogd cholesterolniveau echter een leasie stabiliserende eigenschap, namelijk het sterk verminderen van de hoeveelheid macrofagen in de laesie. Deze effecten zijn minder groot dan de anti-atherosclerotische effecten van vitamine E bij een vergelijkbaar cholesterolniveau in LDLR +/0 muizen (hoofdstuk 5). Dit verschil in effectiviteit zou veroorzaakt kunnen zijn doordat in apoE*3-Leiden muizen vitamine E behandeling tot een kleinere plasma vitamine E verhoging leidt en agressieve cholesterolverlaging met atorvastatine dat leidde tot een sterk verminderde ontwikkeling van atherosclerose, verdere anti-atherosclerotische effecten van vitamine E maskeerde.

Tevens hebben we aanwijzingen dat de cholesterolverlagende werking van atorvastatine verantwoordelijk is voor de anti-atherosclerotische en laesie-stabiliserende eigenschappen van atorvastatine.

List of publications

Full papers:

published

Volger OL, van der Boom H, de Wit EC, van Duyvenvoorde W, Hornstra G, Plat J, Havekes L, Mensink RP, Princen HM. Dietary plant stanol esters reduce VLDL-cholesterol secretion and bile saturation in apoE*3-Leiden transgenic mice. *Arterioscler Thromb Vasc Biol.* 2001;21:1046-1052.

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in preperation

Oscar L. Volger, Richard Draijer , Vivian E.H. Dahlmans, Elly C.M. de Wit, Louis M. Havekes, Hans M.G. Princen. The first two authors have contributed equally to this work HOE 402 lowers serum cholesterol levels by reducing VLDL-lipid production, and not by induction of the LDL receptor, and reduces atherosclerosis in wild-type and LDL receptor-deficient mice.

Oscar L. Volger, Wim van Duyvenvoorde, Erik Offerman, Annet J.C. Roodenburg, Rianne Leenen, Louis M. Havekes, Hans M.G. Princen. Long-term dietary vitamin E reduces atherosclerotic lesion size and increases plaque stability in heterozygous LDL receptor-deficient mice.

Oscar L. Volger, Yvonne van den Berg, Elly de Wit, Jenny van Loon, Jürgen C. Frölich, Louis M. Havekes, Hans M.G. Princen. Vitamin E reduces the macrophage rich area of atherosclerotic lesions at moderate cholesterol levels in apoE*3-Leiden transgenic mice.

Abstracts:

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Abbreviations

ABCA1	ATP-binding cassette transporter 1
ACAT	acyl-coenzyme A:cholesterol-acyltransferase
ANOVA	analysis of variance
Apo	apolipoprotein
CCR-2	CC chemokine receptor-2
ELISA	enzyme-linked immunosorbent assay
eNOS	extracellular NO synthase
FD	familial dysbetalipoproteinaemia
FH	familial hypercholesterolaemia
GC	gass chromatography
GM-CSF	granulocyte macrophage colony stimulating factor
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
iNOS	intracellular NO synthase
LDL	low density lipoprotein
LDLR	LDL receptor
LPL	lipoprotein lipase
LRP	LDL receptor-related protein
mmLDL	minimally modified LDL
MMP	matrix metalloproteinase
MS	mass spectroscopy
NO	nitric oxide
PBS	phosphate buffered saline
oxLDL	oxidized LDL
SAA	serum amyloid A
sICAM-1	soluble intracellular adhesion molecule-1
SR	scavenger receptor
SREBP	sterol regulatory element binding protein
TNF-α	tumor necrosis factor-α
VLDL	very low density lipoprotein
vWf	von Willebrand factor

Curriculum Vitae

Oscar Leonard Volger werd geboren op 13 april 1974 in Wormerveer. In 1992 behaalde hij zijn VWO diploma aan het Saenredam College te Zaandijk. In september van datzelfde jaar begon hij aan de studie Medische Biologie aan de Universiteit van Amsterdam. Het propedeutisch examen werd in augustus van 1993 behaald. Het doctoraal examen Medische Biologie werd afgelegd in mei 1997. In het kader van het doctoraal examen werden twee onderzoeksstages uitgevoerd. De eerste stage werd verricht bij het Instituut voor Antropogenetica aan de Universiteit van Amsterdam (Prof. dr. A. Westerveld), en de tweede stage werd verricht bij de vakgroep Toxicologie aan de Universiteit Leiden (Dr. M. Blom en Dr. J.F. Nagelkerke). Van juni 1997 tot juni 2001 was hij aangesteld als assistent in opleiding aan het Leids Universitair Medisch Centrum op de afdeling Inwendige Geneeskunde op een door de Nederlandse Hartstichting gesubsidieerd project (# 95.057). Tijdens deze periode was hij gedetacheerd bij TNO Preventie en Gezondheid en werkzaam in het Gaubius Laboratorium te Leiden (Prof. dr. ir. L.M. Havekes en Dr. H.M.G. Princen). De resultaten van dit promotie onderzoek staan beschreven in dit proefschrift.