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Interventions in lipid metabolism and atherogenesis as studied in transgenic mouse models

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Interventions in lipid metabolism and atherogenesis as studied in transgenic mouse models

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Stellingen behorende bij het proefschrift
"Interventions in lipid metabolism and atherogenesis as studied in transgenic mouse models"

1. Omdat statines naast een cholesterol-verlagende werking tevens pleiotrope effecten direct op de vaatwand laten zien, is het moeilijk om met behulp van additionele therapieën een extra anti-atherosclerotische werking te bewerkstelligen. *(dit proefschrift;Takemoto and Liao, 2001, ATVB; 21(11):1712-1719)*

2. Subtiele verschuiving van het evenwicht tussen cholesterolverestering en cholesterylester hydrolyse in de macrofaag door middel van ACAT-remming kan atherogenese remmen, terwijl complete afwezigheid van ACAT-activiteit atherosclerose juist verergert. *(dit proefschrift; Accad et al., 2000, J,Clin.Invest,; 105:711-719; Yagyu et al., 2000, J.Biol.Chem.; 275:21324-21330; Fazio et al., 2001, J.Clin.Invest.; 107:163-171)*

3. Cuff-geïnduceerde intima-verdikking in ApoE*3-Leiden muizen op een hoog cholesterol dieet is een goed model voor het bestuderen van vroege processen betrokken bij atherogenese. *(dit proefschrift)*

4. De verwantschap tussen macrofaag schuimcelvorming en adipogenese blijkt uit het feit dat steeds meer genen betrokken blijken te zijn bij beide processen. *(dit proefschrift; O'Rourke et al., 2001, Diabetes; 50:955-961; Makowski et al., 2001, Nat. Med.;7(6):699-705)*

5. De LXR's hebben de touwtjes volledig in handen als het om reversed cholesterol transport gaat. *(Chawla et al., 2001, Science; 294:1866-1870; Lafitte et al., 2001, PNAS; 98(2):507-512)*

6. Atherosclerose wordt vaak veroorzaakt door een probleem in de lever. *(dit proefschrift, R. Davis, 2001, Arterioscler. Thromb. Vasc. Biol.; 21:887)*

7. Omdat atherosclerose een inflammatoire ziekte is, geeft het screenen van de ontstekingsparameter C-reactive protein in plasma naast de traditionele lipidenmetingen een betere indicatie van het risico op cardiovasculaire aandoeningen. *(Blake and Ridker, 2001, Circ. Res.; 89(9):763-771)*

8. In vroegere tijden van varierend voedselaanbod was het ontwikkelen van leptine-resistentie een overlevingsvoordeel vanwege een extra drang tot opslag van energie. Tegenwoordig zorgt deze "survival of the fittest" voor een enorme toename van het voorkomen van obesitas en diabetes en het daaraan gerelateerde verhoogde risico op hart- en vaatziekten. *(Zimmet, 1999, Diabetologia; 42:499-518; Zimmet et al., 2001, Nature; 414:782-787)*

9. Terwijl veel aandoeningen een multi-factoriële oorzaak hebben, wordt bij het wetenschappelijk onderzoek naar mogelijke oplossingen nog teveel in hokjes gedacht.

10. Iedere werkgever zou een carpool-voordeel-regeling moet hebben voor zijn werknemers; mensen gaan namelijk pas aan het milieu denken, als ze zelf in hun portemonnee voelen hoe goed ze bezig zijn.

11. Luidruchtig mobiel bellen zonder daadwerkelijk nuttige informatie over te dragen is één van de meest voorkomende vormen van zinloos geweld.

12. Als het bij de NS loopt als een trein, weet je dat je weer niet op tijd zult zijn.

Leiden, 28 februari 2002 Dianne Delsing

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(1947-1992)

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Chapter 1

General Introduction

Cardiovascular disease has headed the list of primary causes of death in Western society for many decades. The development of these cardiovascular diseases is mainly caused by atherosclerosis, which is characterised by an accumulation of lipids, fibrotic tissue and cellular material in the vessel wall. Apart from smoking, diabetes, lack of physical activity, hypertension, age and genetic predilection, elevated plasma lipid levels are the main risk factor for the development of atherosclerosis. Therefore, lipid metabolism has been one of the main focuses of scientific research aiming at the prevention of cardiovascular disease.

Lipoprotein metabolism

The most important lipids in the circulation are cholesterol and triglycerides. Cholesterol is a constituent of cellular membranes and is essential for synthesis of steroid hormones and bile acids, whereas triglycerides are delivered primarily to adipose tissue and muscle where the fatty acids are stored or oxidized for energy, respectively. For the distribution of these lipids through the body, they have to be transported via aqueous compartments, such as lymph and blood. Therefore, they need to be packaged into water-soluble lipoproteins, which consist of a lipid-rich core containing triglycerides and esterified cholesterol, and a surface layer of free cholesterol, phospholipids and apolipoproteins.

Lipoproteins can be divided into five main subclasses: chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL), depending on their apolipoprotein composition and their distinct densities (table 1)[1]. The metabolism of these lipoproteins consists of three pathways: the exogenous pathway, involving the uptake and processing of dietary lipids; the endogenous pathway, concerning hepatic synthesis and secretion of lipoproteins, and reverse cholesterol transport, which involves transport of cholesterol from peripheral tissues back to the liver[2].

Exogenous pathway

Intestinal cells take up dietary cholesterol and triglycerides and package them into chylomicrons, large triglyceride-rich lipoproteins containing the apolipoproteins ApoB48, ApoA1, ApoA2 and ApoA4. Epithelial cells secrete chylomicrons into the lymph and from there they enter the bloodstream upon which they also acquire

ApoE, ApoC1, ApoC2 and ApoC3. Since chylomicrons are too large to cross the endothelial barrier, they must be metabolised in the bloodstream. Therefore, via the VLDL-receptor they bind to the enzyme lipoprotein lipase (LPL), which is attached to heparan sulphate proteoglycans located on vascular endothelial cells and which hydrolyses the triglyceride core of the chylomicron[3]. The resulting free fatty acids are used as an energy source by muscles or stored as triglycerides in adipocytes. As the triglyceride core is depleted and the particle now called chylomicron remnant is enriched in cholesterol, the chylomicron remnant is rapidly taken up by the liver via ApoE-mediated binding to specific lipoprotein receptors, i.e. the LDL-receptor and LDL-receptor related protein (LRP).

Table 1. Physical properties, lipid and apolipoprotein composition of human plasma lipoproteins.

	Chylomicrons	**VLDL**	**IDL**	**LDL**	**HDL**
Source	Intestine	Liver	VLDL	VLDL	Liver + intestine
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-ß	slow pre-ß	ß	α
Composition**					
Triglycerides	88	56	29	13	15
Phospholipids	8	20	26	28	45
Cholesteryl esters	3	15	34	48	30
Free cholesterol	1	8	9	10	10
Protein	1-2	6-10	11	21	45-55
Apolipoproteins	A1, A2, A4	-	-	-	A1, A2, A4
	B48	B100	B100	B100	-
	C1, C2, C3	C1, C2, C3	C1, C2, C3	-	C1, C2, C3
	E	E	E	-	E

* According to electrophoretic mobility of plasma α- and ß-globulins on agarose gel electrophoresis
** Expressed as a percentage of total weight

Endogenous pathway

Hepatically synthesised cholesterol and triglycerides together with cholesterol and triglycerides derived from incoming chylomicron remnants, IDL, LDL and HDL are packaged into VLDL particles containing ApoB100. Upon entering the bloodstream

they also acquire ApoE and the ApoCs. In the circulation, hydrolysis of VLDL triglycerides by LPL results in the formation of IDL, which can be further hydrolysed or taken up by the liver. LPL and hepatic lipase (HL) are responsible for this further hydrolysis of IDL into LDL. Upon further lipolysis LDL is depleted of most of its apolipoproteins. ApoB100 remains associated with the particle and mediates the binding of LDL to its receptor on different tissues, causing the transport of cholesterol to tissues.

Reverse cholesterol pathway

Reverse cholesterol transport is a process resulting in the net movement of cholesterol from peripheral tissues back to the liver. Cellular cholesterol efflux is mediated by HDL, which serves as a cholesterol acceptor in plasma. Nascent HDL is synthesised by the liver and the intestine and formed from chylomicron remnants and IDL. It removes excess cholesterol from cells after interaction with the ATP-binding cassette transporter-1 (ABCa1)[4]. The enzyme lecithin:cholesterol acyltransferase (LCAT) then esterifies the free cholesterol into cholesteryl esters that accumulate in the core of the HDL particle[5]. Subsequently, HDL cholesterol can follow different routes: 1) selective uptake of HDL cholesteryl ester by the liver and other tissues by scavenger receptor BI[6] and uptake of free cholesterol via ABC-transporters on the liver, 2) HDL particle uptake via an ApoE-mediated receptor uptake in the liver, and 3) transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins as a result of the activity of cholesteryl ester transfer protein (CETP). This latter process allows cholesterol to enter the liver via the LDL-receptor pathway. Once cholesterol enters the liver it can either be re-used for lipoprotein assembly, used for bile acid synthesis or secreted directly into the bile.

Elevated plasma levels of atherogenic lipoproteins, mainly LDL and remnant lipoproteins, predispose to the development of atherosclerosis, whereas a high level of HDL in plasma protects against accelerated atherogenesis[7-9].

Figure 1. Schematic representation of lipid metabolism. Kindly provided by Dr. M.P.J. de Winther.

Pathogenesis of atherosclerosis

Over the years, atherosclerosis has been called a degenerative process coming naturally with age, a proliferative process and an accumulation of lipid in the artery wall slowly occluding the vessel. The most recent "response-to-injury" hypothesis suggests that atherosclerotic lesions develop as a form of protective, inflammatory-fibroproliferative response to various forms of injury to the arterial wall[10-14]. This

response may become excessive, which can ultimately lead to acute clinical events such as myocardial infarction and cerebral stroke.

The healthy vessel wall

Before the onset of atherosclerosis, the intact vessel wall consists of three layers: the tunica intima (intima), the tunica media (media) and the tunica adventitia (adventitia). The intima comprises a single layer of endothelial cells at the luminal side of the vessel, which is separated from the media by an elastic membrane called the internal elastic lamina. The media is made up of smooth muscle cells embedded in an interstitial matrix, mainly composed of elastin, collagen, fibronectin and thrombospondin. The external elastic lamina separates the media from the adventitia, which is composed of collagen, elastin fibres and fibroblasts.

Initiating events: LDL modification

At specific sites in the arteries, such as branches, bifurcations and curvatures, alterations of blood flow induce decreased shear stress, making these sites prone to atherogenesis[13].

Accumulation of atherogenic lipoproteins in the arterial wall as a consequence of elevated plasma lipoprotein concentrations and the condition of the endothelial cell layer, is generally considered to be the first step in the development of atherosclerosis. Reactions with e.g. myeloperoxidase[15], 15-lipoxygenase[16], superoxide anions[17], hydroxyl radicals or peroxynitrite[18] can oxidatively modify the lipid and apoB components of LDL trapped in the subendothelial space. The presence of oxidised lipids in the artery can induce endothelial activation, resulting in an inflammatory response which is suggested to be the start of the development of an atherosclerotic lesion. The fact that intervention with antioxidants, such as probucol and vitamin E, has been shown to reduce the progression of atherosclerosis in several experimental animal studies[19], implies that LDL oxidation is an important process in the onset of atherogenesis.

Monocyte adherence and recruitment

The recruitment of monocytes and T-lymphocytes to lesion-prone sites of the artery is regulated by cell adhesion molecules expressed on the surface of endothelial cells in response to inflammatory stimuli, such as the above-mentioned presence of oxidised LDL (oxLDL) in the artery wall[20]. Chemotaxis of these leukocytes is

mediated by transforming growth factor-β (TGFß), colony-stimulating factors (CSFs), monocyte chemoattractant protein-1 (MCP-1) and oxLDL itself. Subsequently, the interaction of endothelial P- and E-selectin with leukocyte carbohydrate ligands allows leukocytes in the blood to tether and roll along the vessel wall surface. Firm adhesion of leukocytes to the endothelium is induced by the binding of endothelially expressed intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) to the leukocyte integrin very late-acting antigen-4 (VLA-4). Final transmigration of monocytes through the endothelial monolayer is mediated by ß2-integrin and platelet-endothelial cell adhesion molecule-1 (PECAM-1). The importance of these different adhesion molecules in the development of atherosclerosis is illustrated by the fact that several are upregulated in atherosclerotic lesions or atherosclerosis-prone sites on the endothelium[21-23] and that mouse models for atherosclerosis which are deficient for selectins[24] or integrin ligands[25] show diminished atherogenesis.

Foam cell formation

Once monocytes have entered the subendothelial space, they differentiate into macrophages under the influence of various growth factors and cytokines (e.g. interleukin-1 (IL-1), tumour necrosis factor α (TNFα), granulocyte monocyte colony-stimulating factor (GM-CSF), interferon γ) secreted by smooth muscle cells, endothelial cells and other leukocytes. Mediated by scavenger receptors SRA and CD36, these macrophages accumulate modified forms of LDL, a process which is not subject to negative feedback control. Modified LDL-derived cholesterol taken up by the macrophage contains both free cholesterol and cholesteryl esters, which become hydrolysed in lysosomes. Since accumulation of free cholesterol in the cell becomes toxic at a certain threshold[26], the enzyme ACAT (acyl-CoA:cholesterol acyltransferase) esterifies cholesterol so that it can be stored in lipid droplets that characterise foam cells. Cholesteryl esters in turn can be hydrolysed, generating free cholesterol for incorporation into cell membranes and transport out of the cell. Incorporation of excess cholesterol into intracellular membranes inhibits the proteolytic activation of the sterol regulatory element-binding protein (SREBP) transcription factors required for the activation of cholesterol biosynthesis and LDL-receptor expression[27]. The most important way for a macrophage to dispose of excess cholesterol is through efflux via membrane transporters with HDL serving as

the major extracellular cholesterol acceptor, also called the "reverse cholesterol transport" pathway. Recently it was found that the ATP-binding cassette transporter-1 (ABCa1) mediates the transport of cholesterol and phospholipids from cells to extracellular HDL. Patients suffering from Tangier disease, characterised by extremely low levels of HDL and cholesterol accumulations in macrophages, have a null mutation or defect in the ABCa1 gene[28]. When this ABCa1 gene was disrupted in mice, a dramatic increase in lipid-laden macrophages was indeed seen[29,30], indicating that ABCa1 expression may protect the cell from acute lipid overload. Another modulator of cellular cholesterol metabolism is ApoE, which facilitates cholesterol efflux[31,32]. Macrophages isolated from ApoE-deficient mice have been shown to exhibit a diminished capacity to efflux cholesterol to HDL[33,34].

So, although the recruitment of monocytes to the arterial wall and their subsequent differentiation into macrophages initially serves as protection by removing cytotoxic and proinflammatory oxLDL, progressive accumulation of macrophages and their uptake of oxLDL ultimately leads to the development of atherosclerotic lesions.

Figure 2. Initiating events in the development of a fatty streak lesion. LDL is subject to oxidative modification, from minimally modified LDL (mmLDL) to extensively oxidised LDL (oxLDL). Monocytes attach to the endothelium expressing cell adhesion molecules, and then migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL leads to foam cell formation. Reprinted from Cell[14] by kind permission from Prof. C.K. Glass and Cell Press.

Lesion progression

Atherosclerotic lesions consisting mainly of foam cells in the intimal region of the vessel wall, as described above, are also known as fatty streaks. Progression of fatty streaks into fibroproliferative lesions is characterised by the migration and proliferation of smooth muscle cells. Activated macrophages and endothelial cells secrete different chemoattractants, causing migration of smooth muscle cells from the media into the intima. Growth factors and cytokines, such as TGFß, IL-1 and TNFα, produced by the endothelium, macrophages and smooth muscle cells themselves induce proliferation of smooth muscle cells. These cells subsequently start synthesising and secreting connective tissue matrix. This connective tissue forms a protective layer called the fibrous cap, overlying the lipid-filled foam cells of the plaque. Smooth muscle cells in the atherosclerotic lesion are also able to accumulate cholesterol, thereby contributing to the formation of foam cells[13]. Further progression of atherosclerotic lesions involves the formation of a lipid core, as a consequence of foam cell accumulation, macrophage death by necrosis or apoptosis, resulting in extracellular lipid accumulation in the form of free cholesterol crystals[35].

Figure 3. Lesion progression. Interactions between macrophage foam cells, Th1 and Th2 cells establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert pro- and antiatherogenic effects. Smooth muscle cells migrate from the media to the arterial wall, proliferate and secrete extracellular matrix proteins, which form a fibrous cap. Reprinted from Cell[14] by kind permission from Prof. C.K. Glass and Cell Press.

Other important features of further lesion progression are: continued accumulation of macrophages and T-lymphocytes; loss of smooth muscle cells in the media of the vessel wall; cholesterol cleft formation; and calcification.

Plaque rupture

Whereas the slow occlusion of an artery by an atherosclerotic plaque, leading to angina pectoris, can be physiologically circumvented by expansive remodelling, disruption of an atherosclerotic plaque, causing thrombus formation, is the major cause of acute clinical events, such as myocardial infarction and stroke. Ruptured plaques usually have thin, collagen-poor fibrous caps with few smooth muscle cells overlying a large necrotic lipid pool abundant in macrophages and T-lymphocytes[36,37].

Figure 4. Plaque rupture and thrombosis. Necrosis of macrophages and smooth muscle cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol. Macrophage secretion of MMPs contribute to destabilising the plaque. Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets and the formation of a thrombus. Reprinted from Cell[14] by kind permission from Prof. C.K. Glass and Cell Press.

Generally, plaque rupture occurs at the shoulder regions of the lesions, where the concentration of lipid-filled macrophages is high and the fibrous cap is weakest. Matrix metalloproteinases (MMPs) secreted by macrophages, such as collagenases, elastases and stromelysins, can degrade the extracellular matrix synthesised by

smooth muscle cells in the fibrous cap, whereas interferon γ synthesised by T-lymphocytes inhibits the production of collagen by smooth muscle cells[38], resulting in instability of the lesion. The location at which MMP-expression is highest in the plaque is also the site where mechanical stresses are high. This implies that plaque rupture is the interaction between mechanical forces on the one hand and matrix degradation in the shoulder regions of a plaque on the other[39].

After rupture of the fibrous cap, the underlying necrotic core is exposed to the blood flow. Massive tissue factor expression by cells in the necrotic core results in the activation of the coagulation cascade and accumulation of platelets at the site of the rupture, leading to the formation of a thrombus possibly occluding the vessel entirely.

Classification of atherosclerotic lesions

The Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association provided a classification of human atherosclerotic lesions based on their histological composition and structure. A type I lesion is an early lesion consisting of the first microscopically and chemically detectable lipid depositions in the intima. Small isolated groups of lipid-filled macrophages form under the endothelial cell-layer, but histological changes are minimal. A type II lesion is a fatty streak, which consists primarily of macrophage foam cells in layers rather than being present only as isolated groups of cells. The type III lesion is also known as the intermediate lesion. It has microscopically visible extracellular lipid pools lying just below the layers of macrophages and macrophage foam cells, thereby replacing intercellular matrix material and driving smooth muscle cells apart. Lesion types I-III do not cause an occlusion of the artery and are therefore clinically silent. They are the precursors of symptom-producing atherosclerosis[40].

Progression of the aforementioned lesions results in the development of atheroma, also known as a type IV lesion, containing a necrotic lipid core with a dense accumulation of extracellular lipid. These lipid cores thicken the artery wall, though they often fail to narrow the vascular lumen. This indicates that thickening is often associated with an increase in size of the external boundary of the artery, also called expansive arterial remodelling[41,42].

In a type V lesion new fibrous connective tissue has formed, consisting of smooth muscle cells and extracellular matrix. The so-called fibrous cap overlies the lipid core

of the atherosclerotic plaque. The type V lesions can be further subdivided into Va: fibro-atheroma, Vb: calcification and Vc: fibrous without lipid core.
Morbidity and mortality from atherosclerosis is largely due to type IV and V lesions in which disruptions of the lesion surface, and subsequently haematoma or haemorrhage and thrombosis have developed. When one of these additional features occurs in an atherosclerotic lesion, it is called a complicated lesion (type VI)[43].

Treatment of atherosclerosis

Vascular interventions for the treatment of atherosclerosis include percutaneous transluminal coronary angioplasty (PTCA), i.e. balloon dilation of an artery possibly together with the placement of a stent, and coronary artery by-pass grafting (CABG) , where a piece of healthy vessel is used to replace the occluded artery. The initial success of these procedures is very high, but a major problem following these interventions is the occurence of restenosis. Probably as a result of the damage done to the endothelial layer at the site of intervention, smooth muscle cells start to proliferate, inflammation is extended and new thrombus formation is induced. In 30 to 50% of all patients treated by PTCA or CABG restenosis occurs[44].
Since plasma cholesterol levels are a major risk factor for the development of coronary heart disease, prevention of the development of atherosclerosis focusses largely on the use of lipid-lowering drugs. When atherosclerotic lesions are already present, lipid-lowering therapy can be used to inhibit further progression of plaque development or even cause regression of the lesions. Also anti-hypertensive agents[45], such as angiotensin-converting enzyme inhibitors[46], beta-blockers[46] or calcium antagonists[47], and anti-coagulatory drugs[48,49] are important treatments in the reduction of the risk of cardiovascular disease or recurrent events. In this thesis we have mainly focussed on HMG-CoA-reductase inhibitors as lipid-lowering drugs; ACAT-inhibitors as a possible new approach to lipid-lowering and atheroprotection; and calcium antagonists as anti-hypertensive drugs with anti-atherosclerotic properties. Therefore, these drugs are discussed in more detail.

HMG-CoA-reductase inhibitors

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyses the rate-limiting step in cholesterol biosynthesis and has consequently been an important target for the development of cholesterol-lowering agents. Inhibitors of HMG-CoA reductase, or statins, were first introduced into clinical practice in the 1980s. Pravastatin and lovastatin are produced from fungal metabolites, simvastatin is semi-synthetic and fluvastatin, atorvastatin and rosuvastatin are synthetic statins[50]. Pravastatin and rosuvastatin are hydrophyllic agents; the other statins are highly lipophylic. The potent hypolipidemic effects of statins have been established in a number of placebo-controlled clinical trials[51-53]. The mechanism of action is a competitive inhibition of the enzyme HMG-CoA reductase, which reduces cholesterol biosynthesis. This in turn leads to an upregulation of LDL-receptors in the liver mediated by the activation of SREBPs and an enhanced clearance of LDL from the circulation[54]. A number of intervention trials with statins confirmed that by reducing plasma cholesterol levels, the risk of mortality from coronary heart disease and the incidence of non-fatal myocardial infarction were also reduced[55-62].

There is increasing evidence that statins possess additional anti-atherogenic properties beyond their capacity to lower LDL-cholesterol[63]. Statin therapy has been shown to improve the endothelial dysfunction associated with hyperlipidaemia[64]. Several *in vitro* studies revealed that statins can inhibit lipid accumulation in macrophages and proliferation, as well as migration of smooth muscle cells[65,66]. Recent investigations showed that statin treatment reduces recruitment and activation of immune-competent cells, such as macrophages, suggesting that statins may have immunomodulatory effects[67]. Since plasma levels of inflammatory markers have been shown to be predictive of the risk of myocardial infarction [68-71] it is interesting to see that statin therapy reduces CRP levels in patients[72,73]. In addition, in an animal model for atherosclerosis, statin treatment was able to reduce both inflammation and atherogenesis independently of its cholesterol-lowering activity[74].

Other lipid-lowering drugs

Fibrates are a widely used class of lipid-lowering drugs, which decrease plasma triglycerides and also show a moderate decrease in LDL-cholesterol and an increase in HDL-cholesterol[75]. They activate specific transcription factors, termed peroxisome

proliferator-activated receptors (PPARs). Activation of these nuclear hormone receptors causes stimulation of the catabolism of triglyceride-rich lipoproteins via LPL and a reduction in hepatic VLDL-production. Fibrate action on HDL metabolism is mediated by transcriptional induction of ApoAI synthesis via PPARα[76]. Several clinical trials have shown that fibrate treatment reduces the incidence of cardiovascular events[77,78]. Not only are lipid-lowering properties responsible for this effect, but inhibition of coagulation, enhancement of fibrinolysis and inhibition of inflammatory mediators have also been shown to contribute[79].

Bile acid sequestrants interrupt the enterohepatic circulation of bile acids by non-specific binding to the bile acid pool in the intestine, thereby increasing the faecal excretion of bile acids. This decrease in bile acid reabsorption causes increased hepatic production of bile acids. Subsequently, the intrahepatic cholesterol pool is decreased, resulting in an upregulation of the LDL-receptor, which in turn increases plasma clearance of LDL, IDL and VLDL[80]. The clinical benefit of lowering cholesterol by bile acid sequestrant therapy has been demonstrated[81].

Nicotinic acid is a vitamin B that has been shown, in high doses, to lower plasma total cholesterol, LDL-cholesterol and VLDL-triglycerides, while raising HDL-cholesterol. Its exact mechanism of action is not known, but it appears to lower hepatic VLDL-production[82]. Nicotinic acid by itself has been associated with a reduced risk of cardiovascular morbidity in clinical trials[83], but it is mainly used in combination with other cholesterol-lowering agents to maximise lipid-lowering activity[84-86].

ACAT-inhibitors

Another possible approach to reducing plasma cholesterol levels is to inhibit the enzyme acyl-CoA: cholesterol acyltransferase (ACAT), which is responsible for the esterification of cholesterol in the cell. ACAT plays a role in cholesterol absorption through the formation of cholesteryl esters in the enterocytes. Cholesterol esterification has also been shown to be important for the assembly and secretion of ApoB-containing lipoproteins in the intestine and the liver. The enzyme has also been implicated in esterification and thereby storage of cholesteryl esters in monocyte-derived macrophages in the arterial wall[87,88]. Thus, inhibition of ACAT can have lipid-

lowering effects by reducing chylomicron and VLDL secretion. Also, and perhaps more importantly, it can have direct anti-atherogenic potential by inhibiting cholesterol accumulation in macrophages, which is one of the earliest steps in atherogenesis.
Several animal studies with inhibitors of the enzyme ACAT have shown the cholesterol-lowering effects of these drugs[89-96]. However, to distinguish direct anti-atherosclerotic effects on the vessel wall by ACAT-inhibitors from their ability to lower plasma cholesterol levels remains difficult. Reductions in atherosclerotic lesion area have been found after treatment with ACAT-inhibitors, but in most of these studies substantial lipid-lowering was involved[91,94,97-99].
ACAT exists in two isoforms: acat1 and acat2. In murine models it was found that the acat1 gene is predominantly expressed in macrophages, whereas acat2-activity is found in the liver and intestine[100]. In non-human primates acat1 is expressed quite ubiquitously, while acat2 is primarily found in the liver and intestine[101]. Most known ACAT-inhibitors are effective against both acat1 and acat2[102].
Recent studies have shown that acat1 deficiency both in ApoE-deficient as well as LDL-receptor-deficient mice leads to severe xanthomatosis and aggravated atherogenesis[103-105]. It is therefore conceivable that total deficiency of ACAT-activity in macrophages leads to an excessive accumulation of free cholesterol in the cell, since it cannot be esterified. Ultimately, the intracellular free cholesterol concentration becomes toxic to the cell, leading to cell death[26]. As a consequence, free cholesterol clefts are formed extracellularly, which induce inflammatory responses. In the arterial wall these processes can worsen atherogenesis, causing larger and more complex lesions. Subtle ACAT-inhibition by specific drugs, however, could shift the balance of cholesterol esterification and cholesteryl ester hydrolysis slightly, resulting in a net favourable effect on lipid metabolism and atherosclerosis development.
Acat2 deficiency in mice leads to resistance to diet-induced hypercholesterolaemia and gallstone formation, mainly caused by reduced cholesterol esterification in the intestine[106]. These results suggest that it may be more beneficial to develop specific inhibitors of acat2, thus limiting the absorption of dietary cholesterol in the intestine.

Calcium antagonists

Calcium channel blockers, also called calcium antagonists, are essentially used as anti-hypertensive drugs. Their main action is to inhibit calcium transport into the cell

via voltage-gated transmembrane calcium channels, thus decreasing intracellular calcium concentration and inducing smooth muscle relaxation. However, they also have potential anti-atherogenic potency. A number of processes involved in atherogenesis has been shown to be influenced by calcium antagonists, mostly by *in vitro* studies. Calcium antagonists can inhibit vascular smooth muscle cell migration and proliferation, reduce calcium influx and deposition in the arterial wall, inhibit matrix synthesis, enhance removal and degradation of cholesterol-rich lipoproteins, protect LDL from oxidation and preserve endothelial function[107,108]. Since not all cells involved in the above-mentioned effects contain calcium channels, the anti-atherosclerotic activities of calcium antagonists probably involve many additional properties of the compounds.

Clinical trials, such as the INTACT study[109] and the Montreal Heart study[110], investigating the effects of calcium antagonists on the development of atherogenesis reported that these drugs had no effect on progression of existing lesions, but could indeed reduce the number of newly formed lesions. One recent trial demonstrated that treatment with the calcium antagonist amlodipine had no effect on the angiographic progression of coronary atherosclerosis or the risk of cardiovascular events, but was associated with fewer hospitalisations indicating a reduction in vascular morbidity[111].

Thus, direct anti-atherosclerotic effects of calcium antagonists remain disputed, although many investigations suggest that these drugs could possibly influence lesion composition and thereby plaque stability and therefore could be a useful addition to existing drug therapies, such as lipid-lowering.

Mouse models of hyperlipidaemia and atherosclerosis

Although mice have a number of important differences from humans in lipoprotein metabolism, they are frequently used to study lipid metabolism and atherogenesis. In principle, wild-type mice are very resistant to the development of hyperlipidaemia and atherosclerosis, since the main lipoprotein class in plasma is the anti-atherogenic HDL, whereas in humans the predominant lipoproteins are the atherogenic VLDL and LDL. This is probably due to very efficient clearance of VLDL, IDL and LDL from plasma in mice. Other mouse-specific differences include the absence of CETP and a different editing of ApoB100[112].

When the relatively "atherosclerosis-susceptible" mouse strain C57Bl/6 is put on a cholesterol-containing diet, lipoprotein distribution changes toward VLDL and LDL, inducing fatty streak formation after several months of the diet[113,114]. However, with the development of genetically modified mice, by overexpression or knocking-out of specific genes, mouse models have been generated which are more suitable for the study of hyperlipidaemia and atherosclerosis.

ApoE-deficient mice

ApoE is a major component of plasma lipoproteins and has a high affinity for the LDL-receptor and other receptors such as the LRP and VLDL-receptor. It is also an important factor in the efflux of cholesterol from peripheral tissues and hepatic VLDL-production[115,116]. In humans, hyperlipidaemia type III is characterised by elevated plasma levels of cholesterol and triglycerides and can be caused by an impaired receptor binding of the isoform ApoE2 or mutant forms of ApoE, followed by premature development of atherosclerosis[117,118]. One of the most widely used animal models for the study of lipid metabolism and atherosclerosis is the ApoE-deficient mouse. In these mice, plasma cholesterol levels are elevated to about 15-20 mM on a regular chow diet due to the absence of the ligand for clearance pathways of chylomicron and VLDL remnants[119-121]. Atherosclerotic lesions develop within 3 months containing predominantly foam cells, while fibrosis, necrosis, lipid-rich cores and cholesterol clefts can be found much later[122]. The time course of lesion development is dramatically shortened when these mice are fed a Western-type diet, containing cholesterol. Then plasma cholesterol levels easily reach levels of 40 mM[123].

A major drawback of the complete absence of ApoE is the fact that plasma cholesterol levels are quite high in ApoE-deficient mice, even on a regular chow diet. As mentioned before, ApoE plays a very important role in lipoprotein clearance and VLDL-production and ApoE has been shown to mediate the efflux of cholesterol from foam cells in the artery wall which is a very important process in atherogenesis. The absence of this latter effect on cellular cholesterol homeostasis in ApoE-deficient mice is also the reason why massive xanthomatosis is found in various tissues in these mice[124].

*ApoE*3-Leiden transgenic mice*

The rare ApoE*3-Leiden mutation is a dominant negative mutation of ApoE. It is characterised by a tandem duplication of codons 120-126 and yields a mature protein of 306 amino acid residues[125]. This mutation is associated with familial dysbetalipoproteinemia in humans[126,127]. The introduction of this dominant human mutation in a mouse results in a model with a defective clearance of ApoE-containing lipoproteins by reduced binding to the LDL-receptor, resembling human familial dysbetalipoproteinemia. These ApoE*3-Leiden transgenic mice exhibit significant elevations of plasma cholesterol and triglycerides on a regular chow diet[128]. When fed a semi-synthetic sucrose-rich or high fat/cholesterol-rich diet, cholesterol and triglyceride levels in plasma rise considerably, with a prominent increase in VLDL and LDL-sized lipoprotein fractions[128,129]. As compared to the ApoE-deficient mice, the ApoE*3-Leiden transgenic mice represent a moderate mouse model for hyperlipidaemia. Depending on the diet, plasma cholesterol and triglyceride levels can be varied between 3-40 mmol/l and 0.5-4.5 mmol/l, respectively. On a cholesterol-rich diet, ApoE*3-Leiden transgenic mice also develop atherosclerosis which is highly correlated to plasma cholesterol levels[129,130]. Atherosclerosis development starts at the aortic root and progresses along the entire arterial tree in a time-dependent fashion[131]. Depending on the amount of cholesterol in the diet different types of lesions can be found in these animals ranging from fatty streaks to severe lesions[132], showing characteristics of human pathology. When hyperlipidaemia is abolished after the development of atherosclerosis, lesions show regression which is characterised by a decrease in macrophage content and a tendency to reduced lesion size[133]. Because lipoprotein metabolism and atherogenesis in this mouse model highly resemble the human situation, the ApoE*3-Leiden transgenic mouse is a very suitable model for studying hyperlipidaemia and atherosclerosis progression and regression. It was e.g. used to elucidate the cholesterol-raising properties of cafestol, a component of boiled coffee[134]. In addition, ApoE*3-Leiden mice are responsive to anti-hyperlipidaemic treatment. In contrast to the lack of effect of statin treatment in ApoE-deficient mice[135] and LDL-receptor-deficient mice[136], lovastatin therapy in ApoE*3-Leiden mice resulted in plasma cholesterol reductions[137]. Other lipid-lowering agents, such as gemfibrozil[137], fish oil[138] and stanol esters[139] have also been shown to be effective in ApoE*3-Leiden

mice. In addition, the anti-atherosclerotic effect of stanol esters was established in this mouse model[140].

LDL-receptor-deficient mice

In humans, mutations in the gene for the LDL-receptor cause familial hypercholesterolemia, a disease characterised by premature atherosclerosis development and elevated plasma levels of LDL cholesterol and ApoB100[141]. Homozygous LDL-receptor-deficient mice have a two-fold increased plasma cholesterol level compared to their wild-type littermates, mainly caused by an increase in IDL and LDL. Clearance of VLDL and LDL is hampered in this mouse model. When fed a cholesterol-containing diet, LDL-receptor-deficient mice respond with a major increase in cholesterol content of IDL and LDL particles[142]. Consequently, these mice develop massive xanthomatosis, i.e. accumulations of cholesterol in the skin and subcutaneous tissue, and atherosclerosis[143]. The LDL-receptor-deficient mouse represents a more moderate model than the ApoE-deficient mouse, because of the severe hyperlipidaemia and atherosclerosis found in the latter. However, it is not as easy to manipulate plasma cholesterol levels in LDL-receptor-deficient mice as it is in ApoE*3-Leiden transgenic mice. Regarding LDL-receptor-deficient mice fed a chow diet, it was observed that superimposition of LDL-receptor deficiency onto an ApoE deficiency does not increase hypercholesterolaemia beyond the level observed with ApoE deficiency alone, indicating that ApoE deficiency in itself is a very extreme model for hyperlipidaemia and atherosclerosis[144].

ApoC1-deficient and transgenic mice

To investigate the role of ApoC1 in lipoprotein metabolism, mice deficient for ApoC1 and mice overexpressing the human ApoC1 gene were generated. In ApoC1-deficient mice plasma triglyceride levels are elevated both on a chow diet as well as on a mild high fat/cholesterol diet. Only on a very atherogenic diet do these mice develop hypercholesterolaemia. ApoC1 deficiency mainly leads to impaired receptor-mediated clearance of remnant lipoproteins [145,146]. Transgenic mice overexpressing the human ApoC1 gene exhibit plasma triglyceride levels that are 2-3 times higher than in control mice on a chow diet[147]. Again, impaired hepatic uptake of VLDL is the main cause for this hypertriglyceridaemia and hypercholesterolaemia, suggesting

that both an excess and the absence of ApoC1 can inhibit lipoprotein uptake[148-150]. Human ApoC1 transgenic mice also show a dry skin with severely reduced sebum production, a lack of subcutaneous fat, decreased adipose tissue and a hampered fatty acid metabolism in peripheral tissues with increased levels of free fatty acids in plasma[151,152]. The recently discovered protective effect of ApoC1 in the development of obesity and insulin resistance[153] suggests that ApoC1 expression affects cellular lipid homeostasis.

Outline of this thesis

Development of new therapies for the treatment of atherosclerosis has lately shifted from focussing merely on the reduction of plasma cholesterol and triglyceride levels to finding targets for direct anti-atherosclerotic effects in the vascular wall as a possible additional therapy.

In **chapter 2** we investigate the mechanism of cholesterol- and triglyceride-lowering by a newly developed and very potent HMG-CoA-reductase inhibitor, rosuvastatin, in ApoE*3-Leiden transgenic mice. We also addressed why statins reduce plasma cholesterol and triglyceride levels in ApoE*3-Leiden mice as compared to the lack of statin effect found in most other mouse models for hyperlipidaemia.

Retrospective analysis of clinical data revealed that statin treatment together with calcium antagonist therapy reduced progression of atherosclerosis more than statin treatment alone[154]. Consequently, we hypothesised that calcium antagonists can exert their anti-atherosclerotic effects, when administered in combination with lipid-lowering drugs. Therefore, the possible synergistic effect of treatment with the HMG-CoA-reductase inhibitor atorvastatin in combination with the calcium antagonist amlodipine on the development of atherosclerosis in the aortic root in ApoE*3-Leiden mice is investigated in **chapter 3**. To further investigate the extent of atherosclerosis in the aortic arch of these treated mice, the protein, lipid and calcium content of atherosclerotic lesions is determined by a new Raman-spectroscopy technique in **chapter 4**. This technique provides quantitative information about composition of atherosclerotic lesions in a non-invasive manner, potentially opening the way for the monitoring of atherogenesis and the effects of anti-atherosclerotic therapy *in vivo* in future.

Since it is more clinically relevant to investigate atherosclerosis therapy effects on existing lesions, in **chapter 5** treatment with atorvastatin, amlodipine and their combination is applied in ApoE*3-Leiden transgenic mice which had already developed plaques. We examined whether atorvastatin and amlodipine were able to reduce progression or even induce regression of pre-induced atherosclerotic lesions both in the aortic root and arch and whether the combination of atorvastatin and amlodipine was more potent in reducing atherosclerosis than either drug alone.

A possible new approach to reducing plasma cholesterol levels is to inhibit the enzyme ACAT. This enzyme is present in the liver and intestine in the isoform acat2, whereas the isoform acat1 is predominantly expressed in macrophages. Therefore, it has also been suggested that ACAT-inhibitors can have direct anti-atherosclerotic effects on the vessel wall. In **chapter 6**, we investigate the anti-atherosclerotic effects of the ACAT-inhibitor avasimibe, independent of its lipid-lowering properties in ApoE*3-Leiden transgenic mice.

In the search for new targets for anti-atherosclerotic therapy, we investigated the role of apolipoprotein C1 in atherosclerosis development in **chapter 7**. Using macrophages from ApoC1-deficient mice, the effect of ApoC1 on lipid accumulation in the macrophage as one of the earliest steps in atherogenesis was determined.

Chapter 8 describes the early development of atherosclerosis in a new model for atherosclerosis in ApoE*3-Leiden transgenic mice. By placing a non-occlusive perivascular cuff around the femoral artery of a mouse on an atherogenic diet, rapid atherosclerosis was induced in this highly defined area in the vasculature.

The results obtained in these studies and future perspectives are discussed in **chapter 9**.

Reference List

1. Mahley RW, Innerarity TL, Rall SCJ, Weisgraber KH: Plasma lipoproteins: apolipoprotein structure and function. *J Lipid Res* 1984;25:1277-1294
2. Beisiegel U: Lipoprotein metabolism. *Eur Heart J* 1998;19 Suppl A:A20-A23
3. Olivecrona G, Olivecrona T: Triglyceride lipases and atherosclerosis. *Curr Opin Lipidol* 1995;6:291-305
4. Young SG, Fielding CJ: The ABCs of cholesterol efflux. *Nat Genet* 1999;22:316-318
5. Santamarina-Fojo S, Lambert G, Hoeg JM, Brewer HBJ: Lecithin-cholesterol acyltransferase: role in lipoprotein metabolism, reverse cholesterol transport and atherosclerosis. *Curr Opin Lipidol* 2000;11:267-275

6. Williams DL, Connelly MA, Temel RE, Swarnakar S, Phillips MC, de la Llera-Moya M, Rothblat GH: Scavenger receptor BI and cholesterol trafficking. *Curr Opin Lipidol* 1999;10:329-339

7. Miller NE: The evidence for the antiatherogenicity of high density lipoprotein in man. *Lipids* 1978;13:914-919

8. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB: Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. *JAMA* 1986;256:2835-2838

9. Barter PJ, Rye KA: High density lipoproteins and coronary heart disease. *Atherosclerosis* 1996;121:1-12

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

11. Ross R: Rous-Whipple Award Lecture. Atherosclerosis: a defense mechanism gone awry. *Am J Pathol* 1993;143:987-1002

12. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ: Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* 1995;91:2488-2496

13. Ross R: Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999;340:115-126

14. Glass CK, Witztum JL: Atherosclerosis. the road ahead. *Cell* 2001;104:503-516

15. Carr AC, McCall MR, Frei B: Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arterioscler Thromb Vasc Biol* 2000;20:1716-1723

16. Kuhn H, Chan L: The role of 15-lipoxygenase in atherogenesis: pro- and antiatherogenic actions. *Curr Opin Lipidol* 1997;8:111-117

17. Heinecke JW, Baker L, Rosen H, Chait A: Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J Clin Invest* 1986;77:757-761

18. Moore KP, Darley-Usmar V, Morrow J, Roberts LJ: Formation of F2-isoprostanes during oxidation of human low-density lipoprotein and plasma by peroxynitrite. *Circ Res* 1995;77:335-341

19. Steinberg D: Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. *Circulation* 1997;95:1062-1071

20. Dong ZM, Wagner DD: Leukocyte-endothelium adhesion molecules in atherosclerosis. *J Lab.Clin Med* 1998;132:369-375

21. Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R: Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the apoe-deficient mouse. *Arterioscler.Thromb.Vasc.Biol.* 1998;18:842-851

22. Zibara K, Chignier E, Covacho C, Poston R, Canard G, Hardy P, McGregor J: Modulation of expression of endothelial intercellular adhesion molecule-1, platelet-endothelial cell adhesion molecule-1, and vascular cell adhesion molecule-1 in aortic arch lesions of apolipoprotein E-deficient compared with wild-type mice. *Arterioscler Thromb Vasc Biol* 2000;20:2288-2296

23. Ramos CL, Huo Y, Jung U, Ghosh S, Manka DR, Sarembock IJ, Ley K: Direct demonstration of P-selectin- and VCAM-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein E-deficient mice. *Circ Res* 1999;84:1237-1244

24. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD: The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest* 1998;102:145-152

25. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, J.C., Connelly PW, Milstone DS: A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* 2001;107:1255-1262

26. Kellner-Weibel G, Jerome WG, Small DM, Warner GJ, Stoltenborg JK, Kearney MA, Corjay MH, Phillips MC, Rothblat GH: Effects of intracellular free cholesterol accumulation on macrophage viability: a model for foam cell death. *Arterioscler Thromb Vasc Biol* 1998;18:423-431

27. Brown MS, Goldstein JL: A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci U S A* 1999;96:11041-11048

28. Marcil M, Brooks-Wilson A, Clee SM, Roomp K, Zhang LH, Yu L, Collins JA, van Dam M, Molhuizen HO, Loubster O, Ouellette BF, Sensen CW, Fichter K, Mott S, Denis M, Boucher B, Pimstone S, Genest JJ, Kastelein JJ, Hayden MR: Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet* 1999;354:1341-1346

29. Orso E, Broccardo C, Kaminski WE, Bottcher A, Liebisch G, Drobnik W, Gotz A, Chambenoit O, Diederich W, Langmann T, Spruss T, Luciani MF, Rothe G, Lackner KJ, Chimini G, Schmitz G: Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. *Nat Genet* 2000;24:192-196

30. McNeish J, Aiello RJ, Guyot D, Turi T, Gabel C, Aldinger C, Hoppe KL, Roach ML, Royer LJ, de Wet J, Broccardo C, Chimini G, Francone OL: High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc Natl Acad Sci U S A* 2000;97:4245-4250

31. Mazzone T: Apolipoprotein E secretion by macrophages: its potential physiological functions. *Curr Opin Lipidol* 1996;7:303-307

32. Zhang WY, Gaynor PM, Kruth HS: Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J Biol Chem* 1996;271:28641-28646

33. Langer C, Huang Y, Cullen P, Wiesenhutter B, Mahley RW, Assmann G, von Eckardstein A: Endogenous apolipoprotein E modulates cholesterol efflux and cholesteryl ester hydrolysis mediated by high-density lipoprotein-3 and lipid-free apolipoproteins in mouse peritoneal macrophages. *J Mol Med* 2000;78:217-227

34. Van Eck M, Herijgers N, Van Dijk KW, Havekes LM, Hofker MH, Groot PH, van Berkel TJ: Effect of macrophage-derived mouse ApoE, human ApoE3-Leiden, and human ApoE2 (Arg158-->Cys) on cholesterol levels and atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 2000;20:119-127

35. Guyton JR, Klemp KF: Development of the lipid-rich core in human atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.* 1996;16:4-11

36. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, Clinton SK, Sukhova GK, Lee RT: Macrophages and atherosclerotic plaque stability. *Curr.Opin.Lipidol.* 1996;7:330-335

37. Kullo IJ, Edwards WD, Schwartz RS: Vulnerable plaque: pathobiology and clinical implications. *Ann Intern Med* 1998;129:1050-1060

38. Amento EP, Ehsani N, Palmer H, Libby P: Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb* 1991;11:1223-1230

39. Lee RT, Libby P: The increasing significance of the unstable atheroma, in Jacotot B, athé D, Fruchart JC (eds): *Atherosclerosis XI.* Singapore, Elsevire Science, 1998, pp 83-88

40. Stary HC, Chandler AB, Glagov S, Guyton JR, Insull WJ, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW: A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb* 1994;14:840-856

41. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ: Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med* 1987;316:1371-1375

42. Pasterkamp G, de Kleijn DP, Borst C: Arterial remodeling in atherosclerosis, restenosis and after alteration of blood flow: potential mechanisms and clinical implications. *Cardiovasc Res* 2000;45:843-852

43. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull WJ, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW: A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb Vasc Biol* 1995;15:1512-1531

44. Ferrell M, Fuster V, Gold HK, Chesebro JH: A dilemma for the 1990s. Choosing appropriate experimental animal model for the prevention of restenosis. *Circulation* 1992;85:1630-1631

45. Lindholm LH, Hansson L, Ekbom T, Dahlof B, Lanke J, Linjer E, Schersten B, Wester PO, Hedner T, de Faire U: Comparison of antihypertensive treatments in preventing cardiovascular events in elderly diabetic patients: results from the Swedish Trial in Old Patients with Hypertension-2. STOP Hypertension-2 Study Group. *J Hypertens* 2000;18:1671-1675

46. Frishman WH, Cheng A: Secondary prevention of myocardial infarction: role of beta-adrenergic blockers and angiotensin-converting enzyme inhibitors. *Am Heart J* 1999;137:S25-S34

47. Hansson L, Hedner T, Lund-Johansen P, Kjeldsen SE, Lindholm LH, Syvertsen JO, Lanke J, de Faire U, Dahlof B, Karlberg BE: Randomised trial of effects of calcium antagonists compared with diuretics and beta-blockers on cardiovascular morbidity and mortality in hypertension: the Nordic Diltiazem (NORDIL) study. *Lancet* 2000;356:359-365

48. Huynh T, Theroux P, Bogaty P, Nasmith J, Solymoss S: Aspirin, warfarin, or the combination for secondary prevention of coronary events in patients with acute coronary syndromes and prior coronary artery bypass surgery. *Circulation* 2001;103:3069-3074

49. Gensini GF, Comeglio M, Falai M: Advances in antithrombotic therapy of acute myocardial infarction. *Am Heart J* 1999;138:S171-S176

50. Farnier M, Davignon J: Current and future treatment of hyperlipidemia: the role of statins. *Am.J.Cardiol.* 1998;82:J3-J10

51. Bradford RH, Shear CL, Chremos AN, Dujovne CA, Franklin FA, Grillo RB, Higgins J, Langendorfer A, Nash DT, Pool JL: Expanded Clinical Evaluation of Lovastatin (EXCEL) study results: two-year efficacy and safety follow-up. *Am J Cardiol* 1994;74:667-673

52. Jones P, Kafonek S, Laurora I, Hunninghake D: Comparative dose efficacy study of atorvastatin versus simvastatin, pravastatin, lovastatin, and fluvastatin in patients with hypercholesterolemia (the CURVES study). *Am J Cardiol* 1998;81:582-587

53. Nawrocki JW, Weiss SR, Davidson MH, Sprecher DL, Schwartz SL, Lupien PJ, Jones PH, Haber HE, Black DM: Reduction of LDL cholesterol by 25% to 60% in patients with primary hypercholesterolemia by atorvastatin, a new HMG-CoA reductase inhibitor. *Arterioscler.Thromb.Vasc.Biol.* 1995;15:678-682

54. Brown MS, Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986;232:34-47

55. Scandinavian Simvastatin Survival Study Group: Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994;344:1383-1389

56. Tonkin A, Aylward P, Colquhoun D, Glasziou P, Harris P, Macmahon S, Magnus P, Newel D, Nestel P, Sharpe N, Hunt D, Shaw J, Simes RJ, Thompson P, Thomson A, West M, White H, Simes S, Hague W, Caleo S, Hall J, Martin A, Mulray S, Barter P, Beilin L, Collins R, Mcneil J, Meier P, Willimott H, Smithers D, Wallace P, Sullivan D, Keech A: Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. *N.Engl.J Med* 1998;339:1349-1357

57. West of Scotland Coronary Prevention Study Group: Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS). *Circulation* 1998;97:1440-1445

58. Jukema JW, Bruschke AV, van Boven AJ, Reiber JH, Bal ET, Zwinderman AH, Jansen H, Boerma GJ, van Rappard FM, Lie KI: Effects of lipid lowering by pravastatin on progression and regression of coronary artery disease in symptomatic men with normal to moderately elevated serum cholesterol levels. The Regression Growth Evaluation Statin Study (REGRESS). *Circulation* 1995;91:2528-2540

59. MAAS Investigators: Effect of simvastatin on coronary atheroma: the Multicentre Anti-Atheroma Study (MAAS). *Lancet* 1994;344:633-638

60. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E: The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996;335:1001-1009

61. Marschner IC, Colquhoun D, Simes RJ, Glasziou P, Harris P, Singh BB, Friedlander D, White H, Thompson P, Tonkin A: Long-term risk stratification for survivors of acute coronary syndromes. Results from the Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) Study. LIPID Study Investigators. *J Am Coll Cardiol* 2001;38:56-63

62. Gotto AMJ, Boccuzzi SJ, Cook JR, Alexander CM, Roehm JB, Meyer GS, Clearfield M, Weis S, Whitney E: Effect of lovastatin on cardiovascular resource utilization and costs in the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS). AFCAPS/TexCAPS Research Group. *Am J Cardiol* 2000;86:1176-1181

63. Davignon J, Laaksonen R: Low-density lipoprotein-independent effects of statins. *Curr Opin Lipidol.* 1999;10:543-559

64. Vaughan CJ, Murphy MB, Buckley BM: Statins do more than just lower cholesterol. *Lancet* 1996;348:1079-1082

65. Bellosta S, Bernini F, Ferri N, Quarato P, Canavesi M, Arnaboldi L, Fumagalli R, Paoletti R, Corsini A: Direct vascular effects of HMG-CoA reductase inhibitors. *Atherosclerosis* 1998;137 Suppl:S101-S109

66. Kempen HJ, Vermeer M, de Wit E, Havekes LM: Vastatins inhibit cholesterol ester accumulation in human monocyte-derived macrophages. *Arterioscler.Thromb.* 1991;11:146-153

67. Palinski W: Immunomodulation: a new role for statins? *Nat Med* 2000;6:1311-1312

68. Maresca G, Di Blasio A, Marchioli R, Di Minno G: Measuring plasma fibrinogen to predict stroke and myocardial infarction: an update. *Arterioscler Thromb Vasc Biol* 1999;19:1368-1377

69. Haverkate F, Thompson SG, Pyke SD, Gallimore JR, Pepys MB: Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group [see comments]. *Lancet* 1997;349:462-466

70. Morrow DA, Rifai N, Antman EM, Weiner DL, McCabe CH, Cannon CP, Braunwald E: Serum amyloid A predicts early mortality in acute coronary syndromes: A TIMI 11A substudy. *J Am Coll Cardiol* 2000;35:358-362

71. Fyfe AI, Rothenberg LS, deBeer FC, Cantor RM, Rotter JI, Lusis AJ: Association between serum amyloid A proteins and coronary artery disease: evidence from two distinct arteriosclerotic processes. *Circulation* 1997;96:2914-2919

72. Strandberg TE, Vanhanen H, Tikkanen MJ: Effect of statins on C-reactive protein in patients with coronary artery disease. *Lancet* 1999;353:118-119

73. Albert MA, Danielson E, Rifai N, Ridker PM: Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study. *JAMA* 2001;286:64-70

74. Sparrow CP, Burton CA, Hernandez M, Mundt S, Hassing H, Patel S, Rosa R, Hermanowski-Vosatka A, Wang PR, Zhang D, Peterson L, Detmers PA, Chao YS, Wright SD: Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol* 2001;21:115-121

75. Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC: Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* 1998;98:2088-2093

76. Fruchart JC, Duriez P, Staels B: Peroxisome proliferator-activated receptor-alpha activators regulate genes governing lipoprotein metabolism, vascular inflammation and atherosclerosis. *Curr Opin Lipidol* 1999;10:245-257

77. The BIP Study Group: Secondary prevention by raising HDL cholesterol and reducing triglycerides in patients with coronary artery disease: the Bezafibrate Infarction Prevention (BIP) study. *Circulation* 2000;102:21-27

78. Rubins HB, Robins SJ, Collins D, Fye CL, Anderson JW, Elam MB, Faas FH, Linares E, Schaefer EJ, Schectman G, Wilt TJ, Wittes J: Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *N Engl J Med* 1999;341:410-418

79. Watts GF, Dimmitt SB: Fibrates, dyslipoproteinaemia and cardiovascular disease. *Curr Opin Lipidol* 1999;10:561-574

80. Shepherd J: Mechanism of action of bile acid sequestrants and other lipid-lowering drugs. *Cardiology* 1989;76 Suppl 1:65-71

81. Lipid Research Clinics Program: The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. *JAMA* 1984;251:365-374

82. Drood JM, Zimetbaum PJ, Frishman WH: Nicotinic acid for the treatment of hyperlipoproteinemia. *J Clin Pharmacol* 1991;31:641-650

83. Coronary Drug Project Research Group: Clofibrate and niacin in coronary heart disease. *JAMA* 1975;231:360-381

84. O'Keefe JHJ, Harris WS, Nelson J, Windsor SL: Effects of pravastatin with niacin or magnesium on lipid levels and postprandial lipemia. *Am J Cardiol* 1995;76:480-484

85. Brown WV: Review of clinical studies of fenofibrate in combination with currently approved lipid-lowering drugs. *Cardiology* 1989;76 Suppl 1:45-51

86. Kane JP, Malloy MJ, Tun P, Phillips NR, Freedman DD, Williams ML, Rowe JS, Havel RJ: Normalization of low-density-lipoprotein levels in heterozygous familial hypercholesterolemia with a combined drug regimen. *N Engl J Med* 1981;304:251-258

87. Sliskovic DR, White AD: Therapeutic potential of ACAT inhibitors as lipid lowering and anti-atherosclerotic agents. *Trends.Pharmacol.Sci.* 1991;12:194-199

88. Suckling KE, Stange EF: Role of acyl-CoA: cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* 1985;26:647-671

89. OBrien PM, Sliskovic DR, Picard JA, Lee HT, Purchase CF2, Roth BD, White AD, Anderson M, Mueller SB, Bocan T, Bousley R, Hamelehle KL, Homan R, Lee P, Krause BR, Reindel JF, Stanfield RL, Turluck D: Inhibitors of acyl-CoA:cholesterol O-acyltransferase. synthesis and pharmacological activity of (+/-)-2-dodecyl-alpha-phenyl- N- (2,4,6-trimethoxyphenyl)-2H-tetrazole-5- acetamide and structurally related tetrazole amide derivatives. *J.Med.Chem.* 1996;39:2354-2366

90. Krause BR, Pape ME, Kieft K, Auerbach B, Bisgaier CL, Homan R, Newton RS: ACAT inhibition decreases LDL cholesterol in rabbits fed a cholesterol-free diet. Marked changes in LDL cholesterol without changes in LDL receptor mRNA abundance. *Arterioscler.Thromb.* 1994;14:598-604

91. White AD, Purchase CF2, Picard JA, Anderson MK, Mueller SB, Bocan TM, Bousley RF, Hamelehle KL, Krause BR, Lee P, Stanfield RL, Reindel JF: Heterocyclic amides: inhibitors of acyl-CoA:cholesterol O-acyl transferase with hypocholesterolemic activity in several species and antiatherosclerotic activity in the rabbit. *J.Med.Chem.* 1996;39:3908-3919

92. Lee HT, Sliskovic DR, Picard JA, Roth BD, Wierenga W, Hicks JL, Bousley RF, Hamelehle KL, Homan R, Speyer C, Stanfield RL, Krause BR: Inhibitors of acyl-CoA: cholesterol O-acyl transferase (ACAT) as hypocholesterolemic agents. CI-1011: an acyl sulfamate with unique cholesterol-lowering activity in animals fed noncholesterol-supplemented diets. *J.Med.Chem.* 1996;39:5031-5034

93. White AD, Creswell MW, Chucholowski AW, Blankley CJ, Wilson MW, Bousley RF, Essenburg AD, Hamelehle KL, Krause BR, Stanfield RL, Dominick MA, Neub M: Heterocyclic ureas: inhibitors of acyl-CoA:cholesterol O- acyltransferase as hypocholesterolemic agents. *J.Med.Chem.* 1996;39:4382-4395

94. Schaffer SA, Bloom JD, De Vries VG, Dutia M, Katocs ASJr, Largis EE: CL 277,082, a novel inhibitor of cholesterol esterification and cholesterol absorption, in Fidge NH, Nestel PJ (eds): *Atherosclerosis VII.* Amsterdam, Elsevier Science Publishers B.V., 1986, pp 633-636

95. Burnett JR, Wilcox LJ, Telford DE, Kleinstiver SJ, Barrett PH, Huff MW: Inhibition of cholesterol esterification by DuP 128 decreases hepatic apolipoprotein B secretion in vivo: effect of dietary fat and cholesterol. *Biochim Biophys Acta* 1998;1393:63-79

96. Junquero D, Bruniquel F, N'Guyen X, Autin JM, Patoiseau JF, Degryse AD, Colpaert FC, Delhon A: F 12511, a novel ACAT inhibitor, and atorvastatin regulate endogenous hypercholesterolemia in a synergistic manner in New Zealand rabbits fed a casein-enriched diet. *Atherosclerosis* 2001;155:131-142

97. Nicolosi RJ, Wilson TA, Krause BR: The ACAT inhibitor, CI-1011 is effective in the prevention and regression of aortic fatty streak area in hamsters. *Atherosclerosis* 1998;137:77-85

98. Ashton MJ, Bridge AW, Bush RJ, Dron DI, Harris NV, Jones GD, Lythgoe DJ, Riddell D, Smith C: RP-70676: a potent systemically available inhibitor of acyl-CoA:cholesterol o-acyl transferase (ACAT). *Bioorg.Med.Chem.Lett.* 1992;2:375-380

99. Kusunoki J, Hansoty DK, Aragane K, Fallon JT, Badimon JJ, Fisher EA: Acyl-coa:cholesterol acyltransferase inhibition reduces atherosclerosis in apolipoprotein e-deficient mice. *Circulation* 2001;103:2604-2609

100. Meiner V, Tam C, Gunn MD, Dong LM, Weisgraber KH, Novak S, Myers HM, Erickson SK, Farese RVJ: Tissue expression studies on the mouse acyl-CoA: cholesterol acyltransferase gene (Acact): findings supporting the existence of multiple cholesterol esterification enzymes in mice. *J Lipid Res* 1997;38:1928-1933

101. Anderson RA, Joyce C, Davis M, Reagan JW, Clark M, Shelness GS, Rudel LL: Identification of a form of acyl-coa:cholesterol acyltransferase specific to liver and intestine in nonhuman primates. *J.Biol.Chem.* 1998;273:26747-26754

102. Cases S, Novak S, Zheng YW, Myers HM, Lear SR, Sande E, Welch CB, Lusis AJ, Spencer TA, Krause BR, Erickson SK, Farese RV: ACAT-2, a second mammalian acyl-coa:cholesterol acyltransferase - its cloning, expression, and characterization. *J.Biol.Chem.* 1998;273:26755-26764

103. Accad M, Smith SJ, Newland DL, Sanan DA, King LEJ, Linton MF, Fazio S, Farese RVJ: Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. *J Clin Invest* 2000;105:711-719

104. Yagyu H, Kitamine T, Osuga J, Tozawa R, Chen Z, Kaji Y, Oka T, Perrey S, Tamura Y, Ohashi K, Okazaki H, Yahagi N, Shionoiri F, Iizuka Y, Harada K, Shimano H, Yamashita H, Gotoda T, Yamada N, Ishibashi S: Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *J Biol Chem* 2000;275:21324-21330

105. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RVJ: Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest* 2001;107:163-171

106. Buhman KK, Accad M, Novak S, Choi RS, Wong JS, Hamilton RL, Turley S, Farese RVJ: Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat Med* 2000;6:1341-1347

107. Schachter M: Calcium antagonists and atherosclerosis. *Int J Cardiol* 1997;62 Suppl 2:S9-15.:S9-15

108. Keogh AM, Schroeder JS: A review of calcium antagonists and atherosclerosis. *J.Cardiovasc.Pharmacol.* 1990;16 Suppl 6:S28-35

109. Lichtlen PR, Hugenholtz PG, Rafflenbeul W, Hecker H, Jost S, Deckers JW: Retardation of angiographic progression of coronary artery disease by nifedipine.

Results of the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT). INTACT Group Investigators. *Lancet* 1990;335:1109-1113

110. Waters D, Lesperance J, Francetich M, Causey D, Theroux P, Chiang YK, Hudon G, Lemarbre L, Reitman M, Joyal M: A controlled clinical trial to assess the effect of a calcium channel blocker on the progression of coronary atherosclerosis. *Circulation* 1990;82:1940-1953

111. Pitt B, Byington RP, Furberg CD, Hunninghake DB, Mancini GB, Miller ME, Riley W, for TP, I: Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. *Circulation* 2000;102:1503-1510

112. Breslow JL: Mouse models of atherosclerosis. *Science* 1996;272:685-688

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

114. Paigen B, Plump AS, Rubin EM: The mouse as a model for human cardiovascular disease and hyperlipidemia. *Curr Opin Lipidol* 1994;5:258-264

115. Kuipers F, Jong MC, Lin Y, Eck M, Havinga R, Bloks V, Verkade HJ, Hofker MH, Moshage H, Berkel TJ, Vonk RJ, Havekes LM: Impaired secretion of very low density lipoprotein-triglycerides by apolipoprotein E- deficient mouse hepatocytes. *J Clin Invest* 1997;100:2915-2922

116. Mensenkamp AR, Jong MC, Van Goor H, van Luyn MJ, Bloks V, Havinga R, Voshol PJ, Hofker MH, Van Dijk KW, Havekes LM, Kuipers F: Apolipoprotein E participates in the regulation of very low density lipoprotein-triglyceride secretion by the liver. *J Biol Chem* 1999;274:35711-35718

117. Gregg RE, Zech LA, Schaefer EJ, Brewer HBJ: Type III hyperlipoproteinemia: defective metabolism of an abnormal apolipoprotein E. *Science* 1981;211:584-586

118. Rall SCJ, Weisgraber KH, Innerarity TL, Mahley RW: Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc Natl Acad Sci U S A* 1982;79:4696-4700

119. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL: Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992;71:343-353

120. Zhang SH, Reddick RL, Piedrahita JA, Maeda N: Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992;258:468-471

121. van Ree JH, van den Broek WJ, Dahlmans VE, Groot PH, Vidgeon-Hart M, Frants RR, Wieringa B, Havekes LM, Hofker MH: Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis* 1994;111:25-37

122. Reddick RL, Zhang SH, Maeda N: Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. *Arterioscler Thromb* 1994;14:141-147

123. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R: ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb* 1994;14:133-140

124. van Ree JH, Gijbels MJ, van den Broek WJ, Hofker MH, Havekes LM: Atypical xanthomatosis in apolipoprotein E-deficient mice after cholesterol feeding. *Atherosclerosis* 1995;112:237-243

125. van den Maagdenberg AM, de Knijff P, Stalenhoef AF, J.A., Havekes LM, Frants RR: Apolipoprotein E*3-Leiden allele results from a partial gene duplication in exon 4. *Biochem Biophys Res Commun* 1989;165:851-857

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

127. de Knijff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR, Havekes LM: Familial dysbetalipoproteinemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. *J Clin Invest* 1991;88:643-655

128. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der Boom H, Havekes LM, Frants RR: Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol.Chem.* 1993;268:10540-10545

129. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM: Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410

130. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon HM, Havekes LM: Quantitative assessment of aortic atherosclerosis in APOE* 3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arterioscler Thromb Vasc Biol* 1996;16:926-933

131. Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, de Muinck ED: Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276-283

132. Leppanen P, Luoma JS, Hofker MH, Havekes LM, Yla-Herttuala S: Characterization of atherosclerotic lesions in apo E3-leiden transgenic mice. *Atherosclerosis* 1998;136:147-152

133. Gijbels MJ, van der Cammen M, van der Laan LJ, Emeis JJ, Havekes LM, Hofker MH, Kraal G: Progression and regression of atherosclerosis in APOE3- Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* 1999;143:15-25

134. Post SM, de Roos B, Vermeulen M, Afman L, Jong MC, Dahlmans VE, Havekes LM, Stellaard F, Katan MB, Princen HM: Cafestol increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis. *Arterioscler Thromb Vasc Biol* 2000;20:1551-1556

135. Quarfordt SH, Oswald B, Landis B, Xu HS, Zhang SH, Maeda N: In vivo cholesterol kinetics in apolipoprotein E-deficient and control mice. *J Lipid Res* 1995;36:1227-1235

136. Bisgaier CL, Essenburg AD, Auerbach BJ, Pape ME, Sekerke CS, Gee A, W÷lle S, Newton RS: Attenuation of plasma low density lipoprotein cholesterol by select 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in mice devoid of low density lipoprotein receptors. *J.Lipid Res.* 1997;38:2502-2515

137. van Vlijmen BJ, Pearce NJ, Bergö M, Staels B, Yates JW, Gribble AD, Bond BC, Hofker MH, Havekes LM, Groot PH: Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. *Arzneimittelforschung.* 1998;48:396-402

138. van Vlijmen BJ, Mensink RP, van, Offermans RF, Hofker MH, Havekes LM: Effects of dietary fish oil on serum lipids and VLDL kinetics in hyperlipidemic apolipoprotein E*3-Leiden transgenic mice. *J Lipid Res* 1998;39:1181-1188

139. Volger OL, van der Boom H, de Wit EC, van Duyvenvoorde W, Hornstra G, Plat J, Havekes LM, Mensink RP, Princen HM: Dietary plant stanol esters reduce vldl

cholesterol secretion and bile saturation in apolipoprotein e*3-leiden transgenic mice. *Arterioscler Thromb Vasc Biol* 2001;21:1046-1052

140. Volger OL, Mensink RP, Plat J, Hornstra G, Havekes LM, Princen HM: Dietary vegetable oil and wood derived plant stanol esters reduce atherosclerotic lesion size and severity in apoE*3-Leiden transgenic mice. *Atherosclerosis* 2001;157:375-381

141. Brown MS, Goldstein JL: Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. *Science* 1974;185:61-63

142. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J: Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 1993;92:883-893

143. Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK: Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest* 1994;93:1885-1893

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

145. van Ree JH, Hofker MH, van den Broek WJ, van Deursen JM, van Der BH, Frants RR, Wieringa B, Havekes LM: Increased response to cholesterol feeding in apolipoprotein C1-deficient mice. *Biochem J* 1995;305 (Pt 3):905-911

146. Jong MC, van Ree JH, Dahlmans VE, Frants RR, Hofker MH, Havekes LM: Reduced very-low-density lipoprotein fractional catabolic rate in apolipoprotein C1-deficient mice. *Biochem J* 1997;321 (Pt 2):445-450

147. Simonet WS, Bucay N, Pitas RE, Lauer SJ, Taylor JM: Multiple tissue-specific elements control the apolipoprotein E/C-I gene locus in transgenic mice. *J Biol Chem* 1991;266:8651-8654

148. Jong MC, Dahlmans VE, van Gorp PJ, Breuer ML, Mol MJ, van der ZA, Frants RR, Hofker MH, Havekes LM: Both lipolysis and hepatic uptake of VLDL are impaired in transgenic mice coexpressing human apolipoprotein E*3Leiden and human apolipoprotein C1. *Arterioscler Thromb Vasc Biol* 1996;16:934-940

149. Jong MC, Dahlmans VE, van Gorp PJ, Van Dijk KW, Breuer ML, Hofker MH, Havekes LM: In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor-associated protein-sensitive pathway. *J Clin Invest* 1996;98:2259-2267

150. Jong MC, Hofker MH, Havekes LM: Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. *Arterioscler Thromb Vasc Biol* 1999;19:472-484

151. Jong MC, Gijbels MJ, Dahlmans VE, Gorp PJ, Koopman SJ, Ponec M, Hofker MH, Havekes LM: Hyperlipidemia and cutaneous abnormalities in transgenic mice overexpressing human apolipoprotein C1. *J Clin Invest* 1998;101:145-152

152. Koopmans SJ, Jong MC, Que I, Dahlmans VE, Pijl H, Radder JK, Frolich M, Havekes LM: Hyperlipidaemia is associated with increased insulin-mediated glucose metabolism, reduced fatty acid metabolism and normal blood pressure in transgenic mice overexpressing human apolipoprotein C1. *Diabetologia* 2001;44:437-443

153. Jong MC, Voshol PJ, Muurling M, Dahlmans VEH, Romijn JA, Pijl H, Havekes LM: Protection from obesity and insulin resistance in mice overexpressing human apolipoprotein C1. *Diabetes* 2001;in press:

154. Jukema JW, Zwinderman AH, van Boven AJ, Reiber JH, Van der Laarse A, Lie KI, Bruschke AV: Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler Thromb Vasc Biol* 1996;16:425-430

Chapter 2

Rosuvastatin potently reduces plasma total cholesterol and triglyceride levels by inhibition of VLDL-production in ApoE*3-Leiden transgenic mice

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Abstract

Background The present study was designed to investigate the lipid-lowering properties and the mechanism of action of a new HMG-CoA-reductase inhibitor, rosuvastatin, in female ApoE*3-Leiden transgenic mice.

Methods and results Mice received a high fat/high cholesterol (HFC) diet either containing different doses of rosuvastatin (0 (control), 0.00125, 0.0025 or 0.005% (w/w)) or 0.05% (w/w) lovastatin as a control. The highest dose of rosuvastatin reduced plasma cholesterol and triglyceride levels by 39 and 42%, respectively, compared to the HFC control. Lovastatin had no effect on plasma cholesterol and triglyceride levels.

Rosuvastatin treatment in HFC-fed mice resulted in decreased hepatic VLDL-triglyceride and VLDL-ApoB production. VLDL lipid composition remained unchanged, indicating that plasma lowering of cholesterol and triglycerides was caused by a reduction in the amount of VLDL-particles secreted from the liver. Cholesterol and triglyceride synthesis and ß-oxidation of fatty acids in the liver were not found to be reduced by rosuvastatin treatment, while hepatic lipid content also did not change as a result of rosuvastatin therapy. In contrast, biliary excretion of cholesterol, phospholipids and bile acids was increased upon treatment.

Conclusion The present results indicate that rosuvastatin treatment in ApoE*3-Leiden mice leads to a redistribution of cholesterol and triglycerides over the body, both by reduced hepatic VLDL-production and enhanced enterohepatic circulation of cholesterol and phospholipids, leading to potent reductions in plasma cholesterol and triglyceride levels.

Introduction

Hyperlipidaemia has been shown to be a major risk factor for the development of cardiovascular disease. Recent clinical trials with lipid-lowering HMG-CoA-reductase inhibitors (statins) have shown marked reductions in the risk of cardiovascular events[1]. The main mechanism of action of statins is the reduction of cholesterol synthesis by inhibition of HMG-CoA-reductase activity, resulting in an upregulation of the LDL-receptor[2]. However, there are also indications that statin treatment reduces hepatic VLDL-production, both in patients[3] as well as in animal models[4]. The supply

of cholesterol available for incorporation into nascent lipoprotein particles regulates the secretion of apoB by the liver[3]. Reductions in plasma triglyceride levels by statin therapy have also been found[5]. This hypotriglyceridemic effect could possibly be explained by a reduction in VLDL-particle secretion from the liver, but also a higher clearance rate of remnant lipoproteins by upregulated LDL-receptor activity and increases in lipoprotein lipase activity[6,7] have been suggested.

The National Cholesterol Education Program (NCEP) recommends a reduction of LDL-cholesterol-levels to at least 2.6 mmol/l in patients with coronary artery disease, which usually means a desired cholesterol-lowering effect of 30-40%[8]. However, recent clinical trials suggest that larger LDL-cholesterol lowering will achieve even greater event reductions[9]. Since statins have different efficacies in lipid-lowering[10], the development of statins that potently reduce plasma cholesterol levels at a low dosage may be advantageous.

Several animal models have been used to investigate the lipid-lowering effects of statins and have been shown to respond differently to statin therapy. For example, atorvastatin and simvastatin reduced plasma cholesterol levels in guinea pigs, pravastatin had cholesterol-lowering effects in WHHL rabbits, statin treatment in hamsters has shown both cholesterol-lowering and cholesterol-raising effects and in rats mainly a triglyceride-lowering was found by atorvastatin and lovastatin, correlating well to cholesterol-lowering effects in cholesterol-fed rabbits[11]. Even between mouse models for hyperlipidemia responses to statin therapy differ greatly. Recent studies have shown that ApoE*3-Leiden transgenic mice have human-like lipoprotein profiles due to a defect in the clearance of lipoproteins[12] and are highly responsive to lipid-lowering therapies, such as by plant stanol esters[13] and ACAT-inhibitors[14], but also to statin treatment[15,16]. Studies in LDL receptor deficient mice showed that high dosages of atorvastatin lowered total plasma cholesterol by 30-40%, but the decrease was transient in male mice[14]. Simvastatin, lovastatin and pravastatin were much less efficient than atorvastatin in reducing plasma cholesterol levels in these mice. In mice deficient for ApoE simvastatin treatment had no effect on plasma lipid levels[17], whereas lovastatin even caused an increase in plasma total cholesterol concentrations[18].

The present study was designed to investigate the lipid-lowering properties and the mechanism of action of a new statin, rosuvastatin, in ApoE*3-Leiden transgenic mice. This study demonstrates that rosuvastatin treatment at a low dose reduces plasma

cholesterol and triglyceride levels by inhibition of VLDL-production in ApoE*3-Leiden transgenic mice.

Methods

Mice

Heterozygous female ApoE*3-Leiden mice of F16 generations, 9 to 13 weeks old at the start of the experiment were used. Identification of transgenic mice was performed by an ELISA for human apoE, as previously described[19]. All animal experiments were approved by the institutional committee on animal welfare of TNO.

Diets

During a five week run-in period, 12 animals were kept on standard rat/mouse chow (Hope Farms, The Netherlands) and 72 animals received a semi-synthetic high fat/high cholesterol diet (HFC, 15% saturated fat, 0.25% cholesterol, 40.5% sucrose, all weight by weight). After this period, total plasma cholesterol concentrations were measured. On the basis of equal age and cholesterol level, the chow fed mice were divided into two groups of 6 mice. One group was kept on chow diet (control), the other received 0.005% w/w rosuvastatin mixed in the chow diet. The HFC fed mice were divided into five groups. One group of 18 mice was kept on the HFC diet (control), rosuvastatin was administered to the HFC diet in three doses: 0.00125% (12 mice), 0.0025% (12 mice) and 0.005% (18 mice). One group of 12 mice received 0.05% lovastatin added to the HFC diet. All animals had free access to water and food. Body weight and food intake were monitored during the 5 week treatment study.

Analysis of blood parameters

After a 4 hour fasting period from 9 a.m. to 1 p.m., blood samples were obtained from each individual mouse by tail incision. Blood samples were collected in EDTA coated microvettes (Sarstedt, Nümbrecht, Germany) and centrifuged at 5000 rpm for 10 minutes to obtain plasma samples. Total plasma cholesterol and triglyceride levels were measured enzymatically, using commercially available kits: No 236691 (Boehringer Mannheim GmbH, Mannheim, Germany) and No 337-B (Sigma Diagnostics, Deisenhofen, Germany), respectively. For size fractioning of lipoproteins, 40 μl of pooled plasma was injected onto a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia, Uppsala, Sweden) and eluted at a constant flow rate of 50 μl/min with PBS (pH 7.4, containing 1 mmol/l EDTA). Fractions of 50 μl were collected and assayed for total cholesterol and triglycerides as described above. Plasma alanine aminotransferase (ALAT) was measured enzymatically at group level

(GPT, Boehringer Mannheim No 745138). As a measure for in vivo ß-oxidation of fatty acids, plasma ß-hydroxybutyrate was measured enzymatically (Sigma Diagnostics, No 310-A).

Measurement of hepatic lipid composition

Liver samples were homogenized and samples were taken for measurement of protein content. Lipids were extracted and separated by high performance thin layer chromatography[20]. Quantitation of the amounts was performed by scanning the plates and by integrating the density areas using TINA version 2.09 software.

Hepatic mRNA expression

Total mRNA was isolated from liver by a single step method, followed by electrophoresis, Northern Blotting and hybridization as described by Post *et al.*[21]. Specific ^{32}P-labeled cDNA probes for the mouse LDL receptor, HMG-CoA-synthase, sterol regulatory element binding protein 1c (SREBP1c), fatty acid synthase (FAS), acyl-CoA synthase (ACS), stearoyl-CoA desaturase (SCD), acyl-CoA oxidase (ACO), thiolase and bifunctional enzyme were used for hybridization. SB34 and 18S probes were used as internal standards. The intensity of the hybridization signal was quantified with a Phosphor-Imager (Molecular Dynamics). In chow-fed mice only the expression of LDL receptor and HMG-CoA-reductase was measured.

Expression levels of SREBP-2, diacylglycerol:acyltransferase (DGAT) and microsomal triglyceride transfer protein (MTTP) were measured by real time reverse transcriptase-polymerase chain reaction (RT-PCR), essentially as described by Bloks *et al.*[22]. Primers used were CAA TGG CAC GCT GCA GAC CCT TG and ATG GCC TTC CTC AGA ACG CCA G for SREBP-2, TGT TCA GCT CAG ACA GTG GT and AGG TTG TCT GGA TAG CTC AC for DGAT and ATC TGA TGT GGA CGT TGT GT and CCT CTA TCT TGT AGG TAG TG for MTTP. Amplification was done for 30 s at 95°C, annealing for 30 s at 60, 60 and 57°C for the respective primers, and extension for 30 s at 72°C. These steps were repeated 28, 29 and 28 times for SREBP-2, DGAT and MTTP, respectively. Values were normalised to cyclophilin RNA values. The primers used for cyclophilin were ATC TTG TCC ATG GCA AAT GCT G and GCT CTC CTG AGC TAC AGA AGG (annealing at 54°C, 25 cycles).

VLDL-triglyceride and apoB production

At the end of the experiment, mice were injected with 0.1 ml PBS containing 100 µCi Tran^{35}S-label (ICN) 30 minutes before they were treated with Triton WR1339 (500 mg/kg i.v.). Triton virtually completely inhibits VLDL clearance. The accumulation of VLDL-triglyceride in plasma and the incorporation of labelled amino acid into newly synthesised apoB over 90 minutes were measured essentially as described before[21].

Faecal and biliary excretion of neutral sterols and bile acids

In a separate experiment, 7 mice received the HFC diet and another 7 mice were fed the HFC diet with the addition of 0.005% (w/w) rosuvastatin for 5 weeks. Faeces were collected over a period of 3 days at group level at 3 different time points. For extraction of bile acids, dried faeces (5 mg) were incubated in 1 mL alkaline methanol (methanol : 1 mol/L NaOH 3:1 (v/v) for 2 h at 80°C in screw capped tubes using nor-hyodeoxycholate as an internal standard. Then 9 mL of distilled water was added and the tubes were mixed and centrifuged. The supernatant was subsequently applied to a prepared Sep-Pak C18 solid phase extraction cartridge. After a clean up by wash procedures, bile acids were eluted with 75% methanol. The solvent was evaporated to dryness and the bile acids were derivatized by incubation with 50 µL trifluoroacetic anhydride (TFAA) and 30 µL 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) for 1 hr at 60°C. The bile acid derivatives were separated on a 25 m x 0.25 mm capillary GC column (CP Sil 5B, Chrompack International, Middelburg, The Netherlands) in a 3800 GC gas chromatograph (Varian, Walnut Creek, USA) equipped with a flame ionization detector (FID). The injector and the FID detector were kept at 300°C. The column temperature was programmed from 230 to 280°C. Bile acid derivatives were introduced by split-injection (split ratio 20:1). Quantitation was based on the area ratio of the individual bile acid to the internal standard.

To extract neutral sterol from faeces, dried faeces (10 mg) were treated with 1 mL alkaline methanol as described for bile acid measurement using 5α-cholestane as internal standard. After treatment the tubes were cooled to room temperature and the neutral sterols extracted three times with 3 mL petroleum ether. The combined petroleum ether layers were evaporated to dryness and the neutral sterols silylated by DMF-Silprep (Alltech, Deerfield, USA). Analysis of the sterol derivatives was performed by GC applying the same column as for the bile acids. Quantitation was based on the area ratio of the individual neutral sterol to the internal standard 5α-cholestane.

After 5 weeks on the diets, mice were anaesthetised with Hypnorm and Dormicum, after which the common bile duct was ligated and the gallbladder was cannulated. Bile was collected in 15 minutes interval for 105 minutes. Cholesterol and phospholipid in bile were determined after lipid extraction. Total bile acid in bile was determined using a commercial kit from Nycomed (Oslo, Norway) as described previously[23].

Plasma noncholesterol sterol determination

Plasma noncholesterol sterol concentrations were determined essentially as described by Volger *et al.*[13]. Plasma lathosterol relative to plasma cholesterol levels is used as a measure for total body cholesterol synthesis.

Statistics

All data are presented as mean ± SD. For statistical analysis SPSS 8.0 for Windows was used. Non-parametric Mann-Whitney test was used to compare treatment effects with the control group.

Results

Food, body weight and plasma ALAT

Treatment with rosuvastatin or lovastatin had no effect on body weight or food intake. In time, rosuvastatin treatment resulted in an increase in plasma ALAT levels. HFC-fed mice receiving the highest dose of rosuvastatin (0.005%) showed a four times elevated ALAT level (225 U/l) compared to the control (49 U/l).

Figure 1. The effect of rosuvastatin treatment on plasma total cholesterol and triglyceride levels in HFC-fed (panel A) and chow-fed (panel B) ApoE*3-Leiden transgenic mice. *P<0.05 (n=12 for HFC-fed mice, n=6 for chow-fed mice)

Plasma lipids, lipid composition and lathosterol levels

After 4 weeks of treatment on an HFC diet, 0.0025% and 0.005% rosuvastatin reduced plasma total cholesterol levels by 25% and 39%, respectively, as compared to the control group. 0.00125% rosuvastatin and lovastatin treatment had no significant effect on plasma total cholesterol. Treatment of mice on a chow diet with 0.005% rosuvastatin resulted in a 35% decrease of plasma total cholesterol. All rosuvastatin concentrations reduced triglycerides in HFC-fed mice, whereas on chow diet rosuvastatin treatment had no effect on plasma triglycerides (figure 1).

Figure 2. The effect of rosuvastatin on lipoprotein profiles for cholesterol in HFC-fed (panel A) and chow-fed (panel B) ApoE*3-Leiden transgenic mice after 4 weeks of treatment.

Lipoprotein profiles showed that rosuvastatin treatment on an HFC diet reduced plasma total cholesterol mainly by a reduction in the VLDL and IDL fractions. LDL

shifted to a smaller particle size and a slight reduction in HDL was observed. On a chow diet, rosuvastatin caused a decrease in LDL and HDL (figure 2).

Plasma lathosterol levels relative to plasma cholesterol concentrations were 0.04±0.01 μmol/mmol in the HFC control group and 0.14±0.04 μmol/mmol in the HFC-fed mice treated with 0.005% rosuvastatin, indicating that statin treatment did not reduce whole body cholesterol synthesis.

Hepatic mRNA levels and lipid composition

To investigate whether plasma cholesterol lowering by rosuvastatin could be explained by changes in the hepatic levels of LDL receptor and HMG-CoA-synthase, mRNA expression levels of these enzymes were measured. As shown in table 1 the mRNAs were slightly, but mostly not significantly, decreased in HFC-fed mice treated with rosuvastatin or lovastatin. Expression of the regulator of LDL receptor and HMG-CoA-synthase expression, SREBP-2[24], tended to be higher in the HFC-fed mice treated with rosuvastatin than in their controls. The lack of effect of rosuvastatin on LDL-receptor and HMG-CoA-synthase is probably due to the administration of cholesterol in the diet, since on a chow diet, treatment with 0.005% rosuvastatin resulted in a 25% increase of LDL receptor mRNA and a 143% rise in HMG-CoA-synthase mRNA in the liver.

Table 1. Effect of 0.005% (w/w) rosuvastatin on hepatic mRNA levels of LDL-receptor, HMG-CoA-synthase and SREBP-2 (n=6 mice per group)

	Control chow	Chow + rosuvastatin	Control HFC	HFC + rosuvastatin
LDL receptor				
SB34	100 ± 11	125 ± 11*	100 ± 17	58 ± 20*
18S	100 ± 27	126 ± 33	100 ± 23	103 ± 28
HMG-CoA-synthase				
SB34	100 ± 12	243 ± 91*	100 ± 11	65 ± 30
18S	100 ± 22	231 ± 52*	100 ± 26	114 ± 38
SREBP-2				
Cyclophilin			100 ± 39	152 ± 26#

Expression levels of LDL-receptor, HMG-CoA-synthase and SREBP-2 are presented as compared to the control signals of SB34, 18S or cyclophilin. *P<0.05, #P=0.057

As shown in table 2, treatment with rosuvastatin or lovastatin had no significant effects on the hepatic content of free cholesterol, cholesteryl esters and triglycerides in cholesterol and chow-fed mice, suggesting that no accumulation or depletion of lipids occurs in the liver.

Table 2. Effect of rosuvastatin and lovastatin treatment on hepatic lipid content.

Diet	FC	CE	TG
	μg lipid/ mg liver		
Control HFC diet	20.5 ± 2.8	52.8 ± 9.6	114.3 ± 12.3
HFC + 0.005% rosuvastatin	20.4 ± 4.9	58.9 ± 5.9	113.2 ± 34.2
HFC + 0.05% lovastatin	18.5 ± 1.7	49.8 ± 6.2	82.1 ± 24.8
Control chow diet	12.2 ± 1.4	9.5 ± 2.4	54.5 ± 15.0
Chow + 0.005% rosuvastatin	14.5 ± 1.9	11.5 ± 1.6	76.3 ± 7.8

N = 6 mice per group. Data are presented as mean ± SD. No significant differences were observed.
FC: free cholesterol, CE: cholesteryl ester, TG; triglycerides

To investigate the effect of rosuvastatin therapy on triglyceride synthesis, mRNA levels of the transcription factor SREBP1c, which regulates lipidogenic enzymes[25], were measured. Also the mRNA expression levels of enzymes involved in fatty acid and triglyceride synthesis FAS, ACS, SCAD and DGAT as well as of enzymes involved in ß-oxidation of fatty acids ACO, thiolase and bifunctional enzyme were determined. There were no significant differences in the expression of any of these mRNAs between the rosuvastatin or lovastatin treated mice and the control group (data not shown). Plasma ketonbodies (ß-hydroxybutyrate) were also not changed in the treated groups. These data indicate that the decrease in plasma triglycerides in rosuvastatin-treated mice on HFC diet is not caused by changes in fatty acid synthesis and degradation.

VLDL production

In HFC-fed mice treatment with rosuvastatin reduced VLDL-triglyceride and VLDL-ApoB production (figure 3). Only at a concentration of 0.005% rosuvastatin the decrease was significant for VLDL-triglyceride production ($P<0.05$), whereas VLDL-ApoB production showed a strong tendency to a decrease compared to the control

(P=0.063). Lovastatin treatment had no effect on VLDL-triglyceride and VLDL-ApoB production.

Figure 3. The effect of rosuvastatin on VLDL-triglyceride and VLDL-ApoB production after 5 weeks of treatment. Control values for VLDL-triglyceride and VLDL-ApoB production were 2.5 ± 0.4 mmol · h^{-1} and 39000 ± 12400 dpm ApoB · ml $serum^{-1}$, respectively. *P<0.05, #P=0.063 (n=6 mice per group)

Measurement of the lipid composition of nascent VLDL in the VLDL production experiment showed no effect of rosuvastatin or lovastatin treatment on the percentages of free cholesterol, cholesteryl ester, triglycerides and phospholipids (table 3). This indicates that the number of VLDL-particles secreted and not their composition is reduced, resulting in a decrease in plasma cholesterol and triglyceride levels.

Table 3. Lipid composition of nascent VLDL

	FC	CE	TG	PL
Diet		*% of total lipid*		
HFC control	16±1	12±1	56 ±2	16±1
HFC + 0.005% rosuva	13±3	10±2	60±3	17±3
HFC + 0.05% lova	16±2	9±1	60±2	15±1

To investigate whether decreased expression levels of MTTP, which plays a critical role in the assembly and secretion of VLDL in the liver[26], may explain the reduction in VLDL production found in rosuvastatin-treated mice, we determined hepatic expression levels of MTTP. Ratios of MTTP messenger to cyclophilin mRNA were 0.88±0.54 and 0.73±0.43 for HFC-fed mice treated with rosuvastatin and their HFC controls, respectively. This difference was not significant.

Biliary and faecal excretion of neutral sterols and bile acids

Since liver synthesis of cholesterol and triglycerides and hepatic lipid composition are not changed, the decrease in cholesterol and triglyceride excretion via VLDL has to be compensated by another route for elimination from the liver. Therefore, we measured biliary and faecal output of neutral sterols and bile acids (table 4).

Table 4. Effect of rosuvastatin on flow and composition of bile and faecal neutral sterol and bile acid secretion.

	Control HFC	HFC + 0.005% rosuvastatin
Bile flow	3.11 ± 0.41	3.87 ± 0.55*
($\mu l \cdot min^{-1} \cdot 100$ g body weight^{-1})		
Output		
(nmol $\cdot min^{-1} \cdot 100$ g body weight^{-1})		
Bile acid output	130.9 ± 42.7	219.9 ± 70.7*
Cholesterol output	14.3 ± 5.4	21.4 ± 6.6#
Phospholipid output	22.5 ± 5.7	31.7 ± 5.4*
Hepatic bile acid synthesis	104.1 ± 39.9	146.8 ± 55.1*
(nmol $\cdot min^{-1} \cdot 100$ g body weight^{-1})		
Bile acid pool size	2.44 ± 1.10	3.82 ± 1.29##
(μmol$\cdot 100$ g body weight^{-1})		
Faecal neutral sterols	63.5 ± 3.9	69.3 ± 7.2
(μmol $\cdot$ day^{-1} $\cdot$ 100 g body weight^{-1})		
Faecal bile acids	10.7 ± 1.3	9.2 ± 1.4
(μmol $\cdot$ day^{-1} $\cdot$ 100 g body weight^{-1})		

N = 6 mice per group. *P<0.05, #P=0.063, ##P=0.082.

Data from bile cannulation indicated that the bile flow ($P<0.05$) as well as the bile acid ($P<0.05$) and the phospholipid output ($P<0.005$) and the hepatic bile acid synthesis ($P<0.01$) were increased by rosuvastatin treatment. Cholesterol output and bile acid pool size also strongly tended to be increased ($P=0.063$ and $P=0.082$, respectively). These data suggest that rosuvastatin causes enhanced excretion of cholesterol from the liver, both by increased bile acid synthesis and excretion and via free biliary cholesterol excretion.

Faeces measurements revealed no difference in faecal neutral sterol and bile acid excretion between the control group and the rosuvastatin-treated group. This indicates that the excreted bile acids and free cholesterol are efficiently taken up again in the intestine.

Together these data point to an increased enterohepatic circulation of cholesterol and bile acids during rosuvastatin treatment.

Discussion

In the present study, the new HMG-CoA-reductase inhibitor rosuvastatin dose-dependently reduces plasma cholesterol and triglyceride levels in HFC-fed ApoE*3-Leiden transgenic mice. In chow fed animals only a reduction in plasma cholesterol was found. Plasma cholesterol and triglyceride reductions are mainly caused by a decrease in the amount of VLDL-particles produced by the liver and by an increase of biliary bile acid, cholesterol and phospholipids excretion from the liver, together resulting in a redistribution of lipids in the body.

Our results showed that rosuvastatin treatment potently reduces plasma cholesterol levels by 35% in chow-fed ApoE*3-Leiden transgenic mice and by 40% in HFC-fed mice. Treatment of HFC-fed mice with 0.05% lovastatin had no effect on plasma total cholesterol levels. This is in agreement with a previous study in male ApoE*3-Leiden mice, where lovastatin only reduced plasma total cholesterol concentrations at a dosage of 0.1%[15]. In a pilot study, we found that 0.1% lovastatin in the diet had liver-toxic effects in ApoE*3-Leiden female mice, leading us to choose the lower dosage of 0.05% lovastatin. Plasma cholesterol reduction by 0.005% (5 mg/kg/day) rosuvastatin was comparable to a study with atorvastatin in ApoE*3-Leiden mice, which showed reductions of 50% with a dosage of 0.01%[16]. Rosuvastatin treatment

in rats and dogs recently also resulted in very potent 50% cholesterol reductions at respectively 0.8 mg/kg and 0.2 mg/kg[27].

The main lipid-lowering action of statins in man is the up-regulation of LDL-receptors, thereby enhancing the clearance of lipoproteins from the circulation[2]. This was indeed found in the chow-fed mice treated with rosuvastatin. However, when LDL-receptor expression is suppressed by cholesterol feeding in HFC-fed mice no effect on LDL-receptor mRNA was found in the liver anymore. Feeding a high cholesterol diet down-regulates SREBP-2 expression[28], leading to decreased LDL-receptor expression in the liver[29], which can probably not be overruled by statin-treatment. We merely found a slight tendency of an induction of SREBP-2 expression levels by rosuvastatin treatment. SREBP-2 strongly regulates HMG-CoA-synthase expression[24], possibly explaining why we found no effect on HMG-CoA-synthase mRNA levels in the HFC-fed mice treated with rosuvastatin, whereas chow-fed animals respond to rosuvastatin treatment by increasing endogenous cholesterol-synthesis through up-regulation of HMG-CoA-synthase. Another reason why the LDL-receptor is not regulated is that lover cholesterol homeostasis in mice is mainly regulated via cholesterol synthesis rather that by LDL-receptors[30].

Since in HFC-fed mice treated with rosuvastatin plasma cholesterol-lowering could not be explained by up-regulation of LDL-receptor activity, hepatic VLDL-production was measured. VLDL-ApoB production strongly tended to be decreased by rosuvastatin treatment, whereas VLDL lipid composition did not change, indicating that less VLDL-particles were produced and thereby less cholesterol was secreted into the circulation.

Hepatic lipid composition was not changed in the rosuvastatin-treated mice, while cholesterol synthesis was even found to be increased, as measured by plasma lathosterol levels This suggested that the excretion of cholesterol into the bile either as biliary cholesterol or as bile acids had to be increased. We found that rosuvastatin treatment enhanced bile flow and increased biliary bile acid and phospholipid output. Also biliary cholesterol output and bile acid pool size strongly tended to be increased by rosuvastatin, suggesting that rosuvastatin causes enhanced excretion of cholesterol from the liver, both via increased bile acid synthesis and enhanced biliary cholesterol excretion. In studies with rats simvastatin[31] and pravastatin[32] treatment increased the secretion of cholesterol into bile via enhanced expression of hepatic mdr2, which couples biliary cholesterol secretion to that of phospholipids[33]. Our

findings on enhanced biliary phospholipid and cholesterol excretion following statin treatment also suggest higher activity of hepatic mdr2. Faecal bile acid and neutral sterol excretion were not changed in rosuvastatin treated mice, indicating that intestinal re-uptake of the increased bile acids and biliary cholesterol was very efficient. Altogether, these data point out that rosuvastatin treatment in ApoE*3-Leiden mice leads to increased enterohepatic cycling of cholesterol in the form of free cholesterol and bile acids. Hepatic VLDL-production is found to be lower in rosuvastatin treated mice, whereas cholesterol synthesis is not decreased, resulting in a redirection of cholesterol towards increased bile acid synthesis and biliary cholesterol excretion.

Our results also show a reduction in plasma triglyceride levels after rosuvastatin treatment in HFC-fed mice. Several studies have shown before that statins not only improve cholesterol but also triglyceride levels in plasma[5,6], however, the mechanism by which statins reduce plasma triglycerides remains unclear. It has been suggested that statin-therapy increases lipoprotein lipase activity[6,7] or results in higher clearance of remnant particles via the LDL-receptor[34].

In the present study, rosuvastatin-treatment in ApoE*3-Leiden mice had no effect on plasma ketonbodies and also mRNA levels of the enzymes involved in β-oxidation of fatty acids ACO, thiolase and bifunctional enzyme were not changed. This indicates that triglyceride degradation was not changed by rosuvastatin. Rosuvastatin did reduce hepatic VLDL-triglyceride production, resulting in a lower plasma triglyceride level. MTTP is the enzyme responsible for the incorporation of lipids in the nascent VLDL particle[26]. Recently, it was shown that sterol depletion in cells downregulates MTTP mRNA expression via SREBP-1 regulated mechanisms, resulting in reduced lipoprotein production[35]. Incubation of HepG2 cells with atorvastatin also resulted in reduced ApoB-secretion concomitantly with increased intracellular ApoB degradation. These effects were accompanied by a decrease in MTTP mRNA expression[36], suggesting that statin treatment can reduce VLDL-production via downregulation of MTTP. In the present study we did not find an effect of rosuvastatin treatment on hepatic MTTP mRNA expression, but this can again be brought about by the downregulated SREBP expression following high cholesterol feeding. It is possible that activity of MTTP is indeed decreased by the statin treatment, however, this needs further investigation.

Since also less triglycerides entered the plasma via VLDL, but liver lipid composition was not changed in rosuvastatin-treated mice, it was expected that triglyceride synthesis would be reduced. To address this question, mRNA levels of the transcription factor SREBP1c, which regulates lipidogenic enzymes[25], and mRNA levels of the enzymes involved in fatty acid and triglyceride synthesis FAS, ACS, SCAD and DGAT in the liver were measured. No differences were found in the expression levels of SREBP-1c and the enzymes, indicating that triglyceride synthesis was not downregulated by rosuvastatin treatment. However, it has recently been shown that DGAT can also be regulated posttranscriptionally, so hepatic expression levels of DGAT do not fully explain activity of the enzyme[37]. Concomitantly with increased excretion of cholesterol via the bile, we also found enhanced biliary phospholipid secretion. Supposedly, conversion of precursors of triglycerides (e.g. diacylglycerol) into phospholipids is the route via which triglycerides are removed from the plasma compartment into the enterohepatic circulation.

In overview, the current data indicate different mechanisms of action of statin treatment with regard to lipid lowering between humans and our mouse model. In humans, the primary mode of action of statins is the reduction of body cholesterol synthesis, followed by an upregulation of LDL-receptors, resulting in enhanced clearance of lipoproteins from plasma. However, there are also indications that statins reduce VLDL-production in humans[38]. It is known that human liver homeostasis is mainly dependent on dietary cholesterol intake and LDL-receptor activity, whereas in mice endogenous cholesterol synthesis is much more important[30]. Therefore, in cholesterol-fed mice, we did not find an effect of rosuvastatin treatment on LDL-receptor expression levels. Also, in humans decreased total body cholesterol synthesis as a consequence of statin treatment, lowers biliary cholesterol excretion[39]. In contrast, in the present study, we found enhanced excretion of bile after rosuvastatin treatment. This was found to be a compensatory mechanism for the reduced output of cholesterol and triglycerides from the liver by the reduction of VLDL-production. Hence, rosuvastatin treatment in ApoE*3-Leiden mice leads to a redistribution of cholesterol and triglycerides over the body, both by reduced hepatic VLDL-production and enhanced enterohepatic circulation of cholesterol and phospholipids, leading to potent reductions in plasma cholesterol and triglyceride levels.

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Reference List

1. Bucher HC, Griffith LE, Guyatt GH: Systematic review on the risk and benefit of different cholesterol-lowering interventions. *Arterioscler Thromb Vasc Biol* 1999;19:187-195
2. Brown MS, Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986;232:34-47
3. Thompson GR, Naoumova RP, Watts GF: Role of cholesterol in regulating apolipoprotein B secretion by the liver. *J Lipid Res* 1996;37:439-447
4. Burnett JR, Wilcox LJ, Telford DE, Kleinstiver SJ, Barrett PH, Newton RS , Huff MW: Inhibition of HMG-CoA reductase by atorvastatin decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *Arterioscler Thromb Vasc Biol* 1997;17:2589-2600
5. Stein EA, Lane M, Laskarzewski P: Comparison of statins in hypertriglyceridemia. *Am J Cardiol* 1998;81:66B-69B
6. Verd JC, Peris C, Alegret M, Diaz C, Hernandez G, Vazquez M, Adzet T, Laguna JC, Sanchez RM: Different effect of simvastatin and atorvastatin on key enzymes involved in VLDL synthesis and catabolism in high fat/cholesterol fed rabbits. *Br J Pharmacol* 1999;127:1479-1485
7. Schoonjans K, Peinado-Onsurbe J, Fruchart JC, Tailleux A, Fievet C, Auwerx J: 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors reduce serum triglyceride levels through modulation of apolipoprotein C-III and lipoprotein lipase. *FEBS Lett* 1999;452:160-164
8. Expert Panel on Detection EaToHBCiA: Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486-2497
9. Brown WV: Cholesterol lowering in atherosclerosis. *Am J Cardiol* 2000;86:29H-32H
10. Pincus J: Comparative dose efficacy study of atorvastatin versus simvastatin, pravastatin, lovastatin, and fluvastatin in patients with hypercholesterolemia (the CURVES study). *Am.J.Cardiol.* 1998;82:406-407
11. Krause BR, Princen HM: Lack of predictability of classical animal models for hypolipidemic activity: a good time for mice? *Atherosclerosis* 1998;140:15-24
12. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM, Frants RR: Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol.Chem.* 1993;268:10540-10545
13. Volger OL, van der Boom H, de Wit EC, van Duyvenvoorde W, Hornstra G, Plat J, Havekes LM, Mensink RP, Princen HM: Dietary plant stanol esters reduce vldl cholesterol secretion and bile saturation in apolipoprotein e*3-leiden transgenic mice. *Arterioscler Thromb Vasc Biol* 2001;21:1046-1052

14. Delsing DJ, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit EC, Gijbels MJ, van der Laarse A, Jukema JW, Havekes LM, Princen HM: Acyl-coa:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in apoe*3-leiden mice. *Circulation* 2001;103:1778-1786

15. van Vlijmen BJ, Pearce NJ, Bergö M, Staels B, Yates JW, Gribble AD, Bond BC, Hofker MH, Havekes LM, Groot PH: Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. *Arzneimittelforschung.* 1998;48:396-402

16. Delsing DJM, Jukema JW, Van der Laarse A, Emeis JJ, Havekes LM, Princen HMG: Differential effects of amlodipine and atorvastatin treatment and their combination on atherosclerosis in ApoE*3-Leiden transgenic mice. *(submitted)* 2001

17. Sparrow CP, Burton CA, Hernandez M, Mundt S, Hassing H, Patel S, Rosa R, Hermanowski-Vosatka A, Wang PR, Zhang D, Peterson L, Detmers PA, Chao YS, Wright SD: Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol* 2001;21:115-121

18. Quarfordt SH, Oswald B, Landis B, Xu HS, Zhang SH, Maeda N: In vivo cholesterol kinetics in apolipoprotein E-deficient and control mice. *J Lipid Res* 1995;36:1227-1235

19. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM: Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410

20. Havekes LM, de Wit EC, Princen HM: Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity. *Biochem J* 1987;247:739-746

21. Post SM, de Roos B, Vermeulen M, Afman L , Jong MC, Dahlmans VE, Havekes LM, Stellaard F, Katan MB, Princen HM: Cafestol increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis. *Arterioscler Thromb Vasc Biol* 2000;20:1551-1556

22. Bloks VW, Plosch T, Van Goor H, Roelofsen H, Baller J, Havinga R, Verkade HJ, van Tol A, Jansen PL, Kuipers F: Hyperlipidemia and atherosclerosis associated with liver disease in ferrochelatase-deficient mice. *J Lipid Res* 2001;42:41-50

23. Kuipers F, van Ree JM, Hofker MH, Wolters H, In't Veld G, Havinga R, Vonk RJ, Princen HM, Havekes LM: Altered lipid metabolism in apolipoprotein E-deficient mice does not affect cholesterol balance across the liver. *Hepatology* 1996;24:241-247

24. Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H: Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest* 1998;101:2331-2339

25. Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S, Yamada N : Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. *J Biol Chem* 1999;274:35832-35839

26. Shelness GS, Sellers JA: Very-low-density lipoprotein assembly and secretion. *Curr Opin Lipidol* 2001;12:151-157

27. McTaggart F, Buckett L, Davidson R, Holdgate G, McCormick A, Schneck D, Smith G, Warwick M: Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. *Am J Cardiol* 2001;87:28B-32B

28. Shimomura I, Bashmakov Y, Shimano H, Horton JD, Goldstein JL, Brown MS: Cholesterol feeding reduces nuclear forms of sterol regulatory element binding proteins in hamster liver. *Proc Natl Acad Sci U S A* 1997;94:12354-12359

29. Goldstein JL, Brown MS: Regulation of the mevalonate pathway. *Nature* 1990;343:425-430

30. Dietschy JM, Turley SD, Spady DK: Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res* 1993;34:1637-1659

31. Hooiveld GJ, Vos TA, Scheffer GL, Van Goor H, Koning H, Bloks V, Loot AE, Meijer DK, Jansen PL, Kuipers F, Muller M: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) induce hepatic expression of the phospholipid translocase mdr2 in rats. *Gastroenterology* 1999;117:678-687

32. Carrella M, Feldman D, Cogoi S, Csillaghy A, Weinhold PA: Enhancement of mdr2 gene transcription mediates the biliary transfer of phosphatidylcholine supplied by an increased biosynthesis in the pravastatin-treated rat. *Hepatology* 1999;29:1825-1832

33. Elferink RP, Tytgat GN, Groen AK: Hepatic canalicular membrane 1: The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. *FASEB J* 1997;11:19-28

34. Ginsberg HN: Effects of statins on triglyceride metabolism. *Am J Cardiol* 1998;81:32B-35B

35. Sato R, Miyamoto W, Inoue J, Terada T, Imanaka T, Maeda M: Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J Biol Chem* 1999;274:24714-24720

36. Wilcox LJ, Barrett PH, Huff MW: Differential regulation of apolipoprotein B secretion from HepG2 cells by two HMG-CoA reductase inhibitors, atorvastatin and simvastatin. *J Lipid Res* 1999;40:1078-1089

37. Spahr MA, Barkalow D, Dash D, Essenburg AD, Krause BK, Ramharack R: Dietary regulation of diacylglycerol acyltransferase (DGAT) activity and mRNA levels. *Atherosclerosis* 2001;151:285-285(Abstract)

38. Aguilar-Salinas CA, Barrett H, Schonfeld G: Metabolic modes of action of the statins in the hyperlipoproteinemias. *Atherosclerosis* 1998;141:203-207

39. Hanson DS, Duane WC: Effects of lovastatin and chenodiol on bile acid synthesis, bile lipid composition, and biliary lipid secretion in healthy human subjects. *J Lipid Res* 1994;35:1462-1468

Chapter 3

Differential effects of amlodipine and atorvastatin treatment and their combination on atherosclerosis in ApoE*3-Leiden transgenic mice

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Abstract

Background The present study was designed to investigate the antiatherosclerotic effects of the calcium-antagonist amlodipine, the HMG-CoA-reductase inhibitor atorvastatin and the combination of both in ApoE*3-Leiden transgenic mice.

Methods and Results Four groups of 15 female ApoE*3-Leiden mice were put on a high cholesterol diet. One group received 0.002% (w/w) amlodipine mixed in the diet, which had no effect on plasma total cholesterol levels. Another group received the diet with 0.01% (w/w) atorvastatin, resulting in a decrease of plasma total cholesterol by 50% by a reduction in VLDL-production. The combination group received both amlodipine and atorvastatin mixed in the diet. After 28 weeks, atherosclerosis in the aortic root was quantified. Treatment with amlodipine had no significant effect on atherosclerotic lesion area, whereas atorvastatin markedly reduced atherosclerosis by 77% compared to the control group. Atorvastatin also reduced inflammation markers. The combination of amlodipine and atorvastatin tended to reduce lesion area even more, by 61% compared to the atorvastatin only group, however, this effect did not reach statistical significance. Amlodipine treatment significantly reduced calcification in the lesions, whereas atorvastatin alone had no effect. The combination of amlodipine and atorvastatin resulted in a near absence of calcium deposits in the lesions.

Conclusion This study demonstrates that amlodipine treatment alone does not significantly reduce atherosclerotic lesion development. Atorvastatin was shown to have strong antiatherosclerotic effects and co-treatment with amlodipine showed no significant additional effect.

Introduction

The primary action of calcium-channel blockers is to inhibit calcium ion entry through voltage-gated transmembrane channels. Several important processes in atherosclerosis may be influenced by calcium-antagonists since they require calcium-dependent energy. In vitro studies have shown that calcium-antagonists can reduce lipoprotein oxidation, proliferation and migration of smooth muscle cells. However, the antiatherosclerotic activity of calcium-channel blockers probably involves many additional properties of the compounds, because calcium-independent mechanisms,

such as binding of monocytes to the endothelial cell layer, esterification of cholesterol in macrophages and expression of matrix metalloproteinases in vascular endothelial cells have also been shown to be inhibited by calcium-antagonists[1].

The majority of animal studies investigating the antiatherosclerotic effects of calcium-antagonists was performed in cholesterol-fed rabbits, which only develop fatty streaks on a very cholesterol-rich diet. These studies showed a reduction in atherosclerosis[2], but since atherosclerosis development in this model differs from that in the human situation, conclusions are difficult to be drawn from these results.

Two clinical trials - the INTACT-study[3] and the Montreal Heart Study[4] - showed that calcium-antagonists had no effect on the progression of existing lesions but could only reduce the number of newly formed lesions. More recent data from the PREVENT-trial demonstrated that amlodipine-treatment had no effect on progression of atherosclerosis or cardiovascular events, but was only associated with reduction in cardiovascular morbidity[5]. Therefore, the antiatherosclerotic effects of calcium-antagonists remain under debate.

In a recent retrospective study on data from the REGRESS-study, evaluating the effect of pravastatin on progression and regression of coronary artery disease[6], it was found that the combination of pravastatin and a calcium-antagonist was more succesful in retarding the progression of atherosclerosis than pravastatin alone[7]. Therefore, we hypothesized that calcium-antagonists are only able to counteract the adverse effects of cholesterol on the arterial wall, when treated together with a cholesterol-lowering drug, such as an HMG-CoA-reductase inhibitor (statin).

It is well known that statins have antiatherosclerotic effects due to potent lipid-lowering, but also by lipid-independent effects[8], such as antioxidant effects[9], nitric-oxide mediated improvement of endothelial function[10] and anti-inflammatory properties[11]. A number of these effects have thus far only been investigated in vitro and in cell culture experiments. Besides clear lipid-lowering effects[12-14], animal studies on the effects of statins have also shown direct antiatherosclerotic effects[15-18]. Most of these atherosclerosis studies were performed in rabbits, whereas studies in mice have never been published. Also the combination of a statin and a calcium-antagonist has never been investigated in an animal model for atherosclerosis.

ApoE*3-Leiden mice are an established model for hyperlipidemia and atherosclerosis and have been shown to be responsive to lipid-lowering therapy, such as lovastatin and gemfibrozil[13], stanolesters[19] or the ACAT-inhibitor avasimibe[20]. We used

ApoE*3-Leiden transgenic mice to test whether treatment with the calcium-antagonist amlodipine or atorvastatin-treatment have antiatherosclerotic effects in vivo. Additionally, we investigated whether amlodipine treatment acts synergistically to the antiatherosclerotic effect of atorvastatin treatment, i.e. whether an antiatherosclerotic effect of a calcium-antagonist is only seen after lipid-lowering. Therefore, the mice were treated with either amlodipine, atorvastatin or the combination of both, followed by quantification of atherosclerotic lesion area in the aortic root.

This study demonstrates that amlodipine treatment alone does not significantly reduce atherosclerotic lesion development, whereas treatment with atorvastatin decreased lesion area substantially. The combination of amlodipine and atorvastatin demonstrated no additional anti-atherosclerotic effect. Only amlodipine treatment significantly reduced atherosclerotic lesion calcification.

Methods

Experimental design

A group of 60 heterozygous female ApoE*3-Leiden mice of F16 generations, 9 to 13 weeks of age at the start was used. The presence of the transgene was assessed by an ELISA for human apoE[21].

Before the start of the study, animals were kept on a standard rat/mouse chow (Hope Farms, The Netherlands). During a three week run-in period, all animals received a semi-synthetic high fat/high cholesterol diet (HFC) with added cholate to facilitate intestinal uptake of fat and cholesterol, thereby increasing plasma cholesterol levels. The composition of the diet was 40.5% sucrose, 20% casein, 15% cocoa butter, 10% cornstarch, 5.95% cellulose, 5.1% mineral mixture, 1% choline chloride, 1% corn oil, 0.2% methionine, 0.5% cholesterol and 0.05% cholate (all weight by weight). After this period, the animals were divided into four groups of 15 mice each on the basis of age and cholesterol level and were put on the different treatment diets for 28 weeks. The control group received the HFC diet. The atorvastatin group received the HFC diet with 0.01% w/w atorvastatin. The amlodipine group received the HFC diet with the addition of 0.002% w/w amlodipine, which did not reduce blood pressure. The fourth group received the HFC diet with the combination of atorvastatin (0.01% w/w) and amlodipine (0.002% w/w). Atorvastatin and amlodipine were kindly provided by Pfizer Inc., Groton, USA. All animals had free access to water and food. Body weight and food intake were monitored during the study. All animal experiments were approved by the institutional committee on animal welfare of TNO.

Lipid and lipoprotein analysis, measurements of fibrinogen, serum amyloid A and von Willebrand factor

After a 4 hour fasting period from 9 a.m. to 1 p.m., blood samples were obtained by tail incision. Total plasma cholesterol, triglyceride levels and lipoprotein profiles were measured at t = 0,3,6,11,19,24 and 28 weeks as described before19.

Fibrinogen was determined by ELISA, using rabbit-anti-rat fibrin monomer IgG as catching antibody, and peroxidase-conjugated goat-anti-mouse fibrinogen IgG (Nordic, Tilburg, the Netherlands) as tagging antibody. A mouse plasma with gravimetrically-determined fibrinogen content was used for calibration.

Von Willebrand factor was determined by ELISA, using antisera from Dakopatts (Glostrup, Denmark), essentially a described by Ingerslev22 using rat plasma for calibration.

SAA was determined by ELISA, as prescribed by the manufacturer (Biosource International, Nivelles, Belgium).

Measurement of systolic blood pressure

After seven weeks of diet feeding, systolic blood pressure was measured in all groups, to evaluate the effect of amlodipine in the mice. The Blood Pressure System for Rats and Mice (RTBP1001, Harvard Apparatus, Holliston, MA, USA) was used. For each mouse, systolic blood pressure was measured three times.

VLDL-production

In a separate experiment, 6 female ApoE*3-Leiden mice were put on HFC diet and another 6 mice received the HFC diet with 0.01% atorvastatin. After 5 weeks on the diets, hepatic VLDL-triglyceride and –apoB production and VLDL-composition were measured after intravenous [^{35}S]methionine and Triton WR1339 injection as described before[19].

Histological assessment of atherosclerosis

After 28 weeks of diet feeding, mice were sacrificed after anaesthetizing and blood collection as described[20]. The hearts were dissected, stored overnight in phosphate-buffered 3.8% formalin fixation and embedded in paraffin. Serial cross sections (5 μm thick, spaced 30 μm apart) throughout the entire aortic valve area were used for histological analysis. Sections were routinely stained with haematoxylin-phloxine-saffron (HPS).

Per mouse, 4 sections with intervals of 30 μm were used for quantification and qualification of atherosclerotic lesions. All sections were imaged and stored under identical lighting, microscope- (Nikon), camera- (Hitachi) and PC conditions. Total lesion areas were determined using Leica Qwin image analysis software. All analyses were performed by the

same operator, blinded for the experimental group allocation. The degree of calcification in the atherosclerotic lesions was determined by quantification of a Von Kossa staining; collagen content was quantified morphometrically after staining with Sirius Red.
For determination of severity of atherosclerosis, the lesions were classified into five categories as described before[21,23]: I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, V) severe plaque. Per mouse the percentages of all lesions found in the respective lesion categories were calculated.

Statistics

All data are presented as mean ± SD (unless otherwise indicated). For statistical analysis SPSS 8.0 for Windows was used. Overall comparisons between groups were performed with the Kruskall-Wallis test. If a significant difference was found, distribution-free multiple comparisons were performed to find which groups differed from each other. If only two groups were compared, Mann-Whitney rank sum tests were used. Jonckheere-Terpstra test was used to determine a trend in atherosclerosis reduction in ordered groups. P values less than 0.05 were considered significant.

Results

The effect of amlodipine and atorvastatin on plasma cholesterol levels, lipoprotein composition and systolic blood pressure and of atorvastatin on VLDL-production

Amlodipine and/or atorvastatin treatment did not affect body weights or food intake. Average plasma cholesterol levels from 7 blood draws (at t = 0,3,6,11,19,24 and 28 weeks) were 21.8±3.4 mmol/l for the control group, 20.7±1.9 mmol/l for the amlodipine group, 10.3±1.5 mmol/l for the atorvastatin group and 11.1±0.6 mmol/l in the combination group. The effect of amlodipine and atorvastatin treatment on plasma cholesterol levels in time is shown in figure 1A. Amlodipine had no effect on plasma cholesterol levels. Atorvastatin alone or in combination with amlodipine significantly reduced plasma cholesterol by 53% ($P<0.005$) as compared to the control group.
Triglyceride levels did not differ significantly between the groups during the 28 weeks of treatment (1.4±0.4, 1.4±0.4, 0.9±0.2 and 0.9±0.1 mmol/l for the control, amlodipine, atorvastatin and combination group, respectively). Lipoprotein profiles at 25 weeks of treatment showed that atorvastatin mainly reduced VLDL, IDL and LDL cholesterol, whereas HDL cholesterol was not affected (figure 1B).

Figure 1. The effect of treatment with amlodipine, atorvastatin and the combination of both on plasma lipids. **A.** Plasma total cholesterol levels measured in time of 15 mice per group. **B.** Cholesterol profiles of plasma lipoproteins.

To determine the metabolic background for the reduced plasma cholesterol levels in atorvastatin-treated mice, we measured VLDL-production and lipid composition in a separate experiment. Results in table 1 show that atorvastatin treatment significantly reduced VLDL-triglyceride and -apoB production compared to the control group by 66% and 64%, respectively. VLDL lipid composition was not affected in atorvastatin-treated mice. This indicates that the number of VLDL-particles and consequently the amount of triglycerides and cholesterol secreted from the liver was reduced by atorvastatin therapy, causing the decrease in plasma cholesterol levels.

Table 1. Effect of atorvastatin on VLDL-triglyceride and -apoB production and VLDL lipid composition

	Control	Atorvastatin
VLDL-triglyceride production	53.1 ± 16.5	18.2 ± 6.4* (34%)
(μmol·h^{-1}·kg body weight^{-1})		
VLDL-ApoB production	2278 ± 576	817 ± 305* (36%)
(10^3 dpm·h^{-1}·kg body weight^{-1})		
VLDL lipid composition		
(μmol/mg ApoB)		
Cholesteryl ester	28.8 ± 9.1	23.5 ± 10.2
Free cholesterol	13.3 ± 5.2	11.3 ± 3.9
Triglycerides	24.4 ± 9.1	32.1 ± 15.8
Phospholipids	12.8 ± 4.2	10.8 ± 4.1

Data are presented as mean ± SD for 6 mice per group.
Percentages of control values are presented between brackets.
*P<0.05 compared to control.

After 7 weeks on the diets systolic blood pressure was 107±8 mmHg for the control group, 108±13 in the amlodipine group, 105±11 mmHg in the atorvastatin group and 107±10 in the combination group. There were no signifcant differences found between groups. Plasma amlodipine concentrations were not different between the amlodipine and the amlodipine + atorvastatin groups (66.4±9.6 ng/ml and 60.9±4.4 ng/ml, respectively). Since we wanted to study the effects of amlodipine on development of atherosclerosis by direct effect on the vessel wall and not by blood pressure-lowering the amlodipine concentration in the diet was not increased for the remaining 20 weeks of the study.

The effect of amlodipine and atorvastatin on plasma levels of fibrinogen, serum amyloid A and von Willebrand factor

Since there is growing evidence that atherosclerosis is an inflammatory disease[24] and plasma levels of inflammatory markers can be used as predictors of the risk for cardiovascular events, we measured plasma fibrinogen and serum amyloid A (SAA) at the endpoint of the study. Von Willebrand factor (vWf) was measured as a marker for vessel wall patency.

Table 2. Plasma levels of fibrinogen, SAA and vWf at the endpoint of the study.

Group	Fibrinogen (mg/ml)	SAA (μg/ml)	vWf (% of pooled rat plasma)
Control	2.61 (2.45-2.84)	11.1 (8.3-15.0)	178 (152-209)
Amlodipine	2.31 (1.87-2.64)	9.3 (8.0-14.1)	192 (164-218)
Atorvastatin	2.04 (1.67-2.35)*	3.7 (2.3-5.8)*#	159 (129-180)
Atorva + amlo	1.60 (1.32-1.98)*	2.9 (1.9-3.5)*#	146 (102-169)#

Data are presented as median (95% confidence interval) of 14 mice per group.
*P<0.05 as compared to control
#P<0.05 as compared to amlodipine

Data on plasma levels of fibrinogen, SAA and vWf are shown in table 2. Fibrinogen levels were lower in the atorvastatin and the atorvastatin + amlodipine treated groups as compared to the control. This also applies for SAA, where the combination of atorvastatin and amlodipine also resulted in lower SAA levels than in the amlodipine only group. vWf was lowest in the atorvastatin + amlodipine treated group and differed significantly from the amlodipine only group.

The effect of amlodipine and atorvastatin on atherosclerosis development

Atherosclerotic lesions were quantified in cross sections of the aortic valve area in the heart of each individual mouse. Per mouse, 4 sections over a 110 μm interval, representing the area where the aortic valves are clearly visible, were analysed by computer image analysis. Representative photomicrographs of atherosclerotic lesions found in the different groups are shown in figure 2.

In the control group atherosclerotic lesions mostly covered the entire valve area, containing a large necrotic core with calcification and cholesterol clefts. Amlodipine treatment also resulted in large lesions covering almost the entire valve area. Lesions contained large lipid pools and necrosis. In the atorvastatin group, atherosclerotic lesions were smaller and did not cover the entire valve area. Clearly smaller lipid pools and necrotic areas were found in the intima and media as compared to the control and amlodipine groups. In the group treated with both amlodipine and atorvastatin lesions were relatively small, whereas lipid pools and small necrotic areas were found in the intima and media.

The average lesion area per section for the individual groups is shown in figure 3A. For the control group the average lesion area per section was 329±153 μm^2*1000.

Amlodipine had no significant effect on lesion area (256±128 μm^2*1000). Treatment with atorvastatin resulted in a 77% reduction of lesion area (75±78 μm^2*1000, $p<0.005$), whereas the combination of atorvastatin and amlodipine reduced lesion area by 91% as compared to the control (29±22 μm^2*1000, $p<0.005$). As compared to the group treated with atorvastatin only, the combination of atorvastatin and amlodipine reduced atherosclerotic lesion area by 61%, however, this effect did not reach statistical significance ($p=0.25$). Further statistical analysis by the Jonckheere-Terpstra test revealed that the groups, when placed in the order control, amlodipine, atorvastatin and the combination of both, showed a significant trend to a reduction in atherosclerosis ($p=0.001$).

Figure 2. Representative photomicrographs of atherosclerotic lesions found in the four treatment groups (haematoxylin-phloxine-saffron staining). The control and amlodipine examples show a type V lesion (severe plaque), the atorvastatin example a type IV lesion (moderate plaque) and the atorva + amlo photomicrograph shows a type III lesion (mild plaque).

We also evaluated the effect of treatment with atorvastatin and amlodipine on the severity of the lesions. For each animal, the same 4 sections of the aortic valve area

as used for measurement of lesion size were analysed for their types of lesions and the percentages of lesions belonging to the respective lesion categories were calculated. Figure 3B shows the percentages of type I-III lesions (fatty streaks and mild plaques) and type IV-V lesions (moderate and severe plaques). In the control and amlodipine treated groups about 90% of all lesions consisted of severe lesions. In the atorvastatin and atorvastatin + amlodipine groups lesions were almost equally distributed over the two categories of lesions. This indicates that atorvastatin treatment not only reduced plaque size, but also lesion severity. Amlodipine alone or in combination with atorvastatin had no additional effect on lesion severity.

Figure 3. The effect of treatment with amlodipine, atorvastatin and the combination of both on atherosclerosis (n=14 (control), n=10 (amlo), n=13 (atorva), n=10 (amlo+atorva)). **A.** Total atherosclerotic lesion area per section. Data are presented as mean ± SD. ***P<0.005. **B.** Severity of the atherosclerotic lesion as determined by the percentage of lesions classified as type I-III lesions (fatty streaks and mild plaques) and type IV-V lesions (moderate and severe plaques). Data are presented as mean ± SEM. ***P<0.005.

The effect of amlodipine and atorvastatin on calcification and collagen content of the atherosclerotic lesions

To determine whether amlodipine and atorvastatin treatment had an effect on markers of plaque stability, calcium deposits and collagen content in the atherosclerotic plaques in all groups were quantified. There were no quantitative differences in collagen content of the lesions between the treatment groups. About 55% of the lesion area consisted of collagen fibers (data not shown). Figure 4A shows photomicrographs of examples of atherosclerotic lesions with either large or very small calcium deposits.

Figure 4. The effect of treatment with amlodipine, atorvastatin and the combination of both on calcification of atherosclerotic lesions (n=14 (control), n=10 (amlo), n=13 (atorva), n=10 (amlo+atorva)). **A.** Representative photomicrographs of atherosclerotic lesions with a large (left) or small (right) calcification. Black dots indicated by arrows represent calcium deposits in the atherosclerotic lesions. **B.** The total area of calcification per section. Data are presented as mean ± SEM. *P<0.05, ***P<0.005.

As shown in figure 4B, amlodipine decreased calcification significantly as compared to the control group (-64%), whereas atorvastatin treatment had no significant effect on calcification. The combination of atorvastatin and amlodipine significantly reduced the percentage of calcification as compared to the control group (-95%), the atorvastatin group (-87%) and the amlodipine group (-83%). These results indicate that the combination of atorvastatin and amlodipine can reduce the amount of calcification in atherosclerotic plaques and that atorvastatin treatment adds to the effect of amlodipine in reducing calcification.

Discussion

In the present study, treatment with amlodipine has no significant effect on atherosclerosis development, whereas atorvastatin reduces the amount and severity of atherosclerosis in ApoE*3-Leiden transgenic mice. The combination of amlodipine and atorvastatin had no additional effects on atherosclerosis in the aortic valve area as compared to atorvastatin alone. In addition, amlodipine alone and the combination of amlodipine and atorvastatin decrease the amount of calcium depositions in the lesions.

Our results show that atorvastatin treatment effectively and stably reduces plasma total cholesterol levels by reducing VLDL-production. A decreased VLDL-production as a cause of plasma cholesterol lowering by statins has also been reported previously both in patients and animal models[25,26]. The cause for the decreased VLDL-production was shown to be inhibition of hepatic cholesterol synthesis by statin treatment resulting in decreased availability of cholesterol for incorporation into VLDL-particles and consequently decreased apoB-secretion by an increase in intracellular degradation[27].

In contrast to other mouse models for hyperlipidemia, i.e. LDL receptor deficient[12] and ApoE deficient mice[28,29], ApoE*3-leiden transgenic mice respond well to statin treatment[13]. In LDL receptor deficient mice only much higher dosages of atorvastatin lowered plasma cholesterol by 30-40%, but the decrease was transient in male mice most probably due to compensatory mechanisms to maintain cholesterol homeostasis in the liver. Only in female mice cholesterol-lowering was maintained[12]. It has been shown that female patients have a lower capability to inactivate atorvastatin[30], resulting in longer residence times for the drug. In this respect it is also

important to mention that we performed our studies with a high fat containing diet, resulting in an increased bioavailability of atorvastatin as compared to chow (unpublished data). We also used female mice possibly explaining the lack of compensatory cholesterol synthesis through sustained inhibition by atorvastatin. Also the fact that female ApoE*3-Leiden mice produce more VLDL than male mice[31], may contribute to the larger effect of inhibition of VLDL-production in these mice by atorvastatin.

Simvastatin treatment in mice deficient for apoE had no effect on plasma lipids[28] and lovastatin treatment even caused an increase in plasma cholesterol[29]. The respective residence time and bioavailability of the latter statins are reportedly much lower than those for atorvastatin[32]. Probably the induction of HMG-CoA-reductase expression following statin treatment overcomes the inhibitory capability of these less potent statins, resulting in a net increase in cholesterol synthesis. This effect has also been shown to occur in pravastatin-treated rats[33,34].

Since the primary action of statins is the upregulation of LDL-receptors following inhibition of cholesterol synthesis[35], the expected effect of statin-treatment in LDL-receptor and ApoE deficient mice may also be smaller, as was indeed found. The cholesterol lowering effect found in our study also exceeds the decrease in cholesterol found in casein-fed rabbits (-22%)[36] treated with an equal dosage of atorvastatin, i.e. 10 mg/kg bodyweight per day. Amlodipine had no effect on plasma total cholesterol, making atherosclerosis measurements in the atorvastatin and the amlodipine + atorvastatin groups comparable on the basis of equal plasma total cholesterol levels. In other animal studies[37] and clinical trials[5,38] calcium-antagonists have also not been shown to affect plasma lipoprotein concentrations. Since we wanted to study the effects of amlodipine on development of atherosclerosis by a direct effect on the vessel wall and not via its blood pressure-lowering properties, a dosage was used that had no effect on systolic blood pressure (3 mg/kg bodyweight per day).

Since there is growing evidence that atherosclerosis is an inflammatory disease[24] and plasma levels of inflammatory markers can be used as predictors of the risk for cardiovascular events, we measured plasma fibrinogen and serum amyloid A (SAA) at the endpoint of the study. Data from clinical trials indicate that fibrinogen is an independent risk factor for cardiovascular disease[39]. This also holds true for elevated C-reactive protein[40] to which SAA levels are correlated[41,42]. The hemostatic

compound von Willebrand factor (vWf) that is also known as a marker for vessel wall patency has also recently been implicated as an important risk factor for recurrent myocardial infarction[43]. Measurement of vWf, fibrinogen and SAA showed that amlodipine had no effect on these parameters, indicating that the compound has no anti-inflammatory properties. Atorvastatin alone, but mostly the combination of amlodipine and atorvastatin lowered these markers. Regression analysis of plasma cholesterol concentrations and levels of SAA, fibrinogen and vWf showed a correlation for SAA and fibrinogen. However, their levels could only be accounted for by variation in cholesterol levels by 30 and 19%, respectively. In the case of vWf no significant correlation was found with plasma cholesterol. Several clinical trials have also shown that lipid levels or changes in lipids do not correlate to levels of inflammation markers[44,45], so the reductions in markers for inflammatory responses cannot simply be attributed to the cholesterol-lowering effect of atorvastatin. In addition, multivariate regression in the present study revealed that next to plasma cholesterol as the most important factor in determining the extent of atherosclerosis (P=0.001), SAA clearly tended to play a role in the development of atherosclerosis (P=0.086). Therefore, the current data possibly suggest that anti-inflammatory activity of treatment with atorvastatin or the combination of amlodipine and atorvastatin can add to their anti-atherosclerotic effects.

Measurement of atherosclerotic lesion area in the aortic root after 28 weeks of treatment with either amlodipine, atorvastatin or the combination of both, revealed that amlodipine itself had no significant effect on development of atherosclerosis. This is in contrast to studies performed in rabbits, which show that amlodipine can attenuate the early steps in atherosclerosis[2,46]. This discrepancy could be explained by the fact that rabbits only develop fatty streaks consisting of macrophages, whereas the ApoE*3-Leiden mice used in our study develop atherosclerotic lesions that resemble the human pathology in the vessel wall[21,23,47]. Our results are corroborated by results from a recent prospective trial investigating the effect of amlodipine on coronary atherosclerosis progression, which demonstrated that amlodipine had no effect on angiographic progression of coronary atherosclerosis or the risk of major cardiovascular event[5], indicating that the anti-atherosclerotic effects of calcium-antagonists remain under discussion.

Our results show that treatment with atorvastatin alone reduces atherosclerotic lesion area by 77%. A large part of this reduction is attributable to its lipid-lowering effect,

but direct effects on the vessel wall are likely to have contributed to the decrease in atherosclerosis[28], however, this study was not designed to investigate this question. Although several animal models have shown anti-atherosclerotic effects of statins[15-18], only recently the first two studies of such effects of a statin in a mouse model was published. However, one of these studies used C57Bl6 mice, which received poloxamer 407 injections (a surfactant) to induce hyperlipidemia[48] and the other used apoE deficient mice which do not respond to statin therapy with relation to their plasma lipid levels[28]. In our study, transgenic mice were used, which develop diet-induced hyperlipidemia resembling the human situation and show reduced plasma lipids at statin treatment, therefore making the effect of anti-atherosclerotic therapy more comparable to expected effects in patients.

A possible synergistic effect of calcium-antagonists and statins was first proposed by Jukema et al[7], in a retrospective study on data from the REGRESS-study[6] reporting that the combination of pravastatin and a calcium-antagonist was more successful in decreasing progression of atherosclerosis than pravastatin alone. We therefore hypothesized that amlodipine can only exert antiatherosclerotic properties, when treated together with a cholesterol-lowering drug, such as atorvastatin.

The combination of amlodipine and atorvastatin reduced atherosclerosis even further than atorvastatin alone, by 91% as compared to the control group. In comparison with atorvastatin alone, a reduction in atherosclerotic lesion area of 61% was found, suggesting a synergistic effect of amlodipine and atorvastatin. This effect did not reach statistical significance. However, further statistical analysis revealed a significant trend towards a reduction in atherosclerosis in the subsequent groups. Because of the considerable lipid-lowering by atorvastatin it is possible that amlodipine co-treatment could not further improve the antiatherosclerotic effect of atorvastatin. Since there is a tendency to treat coronary artery disease patients with increasingly higher dosages of statins or statins that have more potent lipid-lowering properties to achieve aggressive lipid-lowering, it is highly questionable whether additional therapy with calcium-antagonists would be extra beneficial with respect to development of atherosclerosis in man. This, however, remains to be investigated.

Currently, in atherosclerosis studies attention has shifted from merely measuring lesion size to studying plaque composition as well as plaque area. Typical features of a vulnerable plaque, making it prone to rupture causing clinical symptoms of atherosclerosis, are the presence of a large lipid pool with many macrophages, a thin

collagen-poor fibrous cap and relatively few smooth muscle cells[49]. In the present study, no effect on collagen content in the atherosclerotic lesions by amlodipine, atorvastatin or combination treatment was found. However, amlodipine clearly reduced the amount of calcification in the lesions, whereas atorvastatin had no significant effect on calcification. The combination of amlodipine and atorvastatin resulted in the near absence of calcium deposits found in plaques.

The relation of calcification and plaque vulnerability remains unclear. Some suggest that calcium stabilizes the plaque[50] and may not be a reliable indicator of the risk of coronary events[51]. However, others have shown that coronary or aortic calcification is predictive of coronary artery disease[52-54]. In view of direct effects on plaque stability it has also been suggested that calcification may upregulate synthesis of matrix metalloproteinases, thereby making the plaque more prone to rupture[55]. From this point of view, amlodipine may have plaque stabilizing features.

In conclusion, this study in transgenic ApoE*3-Leiden mice demonstrates that amlodipine treatment alone did not significantly reduce atherosclerotic lesion development, whereas treatment with atorvastatin decreased lesion area substantially. The combination of amlodipine and atorvastatin showed no additional anti-atherosclerotic effect.

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Reference List

1. Schachter M: Calcium antagonists and atherosclerosis. *Int.J.Cardiol* 1997;62 Suppl 2:S9-15.:S9-15
2. Keogh AM, Schroeder JS: A review of calcium antagonists and atherosclerosis. *J.Cardiovasc.Pharmacol.* 1990;16 Suppl 6:S28-35
3. Lichtlen PR, Hugenholtz PG, Rafflenbeul W, Hecker H, Jost S, Deckers JW: Retardation of angiographic progression of coronary artery disease by nifedipine. Results of the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT). INTACT Group Investigators. *Lancet* 1990;335:1109-1113

4. Waters D, Lesperance J, Francetich M, Causey D, Theroux P, Chiang YK, Hudon G, Lemarbre L, Reitman M, Joyal M: A controlled clinical trial to assess the effect of a calcium channel blocker on the progression of coronary atherosclerosis. *Circulation* 1990;82:1940-1953

5. Pitt B, Byington RP, Furberg CD, Hunninghake DB, Mancini GB, Miller ME, Riley W, for TP, I: Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. *Circulation* 2000;102:1503-1510

6. Jukema JW, Bruschke AV, van Boven AJ, Reiber JH, Bal ET, Zwinderman AH, Jansen H, Boerma GJ, van Rappard FM, Lie KI: Effects of lipid lowering by pravastatin on progression and regression of coronary artery disease in symptomatic men with normal to moderately elevated serum cholesterol levels. The Regression Growth Evaluation Statin Study (REGRESS). *Circulation* 1995;91:2528-2540

7. Jukema JW, Zwinderman AH, van Boven AJ, Reiber JH, Van der Laarse A, Lie KI, Bruschke AV: Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler.Thromb.Vasc.Biol.* 1996;16:425-430

8. Davignon J, Laaksonen R: Low-density lipoprotein-independent effects of statins. *Curr.Opin.Lipidol.* 1999;10:543-559

9. Aviram M, Rosenblat M, Bisgaier CL, Newton RS: Atorvastatin and gemfibrozil metabolites, but not the parent drugs, are potent antioxidants against lipoprotein oxidation. *Atherosclerosis* 1998;138:271-280

10. Laufs U, La F, V, Plutzky J, Liao JK: Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 1998;97:1129-1135

11. Strandberg TE, Vanhanen H, Tikkanen MJ: Effect of statins on C-reactive protein in patients with coronary artery disease. *Lancet* 1999;353:118-119

12. Bisgaier CL, Essenburg AD, Auerbach BJ, Pape ME, Sekerke CS, Gee A, W÷lle S, Newton RS: Attenuation of plasma low density lipoprotein cholesterol by select 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in mice devoid of low density lipoprotein receptors. *J.Lipid.Res.* 1997;38:2502-2515

13. van Vlijmen BJ, Pearce NJ, Bergö M, Staels B, Yates JW, Gribble AD, Bond BC, Hofker MH, Havekes LM, Groot PH: Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. *Arzneimittelforschung.* 1998;48:396-402

14. Conde K, Pineda G, Newton RS, Fernandez ML: Hypocholesterolemic effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in the guinea pig: atorvastatin versus simvastatin. *Biochem.Pharmacol.* 1999;58:1209-1219

15. Bocan TM, Mazur MJ, Mueller SB, Brown EQ, Sliskovic DR, O'Brien PM, Creswell MW, Lee H, Uhlendorf PD, Roth BD: Antiatherosclerotic activity of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase in cholesterol-fed rabbits: a biochemical and morphological evaluation. *Atherosclerosis* 1994;111:127-142

16. Shiomi M, Ito T, Tsukada T, Yata T, Watanabe Y, Tsujita Y, Fukami M, Fukushige J, Hosokawa T, Tamura A: Reduction of serum cholesterol levels alters lesional composition of atherosclerotic plaques. Effect of pravastatin sodium on atherosclerosis in mature WHHL rabbits. *Arterioscler.Thromb.Vasc.Biol.* 1995;15:1938-1944

17. Harsch M, Braesen JH, Niendorf A: Effects of low-dose pravastatin sodium on plasma cholesterol levels and aortic atherosclerosis of heterozygous WHHL rabbits fed a low cholesterol (0.03%) enriched diet for one year. *Atherosclerosis* 1997;128:139-147

18. Pauletto P, Puato M, Faggin E, Sartore S: Low-dose cerivastatin inhibits spontaneous atherogenesis in heterozygous watanabe hyper lipidemic rabbits. *J.Vasc.Res.* 2000;37:189-194

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

20. Delsing DJ, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit EC, Gijbels MJ, van der Laarse A, Jukema JW, Havekes LM, Princen HM: Acyl-coa:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in apoe*3-leiden mice. *Circulation* 2001;103:1778-1786

21. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM: Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J.Clin.Invest.* 1994;93:1403-1410

22. Ingerslev J: A sensitive ELISA for von Willebrand factor (vWf:Ag). *Scand.J.Clin.Lab.Invest.* 1987;47:143-149

23. Gijbels MJ, van der Cammen M, van der Laan LJ, Emeis JJ, Havekes LM, Hofker MH, Kraal G: Progression and regression of atherosclerosis in APOE3- Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* 1999;143:15-25

24. Ross R: Atherosclerosis--an inflammatory disease. *N.Engl.J.Med.* 1999;340:115-126

25. Thompson GR, Naoumova RP, Watts GF: Role of cholesterol in regulating apolipoprotein B secretion by the liver. *J.Lipid.Res.* 1996;37:439-447

26. Burnett JR, Wilcox LJ, Telford DE, Kleinstiver SJ, Barrett PH, Newton RS, Huff MW: Inhibition of HMG-CoA reductase by atorvastatin decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *Arterioscler.Thromb.Vasc.Biol.* 1997;17:2589-2600

27. Wilcox LJ, Barrett PH, Huff MW: Differential regulation of apolipoprotein B secretion from HepG2 cells by two HMG-CoA reductase inhibitors, atorvastatin and simvastatin. *J.Lipid.Res.* 1999;40:1078-1089

28. Sparrow CP, Burton CA, Hernandez M, Mundt S, Hassing H, Patel S, Rosa R, Hermanowski-Vosatka A, Wang PR, Zhang D, Peterson L, Detmers PA, Chao YS, Wright SD: Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler.Thromb.Vasc.Biol.* 2001;21:115-121

29. Quarfordt SH, Oswald B, Landis B, Xu HS, Zhang SH, Maeda N: In vivo cholesterol kinetics in apolipoprotein E-deficient and control mice. *J.Lipid.Res.* 1995;36:1227-1235

30. Gibson DM, Bron NJ, Richens A, Hounslow NJ, Sedman AJ, Whitfield LR: Effect of age and gender on pharmacokinetics of atorvastatin in humans. *J.Clin.Pharmacol.* 1996;36:242-246

31. van Vlijmen BJ, van 't Hof HB, Mol MJ, van der Boom H, van der Zee A, Frants RR, Hofker MH, Havekes LM: Modulation of very low density lipoprotein production and clearance contributes to age- and gender- dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. *J.Clin.Invest.* 1996;97:1184-1192

32. Malinowski JM: Atorvastatin: a hydroxymethylglutaryl-coenzyme A reductase inhibitor. *Am.J.Health.Syst.Pharm.* 1998;55:2253-2267

33. Fujioka T, Nara F, Tsujita Y, Fukushige J, Fukami M, Kuroda M: The mechanism of lack of hypocholesterolemic effects of pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, in rats. *Biochim.Biophys.Acta.* 1995;1254:7-12

34. Fujioka T, Tsujita Y: Effects of single administration of pravastatin sodium on hepatic cholesterol metabolism in rats. *Eur.J.Pharmacol.* 1997;323:223-228

35. Brown MS, Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986;232:34-47

36. Krause BR, Newton RS: Lipid-lowering activity of atorvastatin and lovastatin in rodent species: triglyceride-lowering in rats correlates with efficacy in LDL animal models. *Atherosclerosis* 1995;117:237-244

37. Kramsch DM: Limits of lipid-lowering therapy: the potential benefits of amlodipine as an antiatherosclerotic agent. *Int.J.Cardiol.* 1997;62:S119-S124

38. Trost BN, Weidmann P: Metabolic effects of calcium antagonists in humans, with emphasis on carbohydrate, lipid, potassium, and uric acid homeostases. *J.Cardiovasc.Pharmacol.* 1988;12 Suppl 6:S86-92:S86-S92

39. Maresca G, Di Blasio A, Marchioli R, Di Minno G: Measuring plasma fibrinogen to predict stroke and myocardial infarction: an update. *Arterioscler.Thromb.Vasc.Biol.* 1999;19:1368-1377

40. Haverkate F, Thompson SG, Pyke SD, Gallimore JR, Pepys MB: Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet* 1997;349:462-466

41. Morrow DA, Rifai N, Antman EM, Weiner DL, McCabe CH, Cannon CP, Braunwald E: Serum amyloid A predicts early mortality in acute coronary syndromes: A TIMI 11A substudy. *J.Am.Coll.Cardiol.* 2000;35:358-362

42. Fyfe AI, Rothenberg LS, deBeer FC, Cantor RM, Rotter JI, Lusis AJ: Association between serum amyloid A proteins and coronary artery disease: evidence from two distinct arteriosclerotic processes. *Circulation* 1997;96:2914-2919

43. Wiman B, Andersson T, Hallqvist J, Reuterwall C, Ahlbom A, Defaire U: Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von Willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. *Arterioscler.Thromb.Vasc.Biol.* 2000;20:2019-2023

44. Albert MA, Danielson E, Rifai N, Ridker PM: Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study. *JAMA* 2001;286:64-70

45. Ridker PM, Rifai N, Clearfield M, Downs JR, Weis SE, Miles JS, Gotto AMJ: Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. *N.Engl.J.Med.* 2001;344:1959-1965

46. Henry PD, Bentley KI: Suppression of atherogenesis in cholesterol-fed rabbit treated with nifedipine. *J.Clin.Invest.* 1981;68:1366-1369

47. Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, de Muinck ED: Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276-283

48. Johnston TP, Baker JC, Hall D, Jamal S, Palmer WK, Emeson EE: Regression of poloxamer 407-induced atherosclerotic lesions in C57BL/6 mice using atorvastatin. *Atherosclerosis* 2000;149:303-313

49. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, Clinton SK, Sukhova GK, Lee RT: Macrophages and atherosclerotic plaque stability. *Curr.Opin.Lipidol.* 1996;7:330-335

50. Doherty TM, Detrano RC, Mautner SL, Mautner GC, Shavelle RM: Coronary calcium: the good, the bad, and the uncertain . *Am.Heart.J.* 1999;137:806-814

51. Detrano RC, Wong ND, Doherty TM, Shavelle RM, Tang W, Ginzton LE, Budoff MJ, Narahara KA: Coronary calcium does not accurately predict near-term future coronary events in high-risk adults. *Circulation* 1999;99:2633-2638

52. Mintz GS, Pichard AD, Popma JJ, Kent KM, Satler LF, Bucher TA, Leon MB: Determinants and correlates of target lesion calcium in coronary artery disease: a clinical, angiographic and intravascular ultrasound study. *J.Am.Coll.Cardiol.* 1997;29:268-274

53. Iribarren C, Sidney S, Sternfeld B, Browner WS: Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke, and peripheral vascular disease. *JAMA* 2000;283:2810-2815

54. Detrano R, Hsiai T, Wang S, Puentes G, Fallavollita J, Shields P, Stanford W, Wolfkiel C, Georgiou D, Budoff M, Reed J: Prognostic value of coronary calcification and angiographic stenoses in patients undergoing coronary angiography. *J.Am.Coll.Cardiol.* 1996;27:285-290

55. Bini A, Mann KG, Kudryk BJ, Schoen FJ: Noncollagenous bone matrix proteins, calcification, and thrombosis in carotid artery atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.* 1999;19:1852-1861

Chapter 4

Raman spectroscopic investigation of atorvastatin, amlodipine, and both on atherosclerotic plaque development in ApoE*3-Leiden mice

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Abstract

Background ApoE*3-Leiden transgenic mice allow the controlled study of pathogenesis and treatment of atherosclerosis. We quantified the effects of atorvastatin and amlodipine and the potential synergistic effect of their combination on progression of atherosclerosis in the aorta of these mice, using Raman spectroscopic imaging.

Methods and results 28 mice were fed a high fat/ high cholesterol diet for 28 weeks. They were assigned to a control group receiving a cholesterol rich diet (n=7), or to three groups that received the same diet with either 0.01% w/w atorvastatin (n=7), or 0.002% w/w amlodipine (n=7), or a combination of the two drugs (n=7). Atherosclerosis in the excised, longitudinally opened, intact aortic arch was determined by Raman spectroscopy and Oil Red O staining. The tissue was then homogenized and lipid content of the aorta was determined.

Atorvastatin lowered plasma total cholesterol level by 55% ($p<0.001$). Amlodipine alone had no effect on plasma cholesterol. When mice had been treated with atorvastatin, cholesterol accumulation in the aortic arch was reduced by 91%, and reduction in calcification was 98% ($p<0.001$). Amlodipine demonstrated a 69% ($p<0.05$) reduction in calcification, without reducing the size of the lipid pool. The combination of amlodipine and atorvastatin was as effective as atorvastatin alone. The results obtained with Raman spectroscopy strongly correlated with the results of Oil Red O staining and high performance thin layer chromatography lipid analysis ($r=0.88$ and $r=0.86$, $p<0.0001$ respectively).

Conclusions This study demonstrates the strong atheroprotective potential of atorvastatin on plaque development and chemical composition in the aortic arch of transgenic mice. In addition, the study demonstrates that amlodipine significantly reduces mineralization of a plaque, but does not affect the lipid pool. The combination of drugs showed no synergistic effect on atherosclerosis. This study encourages Raman spectroscopic evaluations of anti-atherosclerotic drugs in larger animals and humans *in vivo*.

Introduction

Clinical trials with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have shown highly significant reductions in the risk of acute coronary events in both primary and secondary prevention. The beneficial effect of statin therapy is caused primarily by its ability to reduce circulating cholesterol levels[1-3], although the beneficial effect may include additional cholesterol-independent effects leading to plaque stabilization[4-7].

Apart from lowering plasma cholesterol levels with statins, many investigations have lent support to the view that a number of key processes in atherosclerosis are calcium dependent and may be influenced by calcium channel blockers (CCBs)[8-14]. From these studies performed in animals and humans, we can conclude that apparently some, but not all types or stages of the atherosclerotic process are inhibited by CCBs. In patients allocated to CCB therapy (amlodipine), a recent prospective randomized trial[14] found no angiographic evidence of inhibition of plaque progression. However in this trial, a 31% reduction of any major fatal or morbid cardiovascular event was reported, thereby demonstrating some atheroprotective potential. In addition, early trials in human subjects have observed a reduction in the number of newly formed lesions[11], or a retardation of the progression of minimal lesions[10] in patients with coronary artery disease who were treated with a CCB. The mechanisms responsible for the anti-atherosclerotic effects of CCBs are still under debate and remain to be investigated[15-17].

Besides the individual anti-atherosclerotic effects of CCBs and statins, it was observed that the combination of a statin (pravastatin) and any CCB was more potent in retarding the progression of atherosclerosis than pravastatin alone[3]. Until now, the synergistic effect of CCB and statin therapy has not been studied in detail, particularly not in terms of chemical composition of the plaque. This was the aim of our study.

Recently, we have shown that ApoE*3-Leiden transgenic mice in combination with Raman spectroscopic imaging of the aortic arch, provide powerful potential for the integral, quantitative study of pathogenesis and treatment of atherosclerosis[18]. This approach allows quantification of accumulations of cholesterol and calcium salts, relative to the amount of protein throughout the entire thickness of the arterial wall, without the need of elaborate sectioning and staining of the samples with standard

histological techniques. Moreover, it has the advantage that it can be applied intravascularly *in vivo*[19]. As the thickness of the fibrous cap (*i.e.* the mass of protein) and the size of the lipid pool (*i.e.* the mass of cholesterol) are correlated with plaque vulnerability[4], this approach may prove valuable in assessing the effects of drugs on the progression, regression, and stability of a plaque. Besides the advantage of the non-destructive technique itself, the ApoE*3-Leiden mouse model is a valuable model for studying hyperlipidemia and atherosclerosis and has shown to be responsive to lipid-lowering and anti-atherosclerotic therapy, such as lovastatin, gemfibrozil, ACAT-inhibitors, and stanolesters[20-24]. The effect of calcium channel inhibitors, alone or in combination with a statin, has not yet been studied in this animal model.

We designed a Raman spectroscopic intervention study to quantify and specify the potentially synergistic effect of the HMG-CoA reductase inhibitor atorvastatin with the dihydropyridine CCB amlodipine, as compared to single drug treatment on the development and chemical composition of atherosclerotic plaques in ApoE*3-Leiden transgenic mice.

Methods

Mice

Male transgenic ApoE*3-Leiden mice were crossbred with female C57BL/6 mice, and heterozygous female mice of F16 generations were used for this experiment. Identification of transgenic mice was performed by an ELISA for human apoE[20]. At the start of the study, mice were between 9 and 13 weeks of age. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Diets

Before the start of the study, animals were kept on a standard mouse chow (Hope Farms, The Netherlands). During a three-week run-in period, all animals received a semi-synthetic high fat/ high cholesterol diet (HFC) with 0.05% cholate to facilitate intestinal uptake of fat and cholesterol, thereby increasing plasma cholesterol levels. This diet contained 40.5% sucrose, 20% casein, 15% cocoa butter, 10% cornstarch, 5.45% cellulose, 5.1% mineral mixture, 1% choline chloride, 1% corn oil, 0.2% methionine, 0.5% cholesterol and 0.05% cholate (all w/w). After the run-in period, total plasma cholesterol concentrations were

measured. On the basis of age and cholesterol level the animals were divided into four groups of 7 mice each, with one control group receiving the HFC diet alone. One group received the same diet as the control group, but with an extra addition of 0.01% w/w atorvastatin (Pfizer Inc., Central Research Division, Groton, USA), approximately equaling a daily dose of 10 mg/kg body weight. This dosage was chosen, based on pilot experiments showing a dose-dependent decrease in plasma cholesterol levels with atorvastatin. The third group received the diet with an addition of 0.002% w/w amlodipine (Pfizer Inc.) corresponding to a daily dose of 2 mg/kg body weight. The fourth group received the control diet with a combination of atorvastatin and amlodipine in the concentrations as mentioned above. The concentration of amlodipine was chosen not to lower blood pressure, since we wanted to study the effects of amlodipine on development of atherosclerosis by a direct effect on the vessel wall and not by blood pressure lowering ability: mice that were treated with amlodipine had an average systolic blood pressure of 103.3 ± 3.0 mmHg (mean ± SEM) compared to an average blood pressure of 105.5 ± 2.5 mmHg in mice that did not receive amlodipine. The addition of atorvastatin to amlodipine treatment did not alter the plasma levels of amlodipine: A random sample survey in four mice in the amlodipine and the combined treatment groups indicated plasma levels of 66.4 ± 4.8 ng/ml (mean ± SEM) and 60.9 ± 2.2 ng/ml, respectively (p=0.338).

All animals had free access to water and food. Body weight and food intake were monitored during the study. All mice were sacrificed at t=28 weeks from the start of randomization.

Lipid analysis

At baseline, at t = 3, 6, 11, 19, 24 weeks and post mortem, blood samples from each individual mouse were obtained. These samples were acquired by tail incision, always after a 4-hour fasting period from 9 a.m. to 1 p.m. Total plasma cholesterol and triglyceride levels were measured enzymatically as described before[18]. Total cholesterol exposure was calculated as the product of the plasma values times week of exposure, and is expressed in mmol/L*week.

Tissue handling

Mouse aortas were dissected, snap frozen in liquid nitrogen and stored at –80 °C until use. Prior to spectroscopic investigation, the first 5-8 mm of each aorta just distally from the aortic valve was cleaned from its adventitial fat and cut open through the outer aortic curvature, longitudinally through the brachio-cephalic, carotid and subclavian arteries[18]. The samples were then moistened with PBS and positioned between two calcium-fluoride windows (Crystran Ltd, Dorset, UK) underneath a scanning microscope (Leica, Cambridge, UK) that

was rebuilt for Raman spectroscopic imaging. With this system, Near Infrared Raman spectra were obtained in 0.25 x 0.25 mm steps over the first 5 mm and full width of the opened aorta[18]. Collection time for each measurement was 10 s. Following Raman spectroscopic investigation, all samples were refrozen at –80°C and stored for further analysis. After thawing, the opened mouse aortas were positioned on a silicone surface in a 60 mm ∅ petri dish with steel pins, and stained with Oil Red O for 24 hours. Digital images were made en face with a Hitachi HV-C20M color CCD camera equipped with a 55 mm Nikon lens. From these images, plaque surface was quantified with a standard computerized method, described by Tangirala *et al.*[25]. Subsequently, the first 5 mm of each aorta was excised and homogenized in a volume of 0.5 ml demineralized water using a micro-turrax (Kinematica GmbH, Littau-Luzern, Switzerland). Lipids were extracted and separated by high performance thin layer chromatography (HPTLC)[26]. Quantitation of the amounts was performed by scanning the plates, and by integrating the density areas using TINA® version 2.09 software (Raytest, Straubenhardt, Germany). The amount of total cholesterol was expressed as μg per mm^2 surface in the aortic arch.

Raman spectroscopic processing

All Raman spectra were processed and analyzed as described previously[18]. For each location of the grid, the relative contributions of protein (P), total cholesterol (TC) and calcium salts (CS) in the arterial wall were derived from the Raman spectra. For each component, arterial surface maps were created[18]. A TC contribution of more than 10% (10-100%) was regarded to represent an atherosclerotic plaque. A weight contribution of more than 10% CS was regarded a calcified plaque. The surface area of regions with TC>10% or CS>10% was calculated and expressed in mm^2. As the relative protein content is dependent on the relative contents of calcium and cholesterol, surface maps of protein were not analyzed (See reference 18 for details).

Statistics

All data are presented as mean ± SEM. For statistical analysis SPSS 9.0 for Windows was used. Because the data were not normally distributed, non-parametric tests were used for comparisons between groups. Overall comparison between groups were performed with the Kruskall-Wallis test. If a significant difference was found, distribution-free multiple comparisons were performed to find which groups differed from each other. If only two groups were compared, Mann-Whitney rank sum tests were used. P values less than 0.05 were regarded statistically significant.

Results

Amlodipine and/or atorvastatin treatment did not affect body weights or food intake. Average total cholesterol exposure (plasma cholesterol times weeks of exposure) and the average size of the atherosclerotic lesion present in the aortic arch were determined. The results for each group are shown in table 1.

Table 1. Effect of amlodipine, atorvastatin and the combination of these drugs on early atherosclerotic plaque development in the proximal aortic arch of ApoE*3-Leiden transgenic mice

			Raman Spectroscopy		ORO	HPTLC
	n	exposure	TC	CS	total lipid	TC*
		(mM x week)	(mm^2)	(mm^2)	(mm^2)	μg/(mm^2)
Control	7	404±27	4.21±0.34	1.91±0.50	3.61±0.51	0.38±0.21
Amlodipine	7	385±8.5	3.45±0.48	0.59±0.22#	3.01±0.47	0.24±0.03
Atorvastatin	7	182±8.9#	0.37±0.10#	0.05±0.02#	0.85±0.15#	0.09±0.007#
Combination	7	204±4.4#	0.35±0.09#	0.03±0.01#	0.87±0.17#	0.08±0.006#

TC, area with total cholesterol > 10%; CS, area with calcium salt content >10%; ORO, area with positive Oil Red O staining; HPTLC, High Performance Thin Layer Chromatography; *TC is expressed as μg total cholesterol per mm^2 of surface area in the aortic arch. #$p<0.05$ versus control; Data are presented as mean ± SEM

Atorvastatin lowered total cholesterol exposure by 55% ($p<0.001$). Amlodipine had no effect on cholesterol exposure. There was no significant difference in cholesterol exposure between the group treated with the combination atorvastatin/amlodipine, and the group treated with atorvastatin alone. Average triglyceride levels did not differ significantly between the groups during the 28 weeks of treatment (1.5±0.2, 1.3±0.2, 0.9±0.1 and 0.9±0.1 mmol/l for the control, amlodipine, atorvastatin and combination group, respectively).

Raman spectroscopy in mice treated with amlodipine revealed a 69% ($p<0.05$) reduction in calcification, but no significant reduction in the area of cholesterol depositions. Treatment with atorvastatin was associated with a 91% ($p<0.001$) reduction in area of cholesterol-rich lesions and a 98% ($p<0.001$) reduction in calcified areas (figure 1). The combination of amlodipine and atorvastatin did not further reduce the area of cholesterol-rich lesions, nor the area of calcification, compared to the group treated with atorvastatin alone.

Figure 1. Effect of amlodipine, atorvastatin and a combination of the two drugs on early atherosclerotic plaque development in the proximal aorta of ApoE*3-Leiden transgenic mice studied by Raman spectroscopy. Plaque surface is calculated as the total plaque area in the proximal aorta with more than 10% cholesterol in the Raman spectrum (left) and with more than 10% calcification in the Raman spectrum (right). Data are presented as mean ± SD. **P<0.01, ***P<0.001.

The Raman spectroscopic results were corroborated by the results of Oil Red O staining of the atherosclerotic plaque. Staining of the first 5 mm of the aortic arch of the atorvastatin treated group showed a reduction in the stained area by 76% (table 1).

In the amlodipine treated group, no such reduction could be observed. There were no differences in size of the stained areas between mice treated with atorvastatin alone or with combined treatment. Plaque surface, measured with Oil Red O staining correlated well with total plaque surface measured with Raman spectroscopy (area with total cholesterol content >10%, r=0.88, p<0.01; figure 2A).

Compared to the control group, the aortic total cholesterol content, determined with HPTLC, was 90% less in mice that were treated with atorvastatin (table 1). This reduction was equal for mice that were treated with the combination therapy, thereby corroborating the results of Raman spectroscopy and Oil Red O staining. The 37% reduction in total cholesterol quantity in the amlodipine treated group was not significant (p=0.17). Aortic cholesterol content as measured by HPTLC strongly correlated with total plaque surface as measured by Raman spectroscopic analyses (r=0.86, p<0.05; figure 2B).

Figure 2. Correlation between the plaque surface with more than 10% cholesterol, determined with Raman spectroscopy and plaque surface stained with Oil Red O (ORO) (**A**) and total cholesterol content obtained with high performance thin layer chromatography (**B**). Total cholesterol is expressed as µg total cholesterol per mm^2 of surface area in the aortic arch.

These data show that the combined treatment with atorvastatin and amlodipine had no synergistic effect on atherosclerosis.

Discussion

In this study, we quantified the anti-atherosclerotic effects of atorvastatin and amlodipine on plaque formation in the proximal aorta of ApoE*3-Leiden transgenic mice. Oil Red O lipid staining, quantitative lipid analysis by HPTLC and integral quantitative Raman spectroscopic mapping of the chemical composition of the aortic arch, demonstrate the anti-atherosclerotic effect of atorvastatin on atherosclerosis in the first 28 weeks of mice that were fed with a high cholesterol diet. Raman spectroscopy demonstrates the beneficial effect of atorvastatin on the size of the lipid pool, but shows an even stronger effect on mineralization of atherosclerosis, as calcification is practically absent in the atorvastatin treated group. Amlodipine did not affect the quantity of cholesterol in the artery wall (Raman, Oil Red O, HPTLC) but demonstrated a significant reduction in size of the calcified deposits in the artery. No synergistic effect of the combination of amlodipine and atorvastatin on retarding the progression of atherosclerosis could be observed.

Previous animal studies were not able to specifically elucidate the chemical compositional changes of the atherosclerotic plaque induced by statins or CCBs such as amlodipine. The present study could discriminate specifically between effects on atheroma and mineralization of a plaque, relative to the protein content. Previous Raman spectroscopic studies have demonstrated high correlation between the weight contributions of cholesterol and calcification calculated by Raman spectroscopy and the weight contributions of these components determined with standard chemical assays (maximum error ~5%)[27]. In addition, the correlation between detecting cholesterol deposits and microcalcifications up to 1 mm deep in the arterial wall with Raman spectroscopy and histological techniques was high[28-30]. Recently, it was demonstrated that with specially developed optical fiber catheters, remote Raman spectra could be acquired intravascularly, from arteries of sheep *in vivo*, in the presence of blood flow[19]. The ability to non-destructively acquire data of this kind will not only reduce the number of larger animals required for this type of studies, but will also offer the possibility to cross-correlate data on plaque progression with many individual animal parameters, such as circulating lipoprotein concentrations. By sequential *in vivo* monitoring of compositional changes of atherosclerotic plaques, similar as in the present *ex vivo* study, Raman spectroscopy can be used to study the proposed beneficial clinical effects of statins (*i.e.*

modification of vulnerable plaques in the coronary arteries by strengthening the fibrous cap and decreasing the lipid core)[7] and CCBs. In the long term, this may enable the development of drug prescription regimens that are tailored to the needs of the individual patient.

The reduction in the development of atherosclerotic plaque in the aortic arch by atorvastatin can mainly be ascribed to its powerful cholesterol lowering effect. Plasma cholesterol decreased by 55% upon treatment with atorvastatin. However, the atherosclerotic lesion size in the aortic arch of the atorvastatin fed mice decreased by 91% (total cholesterol) and 98% (calcification) compared to the control group. These profound effects might be due to effects beyond lipid lowering effects of atorvastatin[6], although this can not be concluded from the present study and will need further attention in future studies. The results correspond to previous results with statins in animal models, such as rabbits[5], guinea pigs[31-33],and mice[18,34].
The reduction in calcification in mice that were treated with amlodipine alone, however, is remarkable. As the mechanisms of anti-atherogenic activity of CCBs include decreased lipid peroxidation, inhibition of TNF-induced apoptosis of endothelial cells and attenuation of the effects of growth factors such as PDGF and free cholesterol[17], it was conceivable that the size of the lipid pool and thus the size of the atherosclerotic lesion would decrease upon treatment with CCBs. This has been reported in several animal studies[9,35], but could not be confirmed in other studies[17,36], including randomized trials with human subjects[10,11]. Even in a recent, prospective trial by Pitt *et al.* that was designed to evaluate the (three year) anti-atherosclerotic potential of amlodipine in patients with coronary artery disease, there was no angiographic evidence of inhibiting plaque formation in the coronary arteries by amlodipine. However, in the same group of patients there was a significant 0.013 mm decrease in carotid intima-media thickness in those treated with amlodipine, versus a 0.033 mm increase in the untreated group[14]. Earlier studies have demonstrated that treatment with CCBs could substantially suppress disease progression of early atherosclerotic lesions[10,11].
Thus, the anti-atherosclerotic action of CCBs, including amlodipine is still under debate. Here, in a well-defined animal model of atherosclerosis, we demonstrate that the dihydropyridine CCB amlodipine apparently does have an anti-atherosclerotic effect. However this effect is not on total plaque size, nor on the lipid pool, but

specifically on delaying mineralization of the atherosclerotic plaque. The mechanism by which amlodipine inhibits plaque mineralization, as well as the exact molecular and cellular mechanisms responsible for calcium crystal formation in the artery are unclear[17,37] and need to be further investigated. Possible mechanisms of calcium channel blockade in inhibiting calcification of the plaque are numerous and include the inhibition of smooth muscle cell migration and proliferation and their expression of proteins that are associated with vascular calcification[17,38].

A previous retrospective analysis of the REGRESS trial has revealed a delayed progression of coronary stenosis and a two-fold reduction in the number of new lesions in patients with coronary artery disease, who are treated with a combination of a statin and a CCB compared to patients treated with a statin alone[3]. We could not confirm the advantage of combined treatment on atherosclerosis in this study. The size of the atherosclerotic lesion in the mouse aortic arch was approximately equal for mice treated with atorvastatin alone compared to those treated with the combination of amlodipine and atorvastatin. As plaque was almost absent in mice treated with atorvastatin alone, the dose of atorvastatin (10 mg/kg body weight) may have been too high to permit detection of a potentially synergistic effect of amlodipine on plaque progression. In clinical practice, however, there is a tendency to lower the plasma cholesterol according to the 'the lower the better' opinion and administer statins in much higher dosage. Therefore, this study may have clinical implications, as it is doubtful whether patients treated with high doses of statins are further protected from atherosclerosis when co-treated with a CCB. Nevertheless, for a clinical answer, this remains to be investigated in a randomized, prospective trial in human subjects.

In conclusion, the present study demonstrates the atheroprotective potential of atorvastatin and amlodipine on plaque composition in the aorta of ApoE*3-Leiden transgenic mice. Atorvastatin potently reduced both the size of the lipid pool and the amount of calcification in the aorta. Amlodipine reduced the extent of calcification, but did not affect the size of the lipid pool. No synergistic effect of adding amlodipine to atorvastatin could be observed. With the possibility of performing intravascular, catheter-based Raman spectroscopy, this study encourages similar evaluations of anti-atherosclerotic drugs in larger animals and humans *in vivo.*

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Reference List

1. 4S Study group. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994;344:1383-89.
2. WOSCOPS Study group. West of Scotland Coronary Prevention Study: identification of high-risk groups and comparison with other cardiovascular intervention trials. *Lancet* 1996;348:1339-42.
3. Jukema JW, Zwinderman AH, van Boven AJ, *et al.* Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler.Thromb.Vasc.Biol.* 1996;16:425-30.
4. Kullo IJ, Edwards WD, Schwartz RS. Vulnerable plaque: pathobiology and clinical implications. *Ann.Intern.Med.* 1998;129:1050-60.
5. Shiomi M, Ito T, Tsukada T, *et al.* Reduction of serum cholesterol levels alters lesional composition of atherosclerotic plaques. Effect of pravastatin sodium on atherosclerosis in mature WHHL rabbits. *Arterioscler.Thromb.Vasc.Biol.* 1995;15:1938-44.
6. Vaughan CJ, Murphy MB, Buckley BM. Statins do more than just lower cholesterol. *Lancet* 1996;348:1079-82.
7. Koh KK. Effects of statins on vascular wall: vasomotor function, inflammation, and plaque stability. *Cardiovasc.Res.* 2000;47:648-57.
8. Kramsch DM, Aspen AJ, Rozler LJ. Atherosclerosis: Prevention by agents not affecting abnormal levels of blood lipids. *Science* 1981;213:1511-12.
9. Henry PD, Bentley KI. Suppression of atherogenesis in cholesterol-fed rabbit treated with nifedipine. *J.Clin.Invest.* 1981;68:1366-69.
10. Waters D, Lesperance J, Francetich M, *et al.* A controlled clinical trial to assess the effect of a calcium channel blocker on the progression of coronary atherosclerosis. *Circulation* 1990;82:1940-53.
11. Lichtlen PR, Hugenholtz PG, Rafflenbeul W, Hecker H, Jost S, Deckers JW. Retardation of angiographic progression of coronary artery disease by nifedipine. Results of the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT). INTACT Group Investigators. *Lancet* 1990;335:1109-13.
12. Tulenko TN, Laury-Kleintop L, Walter MF, Mason RP. Cholesterol, calcium and atherosclerosis: is there a role for calcium channel blockers in atheroprotection? *Int.J.Cardiol.* 1997;62 Suppl 2:55-66.
13. Mason RP, Walter MF, Trumbore MW, Olmstead EG, Jr., Mason PE. Membrane antioxidant effects of the charged dihydropyridine calcium antagonist amlodipine. *J.Mol.Cell Cardiol* 1999;31:275-81.

14. Pitt B, Byington RP, Furberg CD, *et al.* Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. *Circulation* 2000;102:1503-10.

15. Thaulow E. Pharmacologic effects of calcium channel blockers on restenosis. *J.Cardiovasc.Pharmacol.* 1999;33 Suppl 2:12-16.

16. Nayler WG. Review of preclinical data of calcium channel blockers and atherosclerosis. *J.Cardiovasc.Pharmacol.* 1999;33 Suppl 2:7-11.

17. Catapano AL. Calcium antagonists and atherosclerosis. Experimental evidence. *Eur.Heart J.* 1997;18 Suppl A:80-86.

18. van de Poll SWE, Römer TJ, Volger OL, *et al.* Raman spectroscopic evaluation of the effects of diet and lipid-lowering therapy on atherosclerotic plaque development in mice. *Arterioscler.Thromb.Vasc.Biol.* 2001;21:1630-35

19. Buschman HP, Marple ET, Wach ML, *et al.* In vivo determination of the molecular composition of artery wall by intravascular Raman spectroscopy. *Anal.Chem.* 2000;72:3771-75.

20. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, *et al.* Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J.Clin.Invest.* 1994;93:1403-10.

21. Delsing DJ, Offerman EH, van Duyvenvoorde W, *et al.* Acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in ApoE*3-Leiden mice. *Circulation* 2001;103:1778-86.

22. van Vlijmen BJ, Pearce NJ, Bergo M, *et al.* Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. *Arzneimittelforschung.* 1998;48:396-402.

23. Volger OL, van der BH, de Wit EC, *et al.* Dietary plant stanol esters reduce VLDL cholesterol secretion and bile saturation in apolipoprotein E*3-Leiden transgenic mice. *Arterioscler.Thromb.Vasc.Biol.* 2001;21:1046-52.

24. Gijbels MJ, van der Cammen M, van der Laan LJ, *et al.* Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* 1999;143:15-25.

25. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J.Lipid Res.* 1995;36:2320-28.

26. Havekes L, de Wit ECM, Princen HMG. Cellular free cholesterol in HepG2 cells is only partially available for downregulation. *Biochem.J.* 1987;247:739-46.

27. Brennan JF, Römer TJ, Lees RS, Tercyak AM, Kramer JR, Jr., Feld MS. Determination of human coronary artery composition by Raman spectroscopy. *Circulation* 1997;96:99-105.

28. Römer TJ, Brennan JF, III, Fitzmaurice M, *et al.* Histopathology of human coronary atherosclerosis by quantifying its chemical composition with Raman spectroscopy. *Circulation* 1998;97:878-85.

29. Römer TJ, Brennan JF, III, Bakker Schut TC, et al Raman spectroscopy for quantifying cholesterol in intact coronary artery wall. *Atherosclerosis* 1998;141:117-24.

30. Römer TJ, Brennan JF, III, Puppels GJ, *et al.* Intravascular ultrasound combined with Raman spectroscopy to localize and quantify cholesterol and calcium salts in atherosclerotic coronary arteries. *Arterioscler.Thromb.Vasc.Biol.* 2000;20:478-83.

31. Harsch M, Braesen JH, Niendorf A. Effects of low-dose pravastatin sodium on plasma cholesterol levels and aortic atherosclerosis of heterozygous WHHL rabbits fed a low cholesterol (0.03%) enriched diet for one year. *Atherosclerosis* 1997;128:139-47.

32. Bocan TM, Mazur MJ, Mueller SB, *et al.* Antiatherosclerotic activity of inhibitors of 3-hydroxy-3- methylglutaryl coenzyme A reductase in cholesterol-fed rabbits: a biochemical and morphological evaluation. *Atherosclerosis* 1994;111:127-42.

33. Conde K, Pineda G, Newton RS, Fernandez ML. Hypocholesterolemic effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in the guinea pig: atorvastatin versus simvastatin. *Biochem.Pharmacol.* 1999;58:1209-19.

34. Johnston TP, Baker JC, Hall D, Jamal S, Palmer WK, Emeson EE. Regression of poloxamer 407-induced atherosclerotic lesions in C57BL/6 mice using atorvastatin. *Atherosclerosis* 2000;149:303-13.

35. Rouleau JL, Parmley WW, Stevens J, *et al.* Verapamil suppresses atherosclerosis in cholesterol-fed rabbits. *J.Am.Coll.Cardiol* 1983;1:1453-60.

36. Ginsburg R, Davis K, Bristow MR, *et al.* Calcium antagonists suppress atherogenesis in aorta but not in the intramural coronary arteries of cholesterol-fed rabbits. *Lab. Invest.* 1983;49:154-58.

37. Proudfoot D, Shanahan CM. Biology of calcification in vascular cells: intima versus media. *Herz* 2001;26:245-51.

38. Wada T, McKee MD, Steitz S, Giachelli CM. Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. *Circ.Res.* 1999;84:166-78.

Chapter 5

Effects of amlodipine, atorvastatin and the combination of both on advanced atherosclerotic plaque in ApoE*3-Leiden transgenic mice

A combined histological and Raman spectroscopic approach

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Abstract

Background In the treatment of atherosclerosis, co-therpay of statins with calcium channel blockers has been supposed to be superior to statin therapy alone. We quantified the anti-atherosclerotic potential of amlodipine, atorvastatin and their combination on existing atherosclerotic plaques in the aorta of ApoE*3-Leiden transgenic mice that were fed a high fat/ high cholesterol diet.

Methods and results After 18 weeks of high cholesterol feeding, 62 mice were assigned to a group receiving the diet alone or to groups that received the diet together with 0.01% w/w atorvastatin, 0.002% w/w amlodipine, or a combination of the drugs for another 18 weeks. One group of mice was sacrificed at the start of the treatment. All other mice were sacrificed at t=36 weeks. Compared to the late control group, treatment with amlodipine, atorvastatin or the combination, reduced atherosclerotic lesion area by respectively 25%, 39% and 46% in the aortic root (histology, $p<0.01$) and by 53, 55 and 60% in the aortic arch (Raman spectroscopy, $p<0.05$). Compared to the late control group, lesion severity, collagen content and calcification of the plaque was unchanged in the treatment groups. Neither treatment resulted in regression of atherosclerotic plaque size.

Conclusion Both atorvastatin and amlodipine can significantly retard the progression of existing atherosclerotic lesions. There is no additional beneficial effect of combining the two therapies.

Introduction

A variety of studies, performed in humans and animals, have indicated that calcium channel antagonism can influence the natural progression of atherosclerosis[1,2]. Prospective randomized trials in human subjects have demonstrated that treatment with calcium channel blockers (CCBs) can reduce the number of newly formed lesions in patients with coronary artery disease[3,4]. More recent studies have reported a reduction in the carotid intima-media thickness or reduction in coronary calcification in patients who were treated with the CCBs verapamil, nicardipine or amlodipine[5-8]. These studies, however, could not observe any treatment difference in the rates of all cause mortality and major cardiovascular events[3,6,9]. In addition, the proposed reduction in early atherosclerotic lesions[3,4] could not be confirmed by others[6,9], and

thus remains subject to controversy. In animal studies that have investigated the anti-atherosclerotic potential of CCBs similar controversies have been observed: some have clearly demonstrated a reduction of atherosclerosis upon treatment with CCBs, whereas in others, the effect is indifferent[1,10]. Therefore, the anti-atherosclerotic potential of CCBs is still under debate.

Recently, a retrospective analysis of the Regression Growth Evaluation of Statin Study (REGRESS), suggested that addition of a CCB to a 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitor might act synergystically in retarding the progression of established coronary atherosclerosis[9]. It was observed that patients who were treated with pravastatin, had less progression of angiographically quantified coronary atherosclerosis if co-treated with a calcium channel blocker. In addition, the occurrence of new angiographic lesions was significantly reduced in this group[9].

The ApoE*3-Leiden transgenic mouse is a well defined atherosclerotic animal model that has been used widely in atherosclerosis research. Compared to other animal models that have been used for the study of CCBs, ApoE*3-Leiden transgenic mice develop complex atherosclerotic lesions upon feeding a cholesterol rich diet[11,12]. These lesions resemble human atherosclerotic lesions in many respects, including a lipid pool, a fibrous cap and calcifications. This mouse strain has proven to be an excellent model for studying the progression of atherosclerosis and the effects of treatment[13,14]. Regression of early atherosclerotic lesions and fatty streaks has also been demonstrated in this model[11]. Recently, the ApoE*3-Leiden mouse model has been used for the prospective evaluation of the effect of CCB treatment and the proposed synergistic effect of combined CCB and statin therapy[15]. In this study, it was observed that the time-dependent development of atherosclerotic lesions did not differ between mice that were fed the combination of amlodipine/ atorvastatin compared to atorvastatin alone[15]. Although the latter study could not confirm the proposed synergistic effect of CCB co-treatment, it was demonstrated that amlodipine alone could significantly delay the development of calcification, both in the aortic root and in the aortic arch. However, the lipid component of the plaque remained unaffected by amlodipine. These results correspond with a recent study in humans[5], demonstrating a reduction in progression of calcification upon treatment with the CCB nifedipine.

The discrepancy between the results regarding the synergistic effect that was observed in humans, but not in mice, is likely be due to differences in experimental

setup between the two studies: The REGRESS trial evaluated the effect of statin and CCB therapy in patients with documented coronary artery disease,[9] whereas the mouse study evaluated the anti-atherosclerotic potential of statin and CCB therapy on plaque development in healthy mouse aorta[15,16]. The present study was designed to detect any synergistic effect of amlodipine on statin therapy in mice with pre-existing atherosclerotic lesions in the aortic root and arch, using standard histology and Raman spectroscopy, respectively.

Methods

Mice

Heterozygous female mice of F16 generations, 10 to 17 weeks of age at the start of the study were used. Identification of transgenic mice was performed by an ELISA for human ApoE, as previously described[12]. All animal experiments were approved by the institutional committee on animal welfare of TNO.

Diets

Before the start of the study, animals were kept on a standard rat/mouse chow (Hope Farms, The Netherlands). 62 female ApoE*3-Leiden transgenic mice were fed a semi-synthetic high fat/high cholesterol diet (HFC) with added cholate to facilitate intestinal uptake of fat and cholesterol, thereby increasing plasma cholesterol levels. The composition of the diet was 40.5% sucrose, 20% casein, 15% cocoa butter, 10% cornstarch, 5.95% cellulose, 5.1% mineral mixture, 1% choline chloride, 1% corn oil, 0.2% methionine, 0.5% cholesterol and 0.01% cholate (all weight by weight). During 18 weeks on this diet, plasma total cholesterol and triglyceride levels were measured regularly and mice were eventually divided into 5 groups with equal average plasma total cholesterol concentration and age. One group (early control, n = 10) was then sacrificed to determine severity and extent of atherosclerosis at the onset of treatment. The other four groups consisted of 13 mice and received different diets for another 18 weeks. The late control group was maintained on the HFC diet. The atorvastatin group received the HFC diet with 0.01% w/w atorvastatin. The amlodipine group received the HFC diet with the addition of 0.002% w/w amlodipine. The last group received the HFC diet with the combination of atorvastatin and amlodipine in the concentrations as mentioned above. Atorvastatin and amlodipine were kindly provided by Pfizer Inc. (Groton, Ct, USA). All animals had free access to water and food. Body weight and food intake were monitored during the study.

Lipid and lipoprotein analysis

After a 4 hour fasting period from 9 a.m. to 1 p.m., blood samples were obtained from each individual mouse by tail incision. Blood samples were collected in heparinized glass capillary tubes (Hawksley & Sons Ltd, Sussex, England) and centrifuged at 15000 rpm for 2 minutes to obtain plasma samples. Total plasma cholesterol and triglyceride levels were measured enzymatically, using commercially available kits (No 236691, Boehringer Mannheim gmbh, Mannheim, Germany and No 337-B, Sigma Diagnostics, Deisenhofen, Germany, respectively). For size fractionation of lipoproteins, 40 μl of pooled plasma was injected onto a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia, Uppsala, Sweden) and eluted at a constant flow rate of 50 μl/min with PBS (pH 7.4, containing 1 mmol/l EDTA). Fractions of 50 μl were collected and assayed for total cholesterol and triglycerides as described above.

Histological assessment of atherosclerosis

After 18 weeks (early control group) or 36 weeks (other groups) of diet feeding, mice were sacrificed. Mice were anaesthetized with an intraperitoneal injection of 2.5 ml/kg Hypnorm^{tm} (Janssen Pharmaceutica, Tilburg, The Netherlands) and 2.5 ml/kg Dormicum® (Roche Netherlands, Mijdrecht, The Netherlands). Approximately five minutes after an intraperitoneal injection with 25 IE heparin (LEO Pharmaceutical Products, Weesp, The Netherlands) the thorax was opened and blood was drawn via the vena cava. Subsequently, the heart and aorta were dissected. The aorta was excised, snap frozen in liquid nitrogen and stored at –80 ºC for upcoming Raman spectroscopic investigation (see below). The hearts were fixed overnight in phosphate-buffered 3.8% formalin and embedded in paraffin. Serial cross sections (5 μm thick, spaced 30 μm apart) throughout the entire aortic valve area were used for histological analysis. Sections were routinely stained with haematoxylin-phloxine-saffron (HPS).

Per mouse, four sections with intervals of 30 μm were used for quantification and qualification of atherosclerotic lesions. The first section that was obtained showed the aortic wall in full circumference, including three intersected aortic valves. All sections were imaged under identical lighting, microscope- (Nikon), camera- (Hitachi) and PC conditions. Total lesion areas were determined using Leica Qwin image analysis software (EIS, Asbury NJ). All analyses were performed by the same operator, blinded for the experimental group allocation.

For determination of severity of atherosclerosis, the lesions were classified into five categories as described before[11,12]: I) early fatty streak: per section up to 10 foam cells present in the intima, II) regular fatty streak: more than 10 foam cells present in the intima,

III) mild plaque: foam cells in the intima with a fibrous cap, IV) moderate plaque: a more progressed lesion that involves the media without loss of architecture, V) severe plaque: the media is severely affected, elastic fibers are broken, cholesterol clefts, calcification and necrosis are frequently observed. The same sections as used for the quantification of atherosclerosis were used for determination of lesion severity, meaning that for each mouse four sections with three aortic valve segments were analyzed. Subsequently, per mouse the percentages of all lesions found in the respective lesion categories were calculated.
To determine whether the different treatments had an effect on endothelial activation, monocytes adhering to the endothelium were counted in the same sections as used for quantification of atherosclerosis. Collagen content of atherosclerotic lesions, as a measure of plaque stability, was quantified morphometrically in serial sections after Sirius Red staining.

Raman spectroscopic assessment of atherosclerosis

For Raman spectroscopic assessment of atherosclerosis in the ascending aorta and aortic arch, the mouse aortas were dissected, snap frozen in liquid nitrogen, and stored at –80 °C until use. Prior to spectroscopic investigation, the first 5-8 mm of each aorta just distally from the aortic valve was cleaned from its adventitial fat and cut open through the outer aortic curvature, longitudinally through the brachio-cephalic, carotid and subclavian arteries[17]. The samples were then moistened with PBS and positioned between two calcium-fluoride windows (Crystran Ltd, Dorset, UK) underneath a scanning microscope (Leica, Cambridge, UK) that was rebuilt for Raman spectroscopic imaging[17]. With this system, Near Infrared Raman spectra were obtained in 0.25 x 0.25 mm steps over the entire length and full width of the opened aorta. Collection time for each measurement was 10 s. All Raman spectra were processed and analyzed as described previously[17]. For each location of the grid, the relative contributions of protein (P), total cholesterol (TC, cholesterol plus cholesteryl esters) and calcium salts (CS) in the arterial wall were derived from the Raman spectra. For each component, arterial surface maps were created[17]. Any location with a weight contribution of more than 10% TC (10-100%) was regarded to be part of an atherosclerotic plaque. Any location with a weight contribution of more than 10% CS (10-100%) was regarded to be part of a calcified plaque. The surface areas of regions with TC>10% or CS>10% are expressed in mm^2. As the relative protein content is dependent on the relative contents of calcium and cholesterol, surface maps of protein were not analyzed. For a more detailed description of the technique, one is referred to reference 17.

Statistics

All data are presented as mean ± SD. For statistical analysis SPSS 8.0 for Windows was used. Because data were not normally distributed, non-parametric tests were used for

comparisons between groups. Overall comparisons between groups were performed with the Kruskall-Wallis test. If a significant difference was found, distribution-free multiple comparisons were performed to find which groups differed from each other. If only two groups were compared, Mann-Whitney rank sum tests were used. P values less than 0.05 were considered significant.

Results

Effects of amlodipine and atorvastatin on plasma lipid

During the first 18 weeks on HFC diet, blood samples were drawn at t = 7, 11, 13, 16 and 18 weeks to determine plasma total cholesterol and triglyceride concentrations. The average plasma total cholesterol level of all mice was 23.16 ± 2.72 mmol/l, the triglyceride level was 1.76 ± 0.09 mmol/l.

Amlodipine and/or atorvastatin treatment did not affect body weights or food intake. Average plasma cholesterol levels during feeding of the study diets (at t = 21, 28, 34 and 36 weeks) were 17.8 ± 2.3 mmol/l for the late control group, 16.7 ± 2.6 mmol/l for the amlodipine group, 7.7 ± 2.3 mmol/l for the atorvastatin group and 6.7 ± 1.8 mmol/l for the combination group. The effects of amlodipine and atorvastatin treatment on plasma cholesterol levels in time are shown in figure 1A. Amlodipine had no effect on plasma cholesterol levels. Atorvastatin alone or in combination with amlodipine significantly reduced plasma cholesterol by 57% ($p<0.005$) compared to the late control group. The total cholesterol exposure was 421±48, 760±88, 738±83, 597±52 and 564±78 mmol/l*week for the early control, late control, amlodipine, atorvastatin and combination groups, respectively.

Triglyceride levels did not differ significantly between the groups during the 28 weeks of treatment (1.3 ± 0.5, 1.2 ± 0.4, 0.9 ± 0.5 and 0.9 ± 0.4 mmol/l for the late control, amlodipine, atorvastatin and combination group, respectively). The effect of amlodipine and atorvastatin treatment on plasma triglyceride levels in time is shown in figure 1B.

Lipoprotein profiles at 28 weeks showed that atorvastatin mainly reduced VLDL and LDL cholesterol, whereas HDL cholesterol was not affected (figure 1C).

Figure 1. Effect of treatment with amlodipine, atorvastatin and the combination of both on plasma lipids. **A.** Plasma total cholesterol levels measured in time. **B.** Plasma triglyceride levels measured in time. **C.** Cholesterol profiles of plasma lipoproteins. Data are presented as average ± SD.

Effects of amlodipine and atorvastatin on pre-existing atherosclerosis in the aortic root

The average lesion area per section for the individual groups is shown in figure 2A. For the early control group the average lesion area per section was 213 ± 73 μm^2 *1000. The late control group showed significantly larger lesions than the early control group (296 ± 68 μm^2 *1000, $p<0.05$). Amlodipine significantly reduced lesion area by 25% as compared to the late control (222 ± 72 μm^2 *1000, $p<0.05$). Treatment with atorvastatin resulted in a 39% reduction of lesion area compared to the late control (182 ± 51 μm^2 *1000, $p<0.005$), and the combination of atorvastatin and amlodipine reduced lesion area by 46% as compared to the late control (161 ± 72 μm^2 *1000, $p<0.005$). These results indicate that both amlodipine and atorvastatin were able to significantly delay the progression of existing lesions. The combination of amlodipine and atorvastatin did not have an additional positive effect on reducing lesion progression. No significant differences in atherosclerotic lesion area were found between the treated groups and the early control, indicating that regression of atherosclerotic lesions did not occur.

Figure 2B shows the severity of the lesions in the aortic root. Only 13% of the lesions in the early control group were fatty streaks and type III lesions. In the late control group, these early atherosclerotic lesions were practically absent, and drig therapy did not modify this results (fig. 2B). Figure 2C shows the percentage of the lesion area that stained positive for collagen. Compared to the early control group, mice in the late control group showed 21% more collagen within the atherosclerotic plaque. Upon treatment with amlodipine, atorvastatin and the combination, the increase in amount of collagen was approximately similar as in the late control group. As collagen content of a plaque is considered to be a parameter for plaque stability, these results indicate that treatment with amlodipine or atorvastatin does not influence the stability of the plaque.

To investigate whether treatment with amlodipine, atorvastatin or the combination of both can reduce atherosclerosis by decreasing monocyte adherence to the endothelium, the number of monocytes attached to the endothelial cell layer were counted. In the early control group, an average of 2.7±1.2 adhering monocytes were

found per aortic valve segment. The late control group showed a significant increase in monocyte attachment (5.4±1.9, $p<0.05$).

Figure 2. Effect of treatment with amlodipine, atorvastatin and the combination of both on atherosclerosis. **A.** Plaque size **B.** Plaque severity **C.** Collagen content. #$P<0.05$ compared to the early control group, *$P<0.05$ compared to the late control group, ***$P<0.005$ compared to the late control group. The dotted line in panel A represents the level of atherosclerosis in the early control group. Data are presented as average ± SD.

Compared to this late control group, amlodipine treatment did not decrease monocyte adherence (4.4±2.6), whereas atorvastatin and combination therapy showed significantly ($p<0.05$) less monocyte adherence: 3.0±1.3 and 2.3±1.9, respectively. No significant differences were found between the treated groups and the early control. These results suggest that atorvastatin and the combination of amlodipine and atorvastatin can delay atherosclerotic plaque progression by inhibiting monocyte adherence to the endothelium, which is generally considered to be a very important step in atherogenesis.

Figure 3. Effect of treatment with amlodipine, atorvastatin and the combination of both on the area of the aortic arch containing **A.** cholesterol and **B.** calcification, as determined with Raman spectroscopy. #P<0.05 compared to the early control group, *P<0.05 compared to the late control group. The dotted line represents the level of atherosclerosis in the early control group. Data are presented as average ± SD.

Effects of amlodipine and atorvastatin on pre-existing atherosclerosis in the aortic arch

The aortic arch was studied with Raman spectroscopic imaging. Per mouse, the first 5 mm of the aortic arch was analyzed with respect to the contribution of total cholesterol and calcification. From these maps, the first 5 mm were selected. The variation in lesion size between the mice per group was large. Average size of the aorta with >10%, >20% or >30% cholesterol or calcification are summarized in the table. With regard to lesions with more than 10% cholesterol, the late control group showed 135% larger lesions than the early control group (figure 3A, $p<0.05$). Amlodipine significantly reduced lesion size by 53% compared to the late control group, as did atorvastatin (-55%) and the combination of atorvastatin and amlodipine (-60%). There was no significant difference in lesion size between mice that were fed atorvastatin or amlodipine alone and mice that were fed the combination of the two drugs ($p=0.98$). Lesion size in mice in the 3 treatment groups did not differ from lesion size in the early control group. In plaques containing higher weight contributions of cholesterol (20-100%, 30-100%), results were similar although statistical significance could not be reached (table 1).

Table 1. Areas of Plaque Surface of the Aortic Arch of Mice Determined with Raman spectroscopy.

Group	Cholesterol (mm^2)			Calcification (mm^2)		
	>10%	>20%	>30%	>10%	>20%	>30%
Early control	1.77±1.85	0.51±0.46	0.18±0.16	0.22±0.20	0.13±0.11	0.09±0.07
Late control	4.18±1.65#	2.29±1.10#	1.51±0.91#	1.14±0.97#	0.89±0.72#	0.74±0.70#
Amlodipine	1.98±0.59*	0.88±0.42	0.41±0.27	1.25±0.69#	0.91±0.66#	0.72±0.51#
Atorvastatin	1.88±1.04*	0.83±0.58	0.36±0.31	1.13±0.50#	0.80±0.50#	0.66±0.48#
Amlo + Atorva	1.69±0.52*	0.84±0.45	0.51±0.43	1.01±0.60	0.71±0.51	0.61±0.47

Data are presented as mean ± SD # $P < 0.05$ versus early control; * $P < 0.05$ versus late control

The results of study treatment on calcification are summarized in figure 3B. In the early control group, only small areas of the aorta contained >10% calcification. In the late control group, a four-fold larger area of calcification had developed ($p<0.005$). Compared to this late control group, therapy with amlodipine, atorvastatin and the combination did not affect development of calcification: the areas containing >10% calcification were 1.25 ± 0.69 mm^2, 1.13 ±0.50 mm^2 and 1.01 ± 0.60 mm^2 for the

amlodipine, atorvastatin and combination groups respectively. These values differed from those of the early control group: significantly for the atorvastatin and amlodipine group ($p<0.005$) and borderline significant for mice in the combined therapy group ($p=0.052$). In areas containing higher quantities of calcification (> 20%, >30%), similar results were obtained (table).

The results obtained with Raman spectroscopy of the aortic arch indicate that treatment with either amlodipine, atorvastatin or the combination of both can delay the progression of cholesterol accumulation in pre-existing lesions. However, development of calcification was not affected.

Discussion

The present investigation examined the effects of the HMG-CoA reductase inhibitor, atorvastatin, the Ca^{2+}-channel blocker amlodipine, and the possible synergistic effect of the combination of both on progression of regression of existing atherosclerotic lesions in cholesterol-fed ApoE*3-Leiden transgenic mice. We demonstrate that, in this model, treatment with atorvastatin and amlodipine delays progression of pre-existing atherosclerotic lesions. Plaque severity, calcification and collagen content was not affected by treatment with amlodipine or atorvastatin. Addition of amlodipine to atorvastatin therapy had no extra effect compared to atorvastatin therapy alone.

So far, many investigators have shown that CCBs can reduce atherogenesis in rabbits, swine, monkeys and humans[1,2]. Based upon these studies, it appeared that CCBs would be most effective if administered concomitantly with atherogenic stimuli (i.e. cholesterol feeding). If the lesions have already begun to form, CCBs usually showed little or no effect. These studies were mostly carried out with prototype CCBs such as nifedipine and verapamil. In contrast to those studies, the present study demonstrates that the second generation dihydropyridine, amlodipine, is able to inhibit progression of pre-existing atherosclerotic plaque. Although many dihydropiridine CCBs have been studied, the experimental evidence on amlodipine is limited to only a few animal studies. Kramsch et al.[18] observed a significant negative correlation between coronary artery lesion involvement and plasma amlodipine concentrations in Cynomolgus monkeys that were fed an atherogenic diet. However, the experimental methods and observations of this study are only briefly described,

and the results have not yet been confirmed by others. In a previous study we have demonstrated that amlodipine, administered to mice along with a cholesterol rich diet, was associated with, on average, an 18% reduction in plaque growth, although this value was not statistically significant[15,16]. In the present study, we observed that amlodipine inhibited the progression of atherosclerosis of pre-existing lesions. This may be explained by the fact that amlodipine is highly lipophylic. Therefore, the drug is rapidly absorbed in the atheroma of atherosclerotic lesions, accumulate locally, and act more effectively in atheromatous than in non-atherosclerotic artery tissue. Many mechanisms by which CCBs can inhibit atherosclerosis have been described[1] as many key processes in atherosclerosis (e.g. recruitment of macrophages, lipid oxidation and proliferation of smooth muscle cells) are calcium dependent and may be influenced by CCBs.

In the present study, the progression of atherosclerosis in the aortic root and arch was arrested in mice that were treated with atorvastatin. Regression of atherosclerosis did not occur. This is in agreement with previous studies, that have demonstrated effects of statin treatment on existing atherosclerotic plaque development in a variety of animal models[19]. Until now, attempts to accomplish regression of complex lesions that were induced by prolonged exposure of the arterial wall to hypercholesterolemia, were only successful as far as reduction of cholesterol content of the plaque was concerned. Regression of the size of the complex atherosclerotic plaque could not be observed, so far. In addition, statins and other lipid-lowering drugs were hardly able to reduce the non-lipid content of the complex lesions. In contrast, complete regression of *early* atherosclerotic lesions has been observed, both in human and animal studies. In atherosclerotic ApoE*3-Leiden mice it was observed that regression of early fatty streak lesions occurred if total cholesterol levels reached plasma levels below 2 mmol/l[11]. In our experiment, atorvastatin reduced cholesterol levels to approximately 6-7 mmol/l, indicating that fatty streaks and atheroma were unlikely to regress in this study.

The combination of atorvastatin and amlodipine did not delay the progression of atherosclerosis compared to amlodipine or atorvastatin therapy alone. As the individual therapies retard the progression of atherosclerosis, due to the cholesterol lowering effects of atorvastatin and due to cholesterol independent effects of amlodipine, we would expect that the combination would do better. Based upon the present study in mice with existing plaques, and upon previous results in mice that

were treated from the start of the high cholesterol feeding[15,16], we conclude that the synergistic effect of the two drugs is absent or minimal. The proposed synergistic effect, proposed by Jukema et al[9], may be caused by the blood pressure lowering effect of calcium channel blockade, although in several studies, the human studies the anti-atherosclerotic potential of CCBs is clearly demonstrated, irrespective of its blood pressure lowering potential[5]. In our study, the dose of amlodipine that was chosen was the highest dose that did not lower systolic blood pressure. Therefore, bloodpressure lowering effects can be denied as a bias in the evaluation of our results.

Atorvastatin, but not amlodipine could reduce the number of adhering monocytes to the atherosclerotic lesion in the aortic root of the mouse. This reduction did not affect the size and severity of the lesion in the aortic root compared to the early control group, but it is not known what the effect of longer duration on plaque progression would have been. Currently, there are no data on the effect of atorvastatin on adhesion of monocytes. However, Gijbels et al. have demonstrated a reduction in monocytes in mice with low cholesterol levels[11]. In our study, the cholesterol levels of the groups that received atorvastatin were significantly lower than those in the control and amlodipine groups. Thus, the reduction in monocyte adherence might be due to a reduction in plasma cholesterol level per se. This issue will need further attention as the adhesion of monocytes to the lesion is generally considered to be a very important step in atherogenesis.

The collagen content of the plaque was approximatley equal in all treated groups, and did not differ from the collagen content in the late control group. In light of the ability of collagen to increase the stability of a plaque, this observation implies that the plaque stabilizing effects of atorvastatin and amlodipine were absent in this animal model.

An interesting observation of our study, however, is that calcification of atherosclerotic plaque appears to be a process that can not be inhibited, neither by amlodipine or atorvastatin alone, nor by their combination. In the treatment groups that were kept on a lipid rich diet, the sizes of aortic calcification were approximately equal to that of the late control group, and four-fold larger compared to the early control group. In contrast, in our previous experiments in ApoE*3-Leiden mice that were fed a high cholesterol diet together with amlodipine, atorvastatin and the

combination, we obseved that the treatments had potent effects on development of aortic calcification[15,16]. In those experiments, amlodipine and atorvastatin were able to slow down progression of calcification by 69% and 96% compared to mice on an atherogenic diet. Clearly, existing complex atherosclerotic plaque responds differently to drug treatment than developing fatty streaks and early atherosclerosis.

The observations of the present study, however, do not conform with human studies evaluating the effect of drug treatment on atherosclerotic plaque progression[5,20,21]. For example, it was demonstrated that in hypertensive patients, progression of calcification (measured by the 'calcium score' with electron beam computed tomography) could be reduced by 38% in patients treated with nifedipine, compared to patients with equal blood pressure on diuretics. Nonetheless, the calcium score that was calculated from the EBCT experiments represents lesions in the entire coronary tree, including parts where plaque had not developed at the time when CCB treatment was started. The calcium score may therefore be a good indicator for the total burden of atherosclerosis, but it does not give insight in individual plaque dynamics, as evaluated in our study.

With regard to statin treatment, there are only limited data of the effects of atorvastatin or other statins on the development of calcification[15-17]. However, in a retrospective human analysis, it was observed that the extent to which atherosclerotic calcification decreased upon statin treatment, was directly related to plasma LDL cholesterol levels[21]. Patients who were treated with a statin, but did not reach target LDL cholesterol levels (<120 mg/ml = 5 mmol/l) showed a similar increase in calcium score compared to patients that were not treated with atorvastatin. Only patients that were treated with atorvastatin and had reached target LDL cholesterol levels showed a decrease in progression of atherosclerotic plaque. Although the calcium score can not This result, in combination with the results of Gijbels et al.[11] who demonstrated that plaque regression in mice may occur if plasma cholesterol levels are below 2 mmol/l, might explain why an effect of atorvastatin on calcification was lacking in the present study.

Upon treatment with CCBs and statins, plaque growth is attenuated although calcification progresses. What can be the explanation for this finding? It might be that plaque that has developed in the first 18 weeks of cholesterol-rich diet, does not progress because cholesterol recruitment is inhibited (CCBs) or because serum cholesterol is low (statins). In the remaining plaque, apoptotic cell death of smooth

muscle cells and macrophages and induction of calcification stimulating proteins by oxidation of lipids may subsequently enhance calcification[22]. Clinically, in terms of plaque stability, the increase in calcification of atherosclerotic lesions, does not necessarily imply a sign of poor prognosis. Recent evidence indicates that an increase or decrease in the amount of calcification of an atherosclerotic plaque does not enhance or reduce its stability[23]. Nevertheless, if an increase in calcification, occurs at the cost of the size of the atheroma, plaque stability may still be affected.

In conclusion, the present study clearly demonstrates the atheroprotective effects of amlodipine and atorvastatin on advanced lesions in ApoE*3-Leiden transgenic mice. No additional benefit of adding amlodipine to treatment with atorvastatin could be observed in this study. The results of the present study are especially important since the human, double-blind, placebo-controlled multicenter trial ENCORE 2[24], a trial that evaluates the anti-atherosclerotic potential of cerivastatin, nifedipine and their combination on atherosclerosis, has been terminated due to undesired side effects of cerivastatin. It will be years from now, before we know whether in humans co-treatment of calcium channel inhibitors with statins is beneficial in the treatment of atherosclerosis.

Reference List

1. Nayler WG. Review of preclinical data of calcium channel blockers and atherosclerosis. *J.Cardiovasc.Pharmacol.* 1999;33 Suppl 2:S7-11.
2. Tulenko TN, Laury-Kleintop L, Walter MF, Mason RP. Cholesterol, calcium and atherosclerosis: is there a role for calcium channel blockers in atheroprotection? *Int.J.Cardiol* 1997;62 Suppl 2:S55-S66.
3. Waters D, Lesperance J, Francetich M, Causey D, Theroux P, Chiang YK, Hudon G, Lemarbre L, Reitman M, Joyal M. A controlled clinical trial to assess the effect of a calcium channel blocker on the progression of coronary atherosclerosis. *Circulation* 1990;82:1940-53.
4. Lichtlen PR, Hugenholtz PG, Rafflenbeul W, Hecker H, Jost S, Deckers JW. Retardation of angiographic progression of coronary artery disease by nifedipine. Results of the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT). INTACT Group Investigators. *Lancet* 1990;335:1109-13.
5. Motro M, Shemesh J. Calcium channel blocker nifedipine slows down progression of coronary calcification in hypertensive patients compared with diuretics. *Hypertension* 2001;37:1410-13.

6. Pitt B, Byington RP, Furberg CD, Hunninghake DB, Mancini GB, Miller ME, Riley W. Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. *Circulation* 2000;102:1503-10.

7. Simon A, Gariepy J, Moyse D, Levenson J. Differential effects of nifedipine and co-amilozide on the progression of early carotid wall changes. *Circulation* 2001;103:2949-54.

8. Zanchetti A, Rosei EA, Dal Palu C, Leonetti G, Magnani B, Pessina A. The Verapamil in Hypertension and Atherosclerosis Study (VHAS): results of long-term randomized treatment with either verapamil or chlorthalidone on carotid intima-media thickness. *J.Hypertens.* 1998;16:1667-76.

9. Jukema JW, Zwinderman AH, van Boven AJ, Reiber JH, van der Laarse A., Lie KI, Bruschke AV. Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler.Thromb.Vasc.Biol.* 1996;16:425-30.

10. Catapano AL. Calcium antagonists and atherosclerosis. Experimental evidence. *Eur.Heart J.* 1997;18 Suppl A:A80-A86.

11. Gijbels MJ, van der Cammen M., van der Laan LJ, Emeis JJ, Havekes LM, Hofker MH, Kraal G. Progression and regression of atherosclerosis in ApoE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* 1999;143:15-25.

12. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J.Clin.Invest.* 1994;93:1403-10.

13. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J.Biol.Chem.* 1993;268:10540-45.

14. Delsing DJ, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit EC, Gijbels MJ, van der Laarse A, Jukema JW, Havekes LM, Princen HM. Acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in ApoE*3-Leiden mice. *Circulation* 2001;103:1778-86.

15. Delsing DJ, Jukema JW, Emeis JJ, van der Laarse A, Havekes LM, Princen HMG. Differential effects of amlodipine na datorvastatin treatment and their combination on atherosclerosis in ApoE*3-Leiden transgenic mice. *(submitted)* 2001

16. van de Poll SWE, Delsing DJM, Jukema JW, Princen HMG, Havekes LM, van der Laarse A, Puppels GJ. Raman spectroscopic investigation of the effects of atorvastatin, amlodipine, and both on plaque progression in the aorta of ApoE*3-Leiden mice. *(submitted)* 2001

17. van de Poll SWE, Romer TJ, Volger OL, Delsing DJM, Bakker Schut TC, Princen HMG, Jukema JW, Havekes L, van der Laarse A, Puppels GJ. Raman spectroscopic evaluation of the effects of diet and lipid-lowering therapy on atherosclerotic plaque development in mice. *Arterioscler.Thromb.Vasc.Biol.* 2001;21:1630-35.

18. Kramsch DM, Sharma RC. Limits of lipid-lowering therapy: the benefits of amlodipine as an anti- atherosclerotic agent. *J.Hum.Hypertens.* 1995;9 Suppl 1:S3-S9.

19. Stein Y, Stein O. Does therapeutic intervention achieve slowing of progression or bona fide regression of atherosclerotic lesions? *Arterioscler.Thromb.Vasc.Biol.* 2001;21:183-88.

20. Budoff MJ, Lane KL, Bakhsheshi H, Mao S, Grassmann BO, Friedman BC, Brundage BH. Rates of progression of coronary calcium by electron beam tomography. *Am.J.Cardiol* 2000;86:8-11.

21. Callister TQ, Raggi P, Cooil B, Lippolis NJ, Russo DJ. Effect of HMG-CoA reductase inhibitors on coronary artery disease as assessed by electron-beam computed tomography. *N.Engl.J.Med.* 1998;339:1972-78.

22. Proudfoot D, Shanahan CM. Biology of calcification in vascular cells: intima versus media. *Herz* 2001;26:245-51.

23. Huang H, Virmani R, Younis H, Burke AP, Kamm RD, Lee RT. The Impact of Calcification on the Biomechanical Stability of Atherosclerotic Plaques. *Circulation* 2001;103:1051-56.

24. Sutsch G, Buchi M, Zeiher AM, Meinertz T, Hugenholtz PG, Jenni R, Rafflenbeul W, Drexler H, Haussmann D, Noll G, Quitzau K, Luscher TF. Effects of calcium antagonism and HMG-coenzyme reductase inhibition on endothelial function and atherosclerosis: rationale and outline of the ENCORE trials. *European Heart Journal Supplements* 1999;1:M27-M32.

Chapter 6

The acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis additionally to its cholesterol-lowering effect in ApoE*3-Leiden mice

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Abstract

Background The present study investigated whether the ACAT-inhibitor avasimibe can reduce atherogenesis independently of its cholesterol-lowering effect in ApoE*3-Leiden mice.

Methods and Results Two groups of 15 female ApoE*3-Leiden mice were put on a high cholesterol (HC) diet of which one group received 0.01% (w/w) avasimibe mixed in the diet. The HC diet resulted in a plasma cholesterol concentration of 18.7±2.6 mM. Addition of avasimibe lowered plasma cholesterol by 56% to 8.1±1.2 mM, mainly caused by a reduction and composition change in VLDL/LDL. In a separate low cholesterol (LC) control group, plasma cholesterol was titrated to a level comparable to that of the avasimibe group (10.3±1.4 mM) by lowering the amount of dietary cholesterol. After 22 weeks of intervention, atherosclerosis in the aortic root area was quantified. Treatment with avasimibe resulted in a 92% reduction of lesion area as compared to the HC control group. In comparison with the LC control, avasimibe reduced lesion area by 78%. After correction for the slight difference in cholesterol exposure between the LC control and avasimibe group, the effect of avasimibe on lesion area (73% reduction) remained highly significant. In addition, monocyte adherence to the endothelium, free cholesterol accumulation and lesion severity were reduced by avasimibe treatment.

Conclusion Treatment with avasimibe potently lowered plasma cholesterol levels in ApoE*3-Leiden mice and considerably reduced atherosclerotic lesion area, additionally to its cholesterol-lowering effect. Because monocyte adherence to the endothelium and lesion severity were also reduced by avasimibe, treatment with avasimibe may result in higher plaque stability and therefore a reduced risk of plaque rupture.

Introduction

The enzyme ACAT (acyl-CoA:cholesterol-*O*-acyltransferase) catalyses the intracellular formation of cholesteryl esters. It is involved in the absorption of cholesterol in the intestine and the secretion of very low-density lipoproteins (VLDL) from the liver. The enzyme has also been implicated in esterification, and, thereby, storage of cholesteryl ester in monocyte-derived macrophages in the arterial wall[1].

Since the accumulation of cholesteryl esters in the latter cells is one of the earliest steps in the development of atherosclerosis[2], it has been suggested that inhibition of the enzyme ACAT has a direct anti-atherogenic potential[3,4]. Moreover, since the stability of the atherosclerotic plaque is increased when less lipid-laden macrophages are present[5], a decrease of the number of lipid-laden macrophages in the arterial wall upon ACAT-inhibition could lead to a higher plaque stability and consequently a reduced risk of plaque rupture.

Several animal studies with various ACAT-inhibitors have shown cholesterol-lowering effects of these drugs[6-8]. However, data on direct anti-atherosclerotic effects of ACAT-inhibitors remain disputed. ACAT-inhibition has been shown to decrease atherosclerotic lesion areas or cholesteryl ester enrichment in the arterial wall of animal models for atherosclerosis, but in most of these studies substantial lipid-lowering was involved[9-12]. Others found less atherosclerosis at a dose that showed some systemic availability but did not lower plasma total cholesterol levels[13,14]. Thus, a number of models has been used to test the direct anti-atherosclerotic activity of ACAT-inhibitors, but so far conclusions that are drawn from these experiments are based on circumstantial evidence[15].

The present study was designed to determine the direct anti-atherosclerotic effects of the ACAT-inhibitor avasimibe (CI-1011), ([[2, 4, 6-tris- (1-ethylethyl) phenyl] acetyl] sulfamic acid, 2, 6-bis (1-methyl-ethyl) phenyl ester) in ApoE*3Leiden transgenic mice, by ruling out the confounding effects of cholesterol-lowering. ApoE*3-Leiden mice were used, because they exhibit elevated plasma cholesterol- and triglyceride levels, mainly confined to the VLDL/LDL sized lipoprotein fraction[16]. Since they are highly responsive in their plasma lipid levels to dietary treatment[17] and their plasma cholesterol levels can easily be titrated to a constant level by the amount of cholesterol in the diet. In addition, upon high fat, high cholesterol feeding these mice develop atherosclerosic lesions resembling those found in humans, depending on their plasma cholesterol levels[18,19]. Since ApoE*3-Leiden mice have been shown to be responsive to a number of cholesterol-lowering therapies, such as lovastatin, gemfibrozil and fish oil[20,21], these mice were considered a suitable model for the testing of the anti-atherosclerotic properties of the ACAT-inhibitor avasimibe.

Materials and methods

Mice

Heterozygous female ApoE*3-Leiden mice of F14 generations, 31 to 41 weeks of age at the start were used. Identification of transgenic mice was performed by an ELISA for human apoE, as previously described[17]. All animal experiments were approved by the institutional committees on animal welfare of TNO Prevention and Health.

Diets

Before the start of the study, animals were kept on a standard rat/mouse chow diet (Hope Farms, The Netherlands). During a three week run-in period, all animals received a semi-synthetic high fat/high cholesterol diet as used before (HFC[17]). After this period, the animals were divided into three groups of 15 mice each on the basis of age and cholesterol level. The high cholesterol (HC) control group received an HFC diet with addition of 0.5% w/w cholesterol and 0.1% w/w cholate, the latter added to facilitate intestinal uptake of fat and cholesterol, thereby increasing plasma cholesterol levels. The avasimibe group received the same diet as the HC control group with an extra addition of 0.01% w/w avasimibe (kindly provided by Dr. Krause, Parke Davis, Ann Arbor, USA), approximately equalling a daily dose of 10 mg/kg body weight. The diet of the low cholesterol (LC) control group was chosen to reach the same plasma cholesterol level in these mice as in the avasimibe group and contained 0.5% w/w cholesterol and no cholate. Animals had free access to water and food.

Lipid and lipoprotein analysis

After a 4 hour fasting period from 9 a.m. to 1 p.m., blood samples were obtained from each individual mouse by tail incision. Total plasma cholesterol and triglyceride levels were measured enzymatically and lipoprotein profiles were obtained by size fractioning as described[17,22]. For the determination of the composition of the apoB-containing lipoproteins, ultracentrifugation of total plasma was performed, VLDL- and IDL/LDL-fractions were pooled and lipids were analyzed as described previously[23]. Total plasma apoB and apoE were measured by gelelectrophoresis followed by Coomassie staining and calculation of the contribution of apoB and E to total protein.

Histological assessment of atherosclerosis

After 24 weeks of diet feeding, mice were sacrificed after anaesthetizing and blood collection as described[17]. The hearts were dissected, stored overnight in phosphate-buffered 3.8% formalin fixation and embedded in paraffin. Serial cross sections (5 μm thick) throughout the entire aortic valve area were used for histological analysis. Sections were routinely stained

with haematoxylin-phloxine-saffron (HPS). Per mouse, 4 sections with intervals of 30 μm were used for quantification and qualification of atherosclerotic lesions. For determination of severity of atherosclerosis, the lesions were classified into five categories (type I-V) as described before[17,24]: I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, V) severe plaque. Mouse macrophages were immunostained with AIA31240 (1:3000, Accurate Chemical and Scientific, New York, USA) and smooth muscle cells were stained with α-actin (Roche, Almere, The Netherlands).
To evaluate whether avasimibe has an effect on the accumulation of free cholesterol in atherosclerotic lesions, the amount of sections containing free cholesterol clefts was counted in each group. To determine whether avasimibe has an effect on endothelial activation, the amount of monocytes adhering to the endothelium was counted in the same 4 slides as used for the quantification of atherosclerosis. The average amount of monocytes attached to the endothelium per section was used for comparison between groups.

ACAT-activity in J774 cells

The murine macrophage cell line J774 was cultured in DMEM, supplemented with 10% (v/v) FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. For each experiment, cells were plated in 12-well plates, under culture conditions as described by Bocan et al[25]. Cells were preincubated with avasimibe 1 hour before the addition of 200 μg protein/ml βVLDL or 50 μg protein/ml acetylated LDL. ACAT-activity was determined by measuring incorporation of ^{14}C-oleate in cholesteryl oleate, essentially as described by Post et al[22].

Statistics

Overall comparisons between groups were performed with the Kruskall-Wallis test. Distribution-free multiple comparisons were performed to find differences between groups. If only two groups were compared, Mann-Whitney rank sum tests were used. Differences in distributions of atherosclerotic lesions over the different lesion categories and of lipids over different lipoproteins were tested with the Fisher's exact test. P values less than 0.05 were regarded significant. All data are presented as mean ± SD, unless mentioned otherwise.

Results

The effect of avasimibe on plasma cholesterol levels and lipoprotein composition

Avasimibe did not affect body weights or food intake. Average plasma cholesterol levels from 8 blood draws (at t = 3, 6, 9, 10, 14, 19, 21 and 24 weeks) were

18.73±2.63 mmol/l for the HC group, 8.17±1.15 mmol/l for the avasimibe group and 10.24±1.46 mmol/l in the LC control.

Figure 1. The effect of avasimibe on lipoprotein profiles and composition. **A.** The lipoprotein profiles for cholesterol. **B.** The effect of avasimibe on lipid composition of VLDL and IDL/LDL. FC = free cholesterol, CE = cholesteryl ester, TG = triglycerides.

As compared to the HC control group, avasimibe lowered plasma total cholesterol concentrations by 56% ($p<0.005$). In comparison with the LC group, the avasimibe group showed a slightly, but significantly, reduced plasma cholesterol level ($p<0.005$). Triglyceride levels did not differ significantly between the groups (1.29±0.60, 1.59±0.47 and 1.01±0.48 mmol/l for the HC, avasimibe and LC group, respectively).

Lipoprotein profiles at 14 weeks of treatment showed that avasimibe mainly reduced VLDL, IDL and LDL cholesterol (figure 1A). Figure 1B shows the composition of VLDL and IDL/LDL in the different groups. VLDL and IDL/LDL in the avasimibe group contained relatively more triglycerides and less cholesteryl esters than in the LC and HC controls. The IDL/LDL lipoproteins in all groups contained relatively less triglycerides as compared to their respective VLDL.

Total apoB was 44% decreased in the avasimibe group as compared to the HC control (210 and 378 µg/ml, respectively). In the LC control group apoB was 55% lower than in the avasimibe group (93 µg/ml). ApoE was not different between the avasimibe group and the LC control (443 and 436 µg/ml, respectively). Plasma apoE in these latter groups was 39% lower as compared to the HC control (720 µg/ml). These data indicate that in the HC control group the amount of VLDL/LDL particles and their total cholesteryl ester content is largest. Treatment with avasimibe causes a reduction of these particles, which become relatively enriched in triglycerides. As compared to the LC control, the avasimibe-treated animals have about twofold more VLDL/LDL particles in their plasma which contain less cholesteryl ester.

The effect of avasimibe on macrophage ACAT-activity

Plasma concentrations of avasimibe were 50±24 nmol/l. To investigate whether esterification of cholesterol is inhibited at these concentrations, the effect of avasimibe on ACAT-activity was measured in the mouse macrophage cell line J774 in the presence of an additional source of cholesterol, to increase the intracellular cholesterol pool. Incubation with avasimibe resulted in a dose-dependent reduction in ACAT-activity, showing a significant 25% reduction at 50 nmol/l (figure 2, $P<0.005$).

The effect of avasimibe on atherosclerosis development

Atherosclerotic lesions were quantified in cross sections of the aortic valve area in the heart of each individual mouse.

Figure 2. The effect of avasimibe on cholesterol esterification in the mouse macrophage cell-line J774 during lipid accumulation with 200 µg protein/ml ßVLDL or 50 µg protein/ml acetylated LDL. Statistically significant differences are indicated ***p<0.005.

The average lesion area per section for the individual groups is shown in figure 3A. For the HC control group the average lesion area per section was 95.5±35.2 μm^2 *1000. Treatment with avasimibe resulted in a 92% reduction of lesion area (7.6±7.0 μm^2 *1000), related to the total effect of avasimibe. Comparison of the avasimibe group and the LC control (34.1±24.5 μm^2 *1000) showed a 78% reduction of atherosclerotic lesion area by avasimibe, suggesting an effect independently of its cholesterol-lowering effect.

While the avasimibe group and the LC control were almost matched for their plasma total cholesterol level, there was a slight difference in cholesterol level between those groups. Because it has been shown previously that there is a linear relationship between atherosclerotic lesion area and exposure of the arterial wall to plasma cholesterol in ApoE*3-Leiden mice[18], total cholesterol burden over time for the latter groups was calculated to correct the lesion areas for differences in these cholesterol exposures. Mean cholesterol exposure in mice treated with avasimibe was 184±26 mmol/l*weeks and for the mice receiving the LC control diet 236±34 mmol/l*weeks (p<0.005). Correction of the lesion areas for cholesterol exposure by dividing the lesion area per mouse by its individual cholesterol exposure showed that the lesion area in the avasimibe group was 73% smaller than in the LC control group (p=0.012

by Mann-Whitney test, figure 3B). These data indicate that avasimibe reduces the development of atherosclerosis independently of its cholesterol lowering effect.

Figure 3. The effect of avasimibe on atherosclerosis in the aortic valve area. **A.** The effect of avasimibe on atherosclerotic lesion area. Kruskall-Wallis test showed a significant overall difference between the groups (p=0.000). **B.** The effect of avasimibe on atherosclerotic lesion area after correction for cholesterol exposure. Statistically significant differences are indicated *p<0.05, ***p<0.005.

We also evaluated the effect of avasimibe-treatment on the severity of the lesions. Figure 4 shows representative pictures. The percentages of lesions classified in the respective lesion categories were calculated (figure 5). In the HC control group the largest percentage of all lesions is constituted of severe lesions. Lesions in the LC control group are almost equally distributed over the fatty streaks and severe lesions. In the avasimibe group, however, most lesions are mere small fatty streaks and only few lesions belong to the severe types of plaques. These results in combination with the stainings for macrophages (figure 4B, E and H) and smooth muscle cells (figure 4C, F and I) indicate that treatment with avasimibe resulted in the development of

mere small numbers of foam cells in the intima of the arterial wall containing small lipid pools as compared to the LC control group where foam cell accumulation with larger lipid pools also occured in the media. In contrast, in the HC control group large lipid pools were found in the intima and media, the presence of smooth muscle cells in the media was diminished and severe necrosis and calcification was found.

Figure 4. Representative photomicrographs of atherosclerotic lesions in the different groups. **A.** Severe plaque from the HC control group (type V). **D.** Mild plaque from the LC control group (type III). **G.** Early fatty streak from the avasimibe group (type I). **B+E+H.** Immunohistochemistry of macrophages; staining with AIA31240. **C+F+I.** Immunohistochemistry of smooth muscle cells; staining with α-actin.

The effect of avasimibe on free cholesterol content of atherosclerotic lesions

Because avasimibe reduced cholesteryl ester accumulation in the cell, it is conceivable that treatment with avasimibe would result in the accumulation of free cholesterol in the atherosclerotic lesions. While free cholesterol above a certain threshold concentration is toxic to the cell[26], accumulations of free cholesterol can

only be found in the form of cholesterol clefts. Therefore, we determined whether treatment with avasimibe increased the number of sections containing cholesterol clefts. However, the opposite was found (figure 6). Treatment with avasimibe significantly reduced the number of atherosclerotic lesions containing cholesterol clefts, indicating that avasimibe does not increase free cholesterol accumulation.

Figure 5. The effect of avasimibe on severity of atherosclerosis. Data are presented as mean ± sem. Fatty streaks = type I-III lesions, Plaques = type IV-V lesions. Statistically significant differences in distributions of atherosclerotic lesions over fatty streaks and plaques between groups are indicated **p<0.01, ***p<0.005.

Figure 6. The effect of avasimibe on the percentage of sections containing free cholesterol clefts in the atherosclerotic lesions. Statistically significant differences are indicated **p<0.01, ***p<0.005.

Figure 7. Photomicrographs of monocytes adhering to the endothelium in the LC control group **(A-B)** and the HC control group **(C-D).** Immunohistochemistry of monocytes in the LC control group **(B)** and the HC control group **(D)**; staining with AIA31240. In the avasimibe group most aortic walls were free of adhering macrophages (data not shown). Arrows indicate monocytes adhering to the endothelium. **E.** The effect of avasimibe on the amount of adhering monocytes to the endothelium. Statistically significant differences are indicated ***$p<0.005$.

The effect of avasimibe on monocyte adhesion to the endothelium

The amount of monocytes adhering to the endothelium, as shown in figure 7A-D, was counted in the same slides as used for quantification of atherosclerosis.

Figure 7E shows that monocyte attachment to the endothelium was not different between the high and low cholesterol control group. Avasimibe, however, showed a significant 80% reduction of the amount of monocytes adhering to the endothelium ($p<0.001$), suggesting that avasimibe reduces endothelial activation, independently of its cholesterol-lowering effect.

Discussion

In the present study, the ACAT-inhibitor avasimibe decreases plasma cholesterol levels by more than 50% in cholesterol-fed apoE*3-Leiden mice by reduction of VLDL/LDL cholesterol and induces a relative enrichment of triglycerides and decrease of cholesterylesters in these lipoproteins, making them potentially less atherogenic. In addition, treatment with avasimibe results in reduced progression and severity of atherosclerosis, additionally to its cholesterol-lowering effect.

The present study was designed to demonstrate a direct effect of avasimibe on the development of atherosclerosis, additionally to its cholesterol-lowering effect. Therefore, a high cholesterol control (HC) group, an HC group receiving avasimibe and a matched low cholesterol (LC) group were used to compare the development of atherosclerosis in the avasimibe group with that in the groups without ACAT-inhibitor at high or at equally low cholesterol levels.

Our results on the lipid-lowering effects of the ACAT-inhibitor avasimibe are in agreement with previous studies showing that avasimibe can potently reduce plasma lipid concentrations in rats, rabbits and hamsters. However, at the dosage used (10 mg/kg body weight per day), the plasma cholesterol reduction observed in the present study (-56%) exceeds that in previous studies in rats (-32%)[27] and hamsters (-37%)[9]. The reduction of plasma cholesterol levels by ACAT-inhibitors can mainly be attributed to a decreased intestinal cholesterol absorption[1]. However, for inhibitors with increased bioavailability as compared to the early non-absorbable hypocholesterolaemic agents[27] a reduction of the secretion of VLDL-apoB from the liver[28-30] and the subsequent proper disposal of cholesterol into the bile acid synthetic pathway[22] may also contribute to the cholesterol-lowering effect. The present study

shows that treatment with avasimibe also changed lipoprotein composition. As compared to the HC and LC controls, avasimibe-treatment resulted in a relative enrichment of VLDL/LDL with triglycerides, probably as a consequence of the decrease in cholesterol esterification in the liver. In comparison with the HC control group, the avasimibe group showed 44% lower total plasma apoB levels, in agreement with data obtained in miniature pigs[30]. However, as compared to the LC control, apoB levels were over twice as high in the avasimibe group. This indicates that plasma from the avasimibe group contains VLDL/LDL particles which are less atherogenic than those in the LC control, but also that there are more of these particles present in plasma, which again adversely contributes to atherogenicity in the avasimibe group.

Since the enzyme ACAT is also responsible for the esterification and storage of cholesterol in the macrophage, it has been hypothesized that ACAT-inhibition may prevent the accumulation of lipid-laden macrophages in the arterial wall[3,4]. Bocan et al.[25] have recently shown that avasimibe reduced the intracellular cholesteryl ester concentration in human monocyte-derived macrophages in a dose-dependent manner. To investigate whether esterification of cholesterol is inhibited in mouse macrophages at concentrations found in plasma of the avasimibe group (50±24 nmol/l), the effect of avasimibe on ACAT-activity was measured in the mouse macrophage cell line J774. At 50 nmol/l avasimibe, cholesterol esterification was significantly inhibited by 25%, suggesting that in our animal study macrophage ACAT in the vessel wall was also inhibited by avasimibe.

Comparison of atherosclerotic lesion areas in the HC control and the avasimibe group shows that avasimibe reduced atherosclerosis by 92% in the apoE*3-Leiden mice. This finding is in line with previous observations with avasimibe in hamsters[9] and other ACAT-inhibitors in rabbits[10,11] showing an anti-atherosclerotic activity of ACAT-inhibitors concomitant with reductions in plasma cholesterol concentrations. After correction of the extent of atherosclerosis for the slight difference in cholesterol exposure between the LC control and the avasimibe group, comparison of these corrected lesion areas showed that avasimibe significantly reduced the development of atherosclerosis by 73%. It has been suggested that an atherogenic diet containing cholate, such as that used in the avasimibe group, may induce an inflammatory response in the liver, resulting in adverse effects on atherogenesis[31]. However, others have found no such effect in C57BL/6J[32] and LDLr-/-[33] mice, indicating that

the effect of cholate on the liver has not been unequivocally established. The fact that we found that avasimibe in a cholate-containing diet significantly reduced atherosclerosis as compared to the low cholesterol control group without cholate, clearly favors the direct anti-atherosclerotic effect of avasimibe.

We also demonstrate that avasimibe reduced lesion severity as compared to the LC and the HC control. In the HC control group, atherosclerotic lesions were characterised by large lipid depositions in the intima and media with severe fibrosis, necrosis, calcification and reduction of smooth muscle cells. Lesions found in the avasimibe group contained only a small number of foam cells and a small lipid pool. The media was hardly ever affected and the amount of smooth muscle cells was not reduced. This is in contrast with the mild plaques found in the LC control, where the lipid pools were larger and foam cells were also found among the smooth muscle cells in the media. It is currently understood that clinical manifestations of atherosclerosis are characterised by disruption of the plaque[34] and that the most important features of a vulnerable and potentially unstable plaque are abundant presence of T-lymphocytes and macrophages, a large lipid pool in the plaque, low density of smooth muscle cells and a thin, collagen-poor fibrous cap[5]. Therefore, our results indicate that treatment with avasimibe can contribute to a higher plaque stability and consequently reduce the risk of plaque rupture, as has recently also been suggested in studies with hypercholesterolemic rabbits[25]. Also, the finding that treatment with avasimibe significantly reduced the number of atherosclerotic lesions containing cholesterol clefts, indicates that avasimibe does not increase free cholesterol accumulation in the lesions. Thus, the additional effect of avasimibe is an attenuated accumulation of lipids in the arterial wall and inhibition of the infiltration of macrophages into the media. In addition, our results show that avasimibe significantly reduced the number of monocytes adhering to the endothelium, which is considered to be one of the first steps in the development of atherosclerosis[5]. This is in agreement with *in vitro* studies that have shown that ACAT-inhibitors can decrease monocyte adhesion to endothelial cells[35]. It suggests that treatment with avasimibe reduces endothelial activation. Whether avasimibe indeed decreases the expression of adhesion- and/or chemoattractant molecules in the endothelium, remains to be investigated.

In conclusion, this study demonstrates that the ACAT-inhibitor avasimibe potently lowers plasma cholesterol levels in transgenic ApoE*3-Leiden mice. Although it

cannot be fully excluded that the change in lipoprotein composition in the avasimibe-treated animals contributes to its anti-atherogenic effect, the dramatic reduction in atherosclerotic lesion area in the avasimibe group clearly appears to be attributable to an effect additional to cholesterol-lowering. Because lesion severity is also reduced by avasimibe, treatment with avasimibe may result in higher plaque stability and therefore a reduced risk of plaque rupture.

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Reference List

1. Suckling KE, Stange EF: Role of acyl-CoA: cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* 1985;26:647-671
2. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993;362:801-809
3. Krause BK, Sliskovic DR, Bocan TMA: Emerging therapies in atherosclerosis. *Exp.Opin.Invest.Drugs* 1995;4:353-387
4. Sliskovic DR, White AD: Therapeutic potential of ACAT inhibitors as lipid lowering and anti-atherosclerotic agents. *Trends.Pharmacol.Sci.* 1991;12:194-199
5. Libby P, Geng YJ, Aikawa M, et al: Macrophages and atherosclerotic plaque stability. *Curr.Opin.Lipidol.* 1996;7:330-335
6. Krause BK, Bocan TMA: ACAT inhibitors: physiologic mechanisms for hypolipidemic and anti-atherosclerotic activities in experimental animals, in Ruffolo RR, Hollinger MA (eds): *Inflammation: Mediators and Pathways.* Boca Raton, FL, CRC Press, 1995, pp 173-198
7. Largis EE, Wang CH, DeVries VG, et al: CL 277,082: a novel inhibitor of ACAT-catalyzed cholesterol esterification and cholesterol absorption. *J Lipid Res* 1989;30:681-690
8. Burnett JR, Wilcox LJ, Telford DE, et al: Inhibition of cholesterol esterification by DuP 128 decreases hepatic apolipoprotein B secretion in vivo: effect of dietary fat and cholesterol. *Biochim Biophys Acta* 1998;1393:63-79
9. Nicolosi RJ, Wilson TA, Krause BR: The ACAT inhibitor, CI-1011 is effective in the prevention and regression of aortic fatty streak area in hamsters. *Atherosclerosis* 1998;137:77-85

10. White AD, Purchase CF2, Picard JA, et al: Heterocyclic amides: inhibitors of acyl-CoA:cholesterol O-acyl transferase with hypocholesterolemic activity in several species and antiatherosclerotic activity in the rabbit. *J.Med.Chem.* 1996;39:3908-3919

11. Ashton MJ, Bridge AW, Bush RJ, et al: RP-70676: a potent systemically available inhibitor of acyl-CoA:cholesterol o-acyl transferase (ACAT). *Bioorg.Med.Chem.Lett.* 1992;2:375-380

12. Schaffer SA, Bloom JD, De Vries VG, et al: CL 277,082, a novel inhibitor of cholesterol esterification and cholesterol absorption, in Fidge NH, Nestel PJ (eds): *Atherosclerosis VII.* Amsterdam, Elsevier Science Publishers B.V., 1986, pp 633-636

13. Kogushi M, Tanaka H, Ohtsuka I, et al: Anti-atherosclerotic effect of E5324, an inhibitor of acyl- CoA: cholesterol acyltransferase, in Watanabe heritable hyperlipidemic rabbits. *Atherosclerosis* 1996;124:203-210

14. Bocan TM, Mueller SB, Uhlendorf PD, et al: Comparison of CI-976, an ACAT inhibitor, and selected lipid- lowering agents for antiatherosclerotic activity in iliac- femoral and thoracic aortic lesions. A biochemical, morphological, and morphometric evaluation. *Arterioscler.Thromb.* 1991;11:1830-1843

15. Bocan TM: Animal models of atherosclerosis and interpretation of drug intervention studies. *Curr.Pharm.Des* 1998;4:37-52

16. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, et al: Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol.Chem.* 1993;268:10540-10545

17. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, et al: Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410

18. Groot PH, van Vlijmen BJ, Benson GM, et al: Quantitative assessment of aortic atherosclerosis in APOE* 3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arterioscler Thromb Vasc Biol* 1996;16:926-933

19. Lutgens E, Daemen M, Kockx M, et al: Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276-283

20. van Vlijmen BJ, Mensink RP, van, et al: Effects of dietary fish oil on serum lipids and VLDL kinetics in hyperlipidemic apolipoprotein E*3-Leiden transgenic mice. *J Lipid Res* 1998;39:1181-1188

21. van Vlijmen BJ, Pearce NJ, Bergö M, et al: Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. *Arzneimittelforschung.* 1998;48:396-402

22. Post SM, Zoeteweij JP, Bos MH, et al: Acyl-coenzyme A:cholesterol acyltransferase inhibitor, avasimibe, stimulates bile acid synthesis and cholesterol 7alpha-hydroxylase in cultured rat hepatocytes and in vivo in the rat. *Hepatology* 1999;30:491-500

23. Havekes LM, de Wit EC, Princen HM: Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity. *Biochem J* 1987;247:739-746

24. Gijbels MJ, van der Cammen M, van der Laan LJ, et al: Progression and regression of atherosclerosis in APOE3- Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis* 1999;143:15-25

25. Bocan TM, Krause BR, Rosebury WS, et al: The ACAT inhibitor avasimibe reduces macrophages and matrix metalloproteinase expression in atherosclerotic lesions of hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol* 2000;20:70-79

26. Kellner-Weibel G, Jerome WG, Small DM, et al: Effects of intracellular free cholesterol accumulation on macrophage viability: a model for foam cell death. *Arterioscler Thromb Vasc Biol* 1998;18:423-431

27. Lee HT, Sliskovic DR, Picard JA, et al: Inhibitors of acyl-CoA: cholesterol O-acyl transferase (ACAT) as hypocholesterolemic agents. CI-1011: an acyl sulfamate with unique cholesterol-lowering activity in animals fed noncholesterol-supplemented diets. *J.Med.Chem.* 1996;39:5031-5034

28. Carr TP, Hamilton RLJ, Rudel LL: ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of African green monkeys. *J Lipid Res* 1995;36:25-36

29. Huff MW, Telford DE, Barrett PH, et al: Inhibition of hepatic ACAT decreases ApoB secretion in miniature pigs fed a cholesterol-free diet. *Arterioscler.Thromb.* 1994;14:1498-1508

30. Burnett JR, Wilcox LJ, Telford DE, et al: Inhibition of ACAT by avasimibe decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *J Lipid Res* 1999;40:1317-1328

31. Liao F, Andalibi A, Qiao JH, et al: Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J Clin Invest* 1994;94:877-884

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

33. Lichtman AH, Clinton SK, Iiyama K, et al: Hyperlipidemia and atherosclerotic lesion development in LDL receptor-deficient mice fed defined semipurified diets with and without cholate. *Arterioscler Thromb Vasc Biol* 1999;19:1938-1944

34. Falk E, Shah PK, Fuster V: Coronary plaque disruption. *Circulation* 1995;92:657-671

35. Saxena U, Ferguson E, Newton RS: Acyl-coenzyme A:cholesterol-acyltransferase (ACAT) inhibitors modulate monocyte adhesion to aortic endothelial cells. *Atherosclerosis* 1995;112:7-17

Chapter 7

Deficiency of apolipoprotein C1 in macrophages increases cholesterol esterification and reduces cholesterol efflux

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Abstract

Background Since apolipoprotein C1 (ApoC1) expression has been shown to be up-regulated during the differentiation from monocyte to macrophage, the present study was performed to investigate whether ApoC1 is involved in lipid homeostasis in macrophages.

Methods and results Peritoneal macrophages from wild-type and ApoC1-deficient mice were isolated and loaded with acetylated LDL (AcLDL) for 48 hours. Uptake of cholesterol was hardly affected by ApoC1 deficiency, however, the ratio of cholesteryl ester (CE) to free cholesterol (FC) tended to be increased in ApoC1-deficient macrophages compared to wild-type cells. After loading the cells with cholesterol by incubation with ^{3}H-[1α,2α(n)]-cholesteryl oleate-labeled AcLDL, ApoC1-deficient macrophages exhibited 41% less efflux of cholesterol from the cells into medium as compared to wild-type cells. This reduced efflux of cholesterol in ApoC1-deficient macrophages is in accordance with the tendency to an increased CE/FC ratio in these cells.

Because acyl-CoA:cholesterol acyl transferase (ACAT) is the enzyme responsible for esterification of cholesterol in the cell, we investigated the effect of ApoC1-deficiency on ACAT activity directly. Incorporation of ^{14}C-oleic acid into cholesteryl esters was increased by more than 70% in ApoC1-deficient macrophages, indicating that ApoC1-deficiency indeed leads to increased ACAT activity. Incorporation of ^{14}C-oleic acid into triglycerides was also strongly increased in ApoC1-deficient macrophages as compared to wild-type macrophages. ApoC1 deficiency might thus lead to an enhanced availability of fatty acids for intracellular use, like triglyceride synthesis and cholesterol esterification.

Conclusion The expression level of ApoC1 in macrophages affects cholesterol esterification and cholesterol efflux and may therefore have a direct effect on foam cell formation and atherogenesis in vivo.

Introduction

After differentiation, macrophages can start accumulating lipids and develop into foam cells, which is considered to be one of the earliest and most important steps in the development of atherosclerosis. Genes that are up-regulated during

differentiation of monocytes into macrophages include e.g. apolipoprotein E (ApoE), ATP-binding cassette (ABC) transporters and fatty acid binding protein (FABP)[1]. ApoE on macrophages has been shown to be an important stimulator of cholesterol efflux from the cell to extracellular cholesterol acceptors[2-4]. Upregulation of ApoE expression therefore protects the cell from acute lipid overload. This is illustrated by the fact that mice deficient for ApoE develop accelerated atherosclerosis[5] and exhibit massive cholesterol depositions in peripheral tissues in the form of macrophage-derived foam cells[6]. Also the ABC transporter ABCa1 mediates the transport of cholesterol and phospholipids from cells to extracellular HDL. Patients suffering from Tangier disease, characterised by extremely low levels of HDL and massive cholesterol accumulations in macrophages, have a null mutation or defect in the ABCa1 gene[7]. When this ABCa1 gene was disrupted in mice, a dramatic increase in lipid-laden macrophages was indeed seen[8; 9]. In the cell, cholesterol is esterified into cholesteryl esters by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT)[10], thereby preventing the accumulation of toxic levels of free cholesterol[11]. FABP expression is also up-regulated in macrophages, thus possibly providing the cell with the fatty acids needed for the esterification process.

Recently, it has been shown that expression of ApoC1 is also up-regulated during the differentiation of monocytes into macrophages *in vitro*[1]. Apolipoprotein C1 (ApoC1) is a constituent of triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins (VLDL)) and high density lipoproteins (HDL). It plays a role in lipoprotein metabolism by inhibiting lipoprotein binding to the VLDL receptor and the LDL receptor-related protein[12]. Mice overexpressing human ApoC1 exhibit reduced adipose tissue[13], reduced fatty acid uptake by adipose tissue[14] and as a consequence protection from obesity and insulin resistance[15]. This indicates that ApoC1 plays an important role in lipid homeostasis in adipocytes. In the present study we investigated whether ApoC1 also plays a role in cellular lipid homeostasis in macrophages.

Materials

Animals

Homozygous ApoC1 deficient mice (ApoC1-/-)[16] and homozygous ApoE deficient mice(ApoE-/-)[17] were generated as described before. C57Bl6 mice were used as wild-type

controls. All mice were housed under standard conditions with free access to water and standard chow food. Animal experiments were approved by the institutional committee on animal welfare of TNO.

Peritoneal macrophage isolation and culture

Four days after an intraperitoneal injection with 1 ml of 3% Brewer's thioglycollate medium, macrophages were isolated by peritoneal lavage with 10 ml icecold PBS. The isolated macrophages were washed with PBS 3 times, counted and plated in 24-wells plates (Corning Inc., USA) at a density of $0.6x10^6$ cells per well in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 5 U/ml mouse GM-CSF (R&D Systems, Wiesbaden-Nordenstadt, Germany). After 2 hours, nonadhering cells were removed by washing. Cells were cultured at 37°C in an atmosphere of 5% CO_2 for 48 hours before the start of an experiment.

Preparation and labeling of acetylated LDL

Human LDL was isolated by ultracentrifugation[18] and acetylated with acetic anhydride (Merck, Darmstadt, Germany)[19]. Acetylated LDL was labeled with ^{3}H-[1α,2α(n)]-cholesteryl oleate (Amersham Pharmacia Biotech. Inc., USA) in the presence of human lipoprotein-deficient serum (LPDS) as a cholesteryl ester transfer protein (CETP) source[20]. Radiolabeled AcLDL was subsequently isolated by ultracentrifugation, after which the specific activity was 30.8±0.2 dpm/ng protein.

Lipid accumulation in cultured peritoneal macrophages

To investigate the effect of ApoC1 on the accumulation of cholesterol in macrophages, peritoneal macrophages from wild-type, ApoC1-/- and ApoE-/- mice were put into culture and incubated 24 hours with or without acetylated low density lipoprotein (AcLDL, 50 µg/ml in DMEM supplemented with 2% fatty acid free-BSA (Sigma Diagnostics, St. Louis, USA), 4 wells per condition). then washed twice with PBS and harvested in 0.5 ml demineralised water. Cellular protein content was measured according to Lowry et al.[21] and cellular lipids were extracted by Bligh and Dyer[22] after the addition of cholesterol acetate as internal standard. Lipids were separated by high performance thin layer chromatography (HPTLC) on silica-gel precoated plates. Quantification of lipid bands was performed by scanning the plates and integrating the density areas using TINA® version 2.09 software (Raytest, Straubenhardt, Germany).

Cholesterol efflux from cultured peritoneal macrophages

Peritoneal macrophages from wild-type, ApoC1-/- and ApoE-/- mice were put into culture (4 wells per condition). ApoE-/- macrophages were used as control, since it is known that these cells have reduced cholesterol efflux compared to wild-type cells[4]. After 48 hours of culture, cells were loaded with cholesterol by incubation for 48 hours with 50 µg/ml radiolabeled AcLDL in DMEM supplemented with 5% LPDS and 5 U/ml GM-CSF. Cells were then washed twice with DMEM supplemented with 0.2% fatty acid free-BSA and cultured for another 2 hours with DMEM supplemented with 0.5% fatty acid free-BSA to allow equilibration of the label. Thereafter, one set of cells was washed twice with PBS and lysed with 0.5 ml 0.2 M NaOH, radioactivity was measured with a scintillation counter and protein content was determined according to Lowry et al.[21] to determine the 100% value of ^{3}H-labeled cholesteryl ester accumulation at the start of the efflux experiment.

The remainder of the cells was washed twice with DMEM supplemented with 0.2% fatty acid free-BSA, after which they were incubated with DMEM supplemented with 5% serum from wild-type mice as extracellular cholesterol acceptor for 3 or 5 hours. Subsequently, the medium was removed and counted for radioactivity to determine cholesterol efflux. Cells were washed twice with PBS, lysed with 0.5 ml 0.2 M NaOH and also counted for radioactivity. Cholesterol efflux was calculated as $[dpm_{medium} / (dpm_{medium} + dpm_{cells})]*100\%$ for each time point.

Incorporation of ^{14}C-oleic acid into cholesteryl esters and triglycerides

Peritoneal macrophages from wild-type and ApoC1-/- mice were put into culture and incubated with 0, 50 or 75 µg/ml AcLDL in DMEM supplemented with 0.2% fatty acid free-BSA and 5 U/ml GM-CSF for 24 hours (6 wells per condition). The last 2 hours, [1-^{14}C]-oleic acid (100 µM, 0.2 µCi, Amersham Pharmacia Biotech. Inc., USA) was added per well. Cells were then washed twice with icecold PBS and harvested in 0.5 ml demineralised water. Cellular protein content was measured according to Lowry et al. [21] and cellular lipids were extracted by Bligh and Dyer[22] after the addition of ^{3}H-labeled cholesterol oleate as internal standard. Lipids were separated by HPTLC and radioactive lipid bands were visualised by autoradiography. Finally, radioactive bands representing cholesteryl esters and triglycerides were scraped off the silica-plates and counted for radioactivity. The amount of radioactivity found in cholesteryl esters and triglycerides are measures for activity of the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) and triglyceride synthesis in the cell, respectively.

In situ hybridizations

To determine whether ApoC1 is expressed in atherosclerotic lesions, *in situ* hybridizations were performed. Paraffin sections of the aortic valve area of hearts from ApoE*3-Leiden

transgenic mice, as a model for atherosclerosis[23], were used. One section with a healthy vessel wall of a mouse that had received a standard chow diet for 27 weeks and one section containing a vessel with an atherosclerotic lesion consisting mainly of lipid-filled macrophages of a mouse that had received a high fat/high cholesterol diet for 27 weeks were examined for the expression of ApoC1.

A riboprobe for the murine apoc1 gene transcript was synthesized by *in vitro* transcription of a cDNA fragment containing the entire ApoC1 coding sequence, cloned in the pGEMt vector (Promega, Madison, WI). The construct was linearised and the riboprobe was synthesised using SP6 RNA polymerase (Promega, Madison, WI) according to the manufacturers instructions and labeled with [^{35}S]-UTP (Amersham, U.K.).

In situ hybridizations were performed as described by Horrevoets *et al.*[24]

Statistics

All data are presented as mean ± SD. For statistical analysis SPSS 8.0 for Windows was used. Non-parametric Mann-Whitney test was used to compare effects of macrophage ApoC1 or ApoE deficiency with effects in wild-type macrophages.

Results

The effect of ApoC1 deficiency on lipid accumulation in macrophages

After incubation with AcLDL, intracellular levels of free cholesterol and cholesteryl esters were measured in peritoneal macrophages from wild-type ApoC1-/- and ApoE-/- mice. Cellular free cholesterol and cholesteryl esters were not significantly different between cell types (figure 1A and 1B). Ratios between cholesteryl ester and free cholesterol were also not significantly different between cell types (figure 1C), although ApoC1-/- and ApoE-/- macrophages seemed to have relatively more intracellular cholesteryl ester compared to wild-type cells.

The effect of ApoC1 deficiency on cholesterol efflux from macrophages

Since measurement of lipid fluxes through cells is much more sensitive to subtle changes than measurements of intracellular lipid masses as mentioned above, we investigated the effect of ApoC1 deficiency on cholesterol efflux. Macrophages from wild-type, ApoC1-/- and ApoE-/- mice were first loaded with cholesterol by incubation with ^{3}H-labeled AcLDL for 48 hours. After washing, the cells were incubated in medium containing 5% serum from wild-type mice as extracellular cholesterol

acceptor. After 3 and 5 hours of incubation, cholesterol efflux was determined by measuring label appearing in the medium.

Figure 1. The effect of macrophage ApoC1 or ApoE deficiency on lipid accumulation in macrophages after a 24 hour incubation with acetylated LDL. **A.** Intracellular free cholesterol concentrations after AcLDL incubation. **B.** Intracellular cholesteryl ester concentrations after AcLDL incubation. **C.** Ratio of cholesteryl ester to free cholesterol in the cell after AcLDL incubation.

Figure 2 shows that ApoC1 deficiency in macrophages resulted in a 40% reduced cholesterol efflux compared to that in wild-type cells after 3 hours of incubation (P<0.05). This effect is comparable to the 40% decreased cholesterol efflux found in ApoE-/- macrophages used as control cells (P<0.05). After 5 hours, cholesterol efflux from ApoC1-/- and ApoE-/- macrophages was reduced by 34% and 42% compared to wild-type macrophages, respectively (P<0.05).

These results indicate that ApoC1 deficiency leads to reduced cholesterol efflux from macrophages.

Figure 2. The effect of ApoC1 deficiency on cholesterol efflux from macrophages. ApoE deficient macrophages served as control cells. Macrophages were incubated 48 hours with ^{3}H-cholesteryl ester labeled AcLDL, after which cholesterol efflux into medium with wild-type serum as extracellular cholesterol acceptor was measured after 3 and 5 hours. *P<0.05

The effect of ApoC1 deficiency on cholesteryl ester and triglyceride synthesis in macrophages

At equal amounts of cholesterol uptake in the different cell types, reduced cholesterol efflux found in ApoC1-/- macrophages will result in an increased accumulation of cholesteryl esters. Since ACAT is the enzyme responsible for the esterification of free cholesterol in the cell, we determined whether ApoC1 deficiency has an effect on ACAT activity in macrophages. Figure 3A shows that ACAT activity was significantly higher in ApoC1-/- macrophages compared to wild-type cells (P<0.005) both under basal conditions (51%) and during incubation with 50 or 75 µg/ml AcLDL (74% and 71% respectively).

Figure 3. The effect of ApoC1 on ACAT activity and triglyceride synthesis in macrophages. Cells were incubated with AcLDL for 24 hours and the last 2 hours coincubated with ^{14}C-oleic acid. **A.** Incorporation of ^{14}C-oleic acid into cholesteryl esters in macrophages. **B.** Incorporation of ^{14}C-oleic acid into triglycerides in macrophages. *P<0.05, ***P<0.005

This increased formation of cholesteryl esters in ApoC1 deficient macrophages can be a consequence of reduced free cholesterol efflux, because the cell protects itself from toxic levels of accumulating free cholesterol by esterification. However, it is also possible that the availability of fatty acids in the cell, as substrate for esterification, is increased in ApoC1-/- macrophages. If this were the case, intracellular triglyceride synthesis should also be higher in ApoC1-/- macrophages. To discriminate between these two possibilities, we measured incorporation of ^{14}C-labeled oleic acid into triglycerides. Under basal conditions, ^{14}C-labeled oleic acid incorporation into triglycerides was increased 2.4-fold in ApoC1-/- macrophages than in wild-type cells

(P<0.005), during incubation with 50 or 75 µg/ml AcLDL this was 31% (P<0.05) and 54% (P<0.005) higher, respectively (figure 3B).

These results indicate that ApoC1 deficiency results in increased cholesterol esterification and enhanced triglyceride synthesis, indicating that ApoC1 deficiency leads to increased availability of fatty acids.

ApoC1 expression in atherosclerotic lesions

Provided that ApoC1 is expressed in atherosclerotic plaques, findings from the current *in vitro* studies postulate an important role of ApoC1 in several processes involved in atherogenesis, i.e. cholesterol efflux and cholesterol esterification. To determine whether ApoC1 is expressed in atherosclerotic lesions, *in situ* hybridisations for ApoC1 were performed in ApoE*3-Leiden transgenic mice as a model for atherosclerosis, using sections from a healthy vessel wall and an atherosclerotic lesion consisting mainly of lipid-filled foam cells. Figure 4A shows that ApoC1 is expressed at a very low level in the healthy vessel wall. However, macrophages in an atherosclerotic lesion express high levels of ApoC1 (figure 4B), corroborating our hypothesis that ApoC1 might play a protective role in atherogenesis.

Figure 4. In situ hybridizations of ApoC1. White spots indicate ApoC1 expression. **A.** A paraffin-section of a healthy vessel wall in the aortic valve area of an ApoE*3-Leiden transgenic mouse. **B.** A paraffin-section of an atherosclerotic lesion in the aortic valve area of an ApoE*3-Leiden transgenic mouse.

Discussion

Macrophage lipid metabolism plays a very important role in the development of atherosclerosis. After differentiation of monocytes into macrophages, the latter cells

start accumulating lipids, resulting in the formation of foam cells. Since this accumulation of lipids is not subject to negative feedback regulation, the macrophage depends on cholesterol efflux to maintain cellular cholesterol homeostasis.

It has been found that during the differentiation of monocytes into macrophages several genes are up-regulated, such as ApoE and ApoC1[1]. ApoE deficiency in macrophages results in diminished capacity to efflux cholesterol [2-4], which is illustrated by the fact that ApoE deficient mice exhibit accelerated accumulation of lipid-filled macrophages in the vessel wall[5]. Also, ApoE-deficient mice are protected from atherosclerosis after replacing their macrophage population by bone marrow transplantation with wild-type bone marrow, expressing normal levels of ApoE[25]. The up-regulation of ApoE during the differentiation from monocytes to macrophages and during the accumulation of cholesterol, can therefore be seen as an attempt to protect the cell from lipid overload and thereby to reduce the development of atherosclerosis. The present study shows that ApoC1-/- macrophages demonstrate a decreased efflux of cholesterol from the cell in a comparable manner as found in ApoE-/- macrophages.

Since we found equal amounts of cholesterol uptake in wild-type and ApoC1-/- macrophages, reduced cholesterol efflux in ApoC1-/- cells will result in an increased accumulation of cholesteryl esters. Because ACAT is the enzyme responsible for the esterification of cholesterol in the cell[10], we investigated the effect of ApoC1 deficiency on the activity of ACAT. In line with the tendency to an increased cholesteryl ester to free cholesterol ratio found in ApoC1-/- macrophages, ACAT activity was found to be increased in ApoC1-/- macrophages.

We recently showed that reduction of ACAT activity by the ACAT-inhibitor avasimibe reduces development of atherosclerosis in ApoE*3-Leiden transgenic mice[26]. On the other hand, recent evidence points out that total absence of ACAT activity in ApoE-deficient and LDL receptor-deficient mouse models aggravates atherogenesis[27-29]. A complete lack of ACAT activity may lead to an accumulation of unesterifiable free cholesterol in the cell, that cannot be counteracted by sufficient cholesterol efflux. This ultimately may lead to cell death and inflammatory processes, thereby enhancing atherogenesis. In wild-type macrophages, expressing normal levels of ApoC1 as compared to ApoC1 deficient cells, cholesterol esterification was found to be relatively decreased. This inhibition of ACAT activity may shift the balance of free cholesterol and cholesteryl ester into the direction of free cholesterol in a subtle way.

The higher ACAT activity found in ApoC1 deficient macrophages can either be caused by increased availability of free cholesterol due to decreased cholesterol efflux, or by increased intracellular fatty acid levels as substrate for the esterification of cholesterol. To discriminate between these two possibilities, also incorporation of ^{14}C-oleic acid into triglycerides was determined in macrophages with ApoC1 deficiency. ApoC1-/- macrophages exhibited strongly increased triglyceride synthesis compared to wild-type macrophages. These observations are in agreement with previous research showing that ApoC1 overexpression prevents uptake or promotes efflux of fatty acids from adipose tissue[14; 15]. Thus, ApoC1 deficiency, on the other hand, might lead to an enhanced availability of fatty acids for intracellular use, like triglyceride synthesis and cholesterol esterification.

Recently, a possible mechanism explaining the effect of ApoC1 on *in vivo* fatty acid metabolism in adipocytes was postulated by Tacken *et al.*[30]. They suggested that the VLDL receptor might regulate lipoprotein lipase (LPL) activity, the principle enzyme responsible for the extracellular hydrolysis of triglycerides. Since it is also known that ApoC1 strongly inhibits the VLDL receptor[31], it is possible that via this route ApoC1 lowers triglyceride hydrolysis and thereby reduces intracellular availability of fatty acids in adipocytes. A similar mechanism may hold for macrophages, because expression of the VLDL receptor has been demonstrated in macrophages[32]. In the present *in vitro* study, though, we incubated macrophages with free fatty acids, ruling out the possibility that in this setting effects of ApoC1 on LPL activity can affect the availability of intracellular fatty acids. Whether ApoC1 could possibly bind fatty acids itself or have an effect on transmembrane fatty acid transport proteins in the cell is subject to current investigation.

Recently, we found that overexpression of ApoC1 in mice protects against the development of obesity and insulin resistance by inhibiting adipose mass formation[15]. Similarly, Makowski *et al.* recently found that genetic deficiency of the adipocyte fatty-acid-binding protein aP2, a member of the intracellular fatty-acid-binding protein (FABP) family, protects against systemic insulin resistance and obesity[33]. In addition, a lack of macrophage aP2 expression reduces foam cell formation *in vivo*, caused by decreased intracellular cholesteryl ester accumulation as studied *in vitro*[34]. This observation is in line with our data showing that reduced intracellular free fatty acid levels reduce the synthesis of cholesteryl esters and triglycerides in macrophages.

Taken together, we found that presence of ApoC1 in macrophages, in contrast to deficiency of ApoC1, enhances cholesterol efflux from the cell and inhibits intracellular cholesterol esterification. Because we found high expression levels of ApoC1 in atherosclerotic lesions, as determined by *in situ* hybridisations, these findings warrant investigations on the potential protective effect of ApoC1 in foam cell formation and atherogenesis *in vivo*.

Reference List

1. Sugiyama A, Ishii M , Kohro T, Tsutsumi S, Hamakubo T, Kodama T, Hashimoto S, Matsushima K, Aburatani H: Human genes induced during monocyte/macrophage differentiation. *Atherosclerosis* 2000;151:190-190(Abstract)
2. Mazzone T: Apolipoprotein E secretion by macrophages: its potential physiological functions. *Curr Opin Lipidol* 1996;7:303-307
3. Zhang WY, Gaynor PM , Kruth HS: Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J Biol Chem* 1996;271:28641-28646
4. Langer C, Huang Y, Cullen P, Wiesenhutter B, Mahley RW, Assmann G, von Eckardstein A: Endogenous apolipoprotein E modulates cholesterol efflux and cholesteryl ester hydrolysis mediated by high-density lipoprotein-3 and lipid-free apolipoproteins in mouse peritoneal macrophages. *J Mol Med* 2000;78:217-227
5. Reddick RL, Zhang SH, Maeda N: Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. *Arterioscler Thromb* 1994;14:141-147
6. van Ree JH, Gijbels MJ, van den Broek WJ, Hofker MH, Havekes LM: Atypical xanthomatosis in apolipoprotein E-deficient mice after cholesterol feeding. *Atherosclerosis* 1995;112:237-243
7. Marcil M, Brooks-Wilson A, Clee SM, Roomp K, Zhang LH, Yu L, Collins JA, van Dam M, Molhuizen HO, Loubster O, Ouellette BF, Sensen CW, Fichter K, Mott S, Denis M, Boucher B, Pimstone S, Genest JJ, Kastelein JJ, Hayden MR: Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet* 1999;354:1341-1346
8. Orso E, Broccardo C , Kaminski WE, Bottcher A, Liebisch G, Drobnik W, Gotz A, Chambenoit O, Diederich W, Langmann T, Spruss T, Luciani MF, Rothe G, Lackner KJ, Chimini G, Schmitz G: Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. *Nat Genet* 2000;24:192-196
9. McNeish J, Aiello RJ, Guyot D, Turi T, Gabel C, Aldinger C, Hoppe KL, Roach ML, Royer LJ, de Wet J, Broccardo C, Chimini G, Francone OL: High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc Natl Acad Sci U S A* 2000;97:4245-4250
10. Suckling KE, Stange EF: Role of acyl-CoA: cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* 1985;26:647-671
11. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, Clinton SK, Sukhova GK, Lee RT: Macrophages and atherosclerotic plaque stability. *Curr.Opin.Lipidol.* 1996;7:330-335

12. Jong MC, Dahlmans VE, van Gorp PJ, Van Dijk KW, Breuer ML, Hofker MH, Havekes LM: In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor-associated protein-sensitive pathway. *J Clin Invest* 1996;98:2259-2267

13. Jong MC, Gijbels MJ, Dahlmans VE, Gorp PJ, Koopman SJ, Ponec M, Hofker MH, Havekes LM: Hyperlipidemia and cutaneous abnormalities in transgenic mice overexpressing human apolipoprotein C1. *J Clin Invest* 1998;101:145-152

14. Koopmans SJ, Jong MC, Que I, Dahlmans VE, Pijl H, Radder JK, Frolich M, Havekes LM: Hyperlipidaemia is associated with increased insulin-mediated glucose metabolism, reduced fatty acid metabolism and normal blood pressure in transgenic mice overexpressing human apolipoprotein C1. *Diabetologia* 2001;44:437-443

15. Jong MC, Voshol PJ , Muurling M, Dahlmans VEH, Romijn JA, Pijl H, Havekes LM: Protection from obesity and insulin resistance in mice overexpressing human apolipoprotein C1. *Diabetes* 2001;50:2779-2785

16. van Ree JH, Hofker MH, van den Broek WJ, van Deursen JM, van Der BH, Frants RR , Wieringa B, Havekes LM: Increased response to cholesterol feeding in apolipoprotein C1-deficient mice. *Biochem J* 1995;305 (Pt 3):905-911

17. van Ree JH, van den Broek WJ, Dahlmans VE, Groot PH, Vidgeon-Hart M, Frants RR , Wieringa B, Havekes LM, Hofker MH: Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis* 1994;111:25-37

18. Redgrave TG, Roberts DC, West CE: Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem* 1975;65:42-49

19. Basu SK, Goldstein JL, Anderson GW, Brown MS: Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci U S A* 1976; 73:3178-3182

20. Van Eck M, Herijgers N, Van Dijk KW, Havekes LM, Hofker MH, Groot PH, van Berkel TJ: Effect of macrophage-derived mouse ApoE, human ApoE3-Leiden, and human ApoE2 (Arg158-->Cys) on cholesterol levels and atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 2000;20:119-127

21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *Anal Biochem* 1951;65:42-49

22. Bligh EG, Dyer WJ: A rapid method for total lipid extraction and purification. *Can.J.Biochem.* 1959;193:911-917

23. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM : Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410

24. Horrevoets AJ, Fontijn RD, van Zonneveld AJ, de Vries CJ, ten Cate JW, Pannekoek H: Vascular endothelial genes that are responsive to tumor necrosis factor-alpha in vitro are expressed in atherosclerotic lesions, including inhibitor of apoptosis protein-1, stannin, and two novel genes. *Blood* 1999;93:3418-3431

25. Linton MF, Atkinson JB, Fazio S: Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science* 1995;267:1034-1037

26. Delsing DJ, Offerman EH, van Duyvenvoorde W, van der Boom H, de Wit EC, Gijbels MJ, van der Laarse A., Jukema JW, Havekes LM, Princen HM: Acyl-coa:cholesterol

acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in apoe*3-leiden mice. *Circulation* 2001;103:1778-1786

27. Accad M, Smith SJ, Newland DL, Sanan DA, King LEJ, Linton MF, Fazio S, Farese RVJ: Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. *J Clin Invest* 2000;105:711-719

28. Yagyu H, Kitamine T, Osuga J, Tozawa R, Chen Z, Kaji Y, Oka T, Perrey S, Tamura Y, Ohashi K, Okazaki H, Yahagi N, Shionoiri F, Iizuka Y, Harada K, Shimano H, Yamashita H, Gotoda T, Yamada N, Ishibashi S: Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *J Biol Chem* 2000;275:21324-21330

29. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RVJ: Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest* 2001;107:163-171

30. Tacken PJ, Hofker MH, Havekes LM, Van Dijk KW: Living up to a name: the role of the VLDL receptor in lipid metabolism. *Curr Opin Lipidol* 2001;12:275-279

31. Brown MS, Goldstein JL: Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. *Science* 1974;185:61-63

32. Hiltunen TP, Luoma JS, Nikkari T, Yla-Herttuala S: Expression of LDL receptor, VLDL receptor, LDL receptor-related protein, and scavenger receptor in rabbit atherosclerotic lesions: marked induction of scavenger receptor and VLDL receptor expression during lesion development. *Circulation* 1998;97:1079-1086

33. Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM: Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 1996;274:1377-1379

34. Makowski L, Boord JB, Maeda K, Babaev VR, Uysal KT, Morgan MA, Parker RA, Suttles J, Fazio S, Hotamisligil GS, Linton MF: Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med* 2001;7:699-705

Chapter 8

Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3-Leiden transgenic mice

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Abstract

Background Intimal hyperplasia after vascular injury is usually studied in animal models with healthy, normocholesterolemic animals. Here, we assess the effect of diet-induced hypercholesterolemia on the induction of intimal hyperplasia in ApoE*3-Leiden mice.

Methods and results A non-constrictive polyethylene cuff was placed around the femoral artery of APOE3*Leiden mice fed a high cholesterol-rich diet, a mild cholesterol-rich diet or a chow diet for 4 weeks. Diets were continued after cuff placement until sacrifice. At several time points (1 to 14 days) mice were sacrificed and the intimal hyperplasia in the cuffed arteries was analyzed. In mice fed a chow diet a 2 to 4 cell-layer thick intima, predominantly consisting of alpha smooth muscle cell actin positive cells was observed after 14 days. A mild cholesterol rich-diet (mean plasma-cholesterol level: 10.5 mmol/l) resulted in a 2.7-fold increase of total intimal area and a high cholesterol-rich diet (mean plasma cholesterol level 28.6 mmol/l) in a 7.8-fold increase. In the high cholesterol group, the intima consisted predominantly of lipid-loaded foam cells and alpha smooth muscle cell actin positive cells. Foam cell accumulation could be observed by as early as 3 days, resulting in a near-total occlusion of the lumen after 14 days. Hypercholesterolemia resulted in a rapid, cholesterol-dependent induction of foam cell-rich intimal hyperplasia in cuffed femoral arteries of ApoE*3-Leiden mice.

Conclusion The present data show that the combination of a local (cuff placement) and a systemic (hypercholesterolemic) risk factor of atherosclerosis results in a rapid induction (within 14 days) of atherosclerotic-like lesions in ApoE*3-Leiden mice.

Introduction

Intimal thickening is an early and essential stage in the development of atherosclerotic lesions[1]. In patients undergoing a venous bypass graft procedure, accelerated vein graft atheroma together with intimal hyperplasia is frequently observed[2]. In these patients a prolonged history of hypercholesterolemia is often present. Therefore, studying the effect of hypercholesterolemia on intimal hyperplasia after a vascular intervention in an atherosclerotic animal model is indispensable to clarify the underlying mechanisms of accelerated atherosclerosis in patients after a

vascular intervention. The process of intimal thickening has been extensively evaluated in animal models using several techniques. Experimental intimal hyperplasia can be induced by balloon denudation of the endothelium[3], by ligation of the vessel[4], by placement of a rigid polyethylene cuff[5,6], by placement of a flexible silicone collar[7,8] or by electrical injury[9]. Intimal thickening induced by these techniques, results from excessive accumulation of smooth muscle cells and deposition of extracellular matrix in the intimal layer of the vessel wall[3,10,11]. Recently it has been reported that placement of a non-constrictive collar around an arterial segment in mice results in a reproducible, concentric intimal hyperplasia within 21 days[6]. This intimal hyperplasia predominantly consists of alpha smooth muscle cell actin positive cells, as is also reported for rabbits by several other groups[6,8,12]. However, the species (mice, rats, rabbits) used in these studies were predominantly young, healthy and normocholesterolemic animals.

Mice are one of the most useful experimental animals, due to their small size, easy maintenance, short breeding time and the possibilities for carrying out advanced genetic studies such as transgenesis and gene targeting. Transgenic technologies have provided numerous different murine strains to study hyperlipidemia and atherosclerosis. The ApoE*3-Leiden transgenic mice[13] develop diet-dependent hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. In these mice, when fed a mild cholesterol, high-fat diet, early fatty-streak formation (Stage I, AHA classification[14]) can be observed in the aortic arch after three months of treatment and late complex atherosclerotic lesions, consisting of plaques with a necrotic core and a fibrous cap (Stage IV and V, AHA classification[14]) can be registered after 3-6 months of feeding a severe cholesterol-rich diet. This animal model, in which the atherosclerotic lesions have many features in common with human atherosclerotic lesions[15,16], is currently considered as one of the animal models of atherosclerosis closest to that occurring in humans.

In the current paper, this animal model of cholesterol-induced atherosclerosis has been used to evaluate the effect of plasma cholesterol on the development of intimal thickening by placing a polyethylene cuff around the femoral artery of ApoE*3-Leiden mice.

We found that the combination of systemic and local risk factors resulted in an accelerated formation of atherosclerotic-like lesions in mice. After the placement of a non-constrictive cuff, a plasma cholesterol level dependent intimal thickening was

induced within 14 days. This intimal thickening started within 3 days after cuff placement with monocyte adhesion and foam cell accumulation and progressed until near total occlusion of the lumen after 14 days.

Methods

Mice

All experiments were approved by the committee on animal welfare of TNO. Specific Pathogen-Free Transgenic ApoE*3-Leiden mice were crossbred for 18 generations with C57BL/6 mice. Male animals, aged 8-10 weeks, were allocated randomly to one of the 3 experimental diets on the basis of age and litter.

Diets

During the experimental period, animals were fed either a chow-diet, a cholesterol-enriched high-fat diet containing 0.5% cholate to improve intestinal cholesterol uptake and surpress bile acid synthesis, both leading to increased plasma cholesterol levels (High Fat and Cholate enriched diet 0.5%: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0,5%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn-oil 1%, cellulose 5.1%, mineral mixture 5.1%) or a cholesterol enriched/high fat diet containing 0.05% cholate (HFC 0.05%: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0,05%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn-oil 1%, cellulose 4.7%, mineral mixture 5.1%) 4 weeks prior to operation and continued after operation.

Placement of cuff

After 4 weeks of chow, HFC 0.05%, or HFC 0.5%, mice were anesthetized with Hypnorm (Bayer, 25mg/kg) and Dormicum (Roche, 25mg/kg) i.p. The right femoral artery was dissected from its surroundings. A non-constrictive polyethylene cuff (Portex, 0.40mm inner diameter, 0.80mm outer diameter, 1.5mm length), was placed loosely around the right femoral artery[6].

Lipids and lipoprotein analysis

Blood samples were taken under general anesthesia from the tail vein at the time of operation and sacrifice. Total plasma cholesterol (Boehringer Mannheim GmbH, kit 236691) and triglyceride (Sigma Diagnostics, kit. 337-B) concentrations were measured enzymatically using commercially available kits.

Histological assessment of intimal lesions

At sacrifice, mice were anesthetized with Hypnorm/Dormicum. The thorax was opened and mild pressure-perfusion (100mmHg) with 3.7% formaldehyde in 0.9% NaCl (w/v) for 10 minutes was performed by cardiac puncture. After perfusion, the femoral artery was harvested, fixated overnight in 3.7% formaldehyde in phosphate buffered saline and paraffin-embedded. Serial cross sections (5-μm thick) were used throughout the entire length of the cuffed femoral artery for histological analysis. Cryosections were made of 2 mice in each condition. All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert`s elastin staining was used to visualize elastic laminae. Smooth muscle cells were visualized with α-smooth muscle cell actin staining (Boehringer Mannheim) and Mac-3 (Accurate Chemical) macrophage staining was used to detect monocytes/macrophages. Lipid deposition was visualized with Oil-red-O (Boehringer Mannheim) staining. Anti-Pecam-1 antibodies (Sigma) were used as endothelial cell marker.

Quantification of intimal lesions in sections of cuffed femoral artery

Ten equally spaced cross-sections were used in all mice to quantify intimal lesions. Using image analysis software (Leica), total cross sectional medial area was measured between the external and internal elastic lamina; total cross sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Statistics

All data are presented as mean ± S.E.M. Overall comparisons between groups were performed with the Kruskal-Wallis test. If a significant difference was found, groups were compared to their control using Mann-Whitney rank sum tests. P-values less than 0.05 were regarded significant.

Results

Plasma lipid levels

Body weights were monitored at the day of the placement of the cuff and at sacrifice. No significant changes in body weights were registered in any of the animals. Plasma cholesterol and triglyceride concentrations at sacrifice are shown in figure 1. Compared with the standard mouse diet (chow), both diets HFC 0.05% and HFC 0.5% increased plasma cholesterol concentrations significantly, while serum-triglyceride concentrations were decreased as described earlier[17,18]. The distribution of lipids among the different lipoprotein classes in APOE3*Leiden mice on a HFC

0.5% diet are highly comparable to the human situation. A shift in lipoprotein profile from the HDL-sized fraction towards the VLDL/LDL-sized fractions, especially when fed HFC 0.5% diet, was observed as reported previously [15,18].

Figure 1. Plasma cholesterol and triglyceride levels at sacrifice in ApoE*3-Leiden mice fed a chow diet (n = 10), HFC 0.05% diet (n=10) and HFC 0.5% diet (n=10) for 6 weeks. ***p<0.001. No significant changes in triglyceride levels were detected.

Time course of development of intimal lesions

To study the effect of hypercholesterolemia on the development of intimal hyperplasia in time, 5 mice on the chow diet and 5 mice on HFC 0.5% diet were killed at respectively 1, 3, 7, 10 and 14 days after the placement of the cuff. In the animals on a chow diet, alpha smooth muscle cell actin positive cells invaded in the intimal region within 7 days after the placement of the cuff. After 14 days, the intimal lesion area was approximately 2 to 4 cell layers thick. This is in accordance with the intimal lesions observed in C57Bl/6 and SV 129 mice after cuff placement on a chow diet published by Moroi and also found by us (data not shown). However, in the mice fed diet HFC 0.5 %, already three days after the placement of the cuff, intimal thickening was observed on the luminal side of the inner elastic lamina. This intimal thickening progressed in time until near-total occlusion of the lumen after 14 days (Fig.2) In these hypercholesterolemic animals, monocyte adhesion to the endothelial layer was observed as early as one day after the placement of the cuff. After three days, monocyte adhesion became more distinct and the first foam cells could be detected. Seven days after the cuff placement macrophages were present in the media, in addition to the intimal lesion (figure 3, panel A.). Endothelial cells, monitored by

PECAM-1 expression, were observed three and seven days after the cuff placement (figure 3, panel B).

Figure 2. Cross-section of murine femoral artery on several timepoints (1, 3, 7, 10, 14, days) after placement of cuff in mice on a chow diet and HFC 0.5% diet.
In mice on a chow diet first signs of neointima formation were observed 7 days after placement of the cuff. After 14 days, intimal hyperplasia was 2 to 4 cell layers thick. In mice on the HFC 0.5 % diet however, cell adhesion was observed on the luminal side of the inner elastic lamina one day after cuff placement. After 3 days, foam cell deposition on the inner elastic lamina was detectable in mice on a HFC 0.5% diet. Seven days after cuff placement in mice on a HFC 0.5% diet, foam cell accumulation in the intimal lesion progressed and foam cells were observed in the media. Foam cell accumulation progressed in time until near-total occlusion of the lumen 14 days after cuff placement in mice on a HFC 0.5% diet. Magnification 10x.

Figure 3. A. Cross-sections of murine femoral artery on 3 and 7 days after placement of cuff in mice on HFC 0,5 % diet stained for monocyte/macrophage using the monoclonal antibody Mac 3. Monocyte adhesion is detectable on luminal side of inner elastic lamina already after 3 days after cuff placement. Profound macrophage infiltration both in the neointima as in the media was detectable after 7 days.
B. Cross-section of murine femoral artery on 3 and 7 days after placement of cuff in mice on HFC 0,5 % diet stained for PECAM. Magnification 10x, insets 20x.

Characterization and quantification of intimal lesions

To characterize the effect of hypercholesterolemia on intimal hyperplasia, 20 male ApoE*3-Leiden mice were randomized in 2 groups. One group (n=10) was fed a chow diet, and one group (n=10) fed a HFC 0.5% diet, 4 weeks prior to the placement of the cuff in order to obtain stable plasma cholesterol-levels. Fourteen days after the placement of the cuff, light microscopy of transverse sections through the cuffed femoral artery revealed that in the mice on a chow diet a thickening of the intimal region occurred (figure 4), while proximal and distal sections of the cuffed femoral artery possessed normal histology (data not shown). This intimal thickening was approximately 2 to 4 cell-layers thick and consisted predominantly of alpha smooth muscle cell actin positive cells and to a lesser extent monocytes/macrophages. An intact endothelial layer and inner elastic lamina was observed in the arteries 14 days after cuff placement. No foam cells were detected in

Figure 4. Cross-section of the murine femoral artery 14 days after placement of cuff in mice on a chow diet and HFC 0.5% diet; Haemotoxylin-phloxine-saffron staining. In mice on a chow diet, a multiple cell layer thick intimal hyperplasia is observed. In mice on a HFC 0.5% diet significant increase in intimal hyperplasia is observed; PECAM staining for endothelial cells. Both in the mice on a chow diet as the mice on the HFC 0.5% diet endothelial cells are present at the luminal side of the intimal lesion; Alpha smooth muscle cell actin staining for smooth muscle cells. Intimal hyperplasia in mice on a chow diet predominantly consists of alpha smooth muscle cell positive cells. In mice fed a HFC 0.5% diet, smooth muscle cells occur mainly on the luminal side of the foam cell accumulation; MAC-3 staining for macrophages. Intimal lesions of mice on the HFC 0.5% diet are predominantly consisting of lipid loaded foam cell macrophages. Macrophages are also present in granulation tissue within the cuff of both mice on a chow diet as in mice on a HFC 0.5 % diet; Oil red O lipid staining. Abundant lipid deposition in foam cells in mice on a HFC 0.5% diet, both in the intimal lesion as well as in the media.
Arrow indicates the inner elastic lamina, the polyethelyne cuff is visible as a white band. Magnification 10x, insets 20x.

any of the sections taken from the cuffed arteries of normo-cholesterolemic animals at 14 days.

In the group of animals fed a high cholesterol, cholate-containing, diet (HFC 0.5%) for 4 weeks, atherosclerotic-like lesions, predominantly consisting of a massive accumulation of macrophage foam cells in the intimal region, were observed 14 days after the placement of the cuff. At the luminal side of this foam cell accumulation, deposition of alpha smooth muscle cell actin positive cells partly on top of the macrophages could be observed as a sign of the start of lesion cap formation. The deposition of foam cells and smooth muscle cells occurred underneath an apparently intact endothelial layer and upon an intact inner elastic lamina. These spindle-like macrophage foam cells were lipid-filled and primarily present in the intimal region but also to a lesser extent in the medial region.

The abundant accumulation of macrophage foam cells and smooth muscle cells in the mice fed HFC 0.5% resulted in a profound luminal narrowing compared to mice on a chow diet. A near-total occlusion of the lumen was frequently observed in the mice on HFC 0.5% 14 days after placement of the cuff. However, a total occlusion due to intimal hyperplasia or thrombosis never occurred in any of the animals.

Correlation between plasma cholesterol levels and intimal lesion development

To evaluate the correlation between serum cholesterol levels and the development of atherosclerotic lesions in the cuffed femoral artery three groups of 9 ApoE*3-Leiden mice received either chow diet, HFC 0.05% diet or HFC 0.5% diet 4 weeks prior to the cuff placement. At time of the placement of the cuff these mice had a mean serum cholesterol level of 2.3 ± 1.2, 13 ± 5.6, 32 ± 3.2 mmol/L respectively. Quantification of total intimal areas of three groups of ApoE*3-Leiden mice revealed a significant 2.3-fold increase in mean total intimal area in mice fed HFC 0.05% diet ($p<0.001$) and a 7.8-fold increase in mice fed HFC 0.5% diet ($p<0.001$) compared to mice on a chow diet. Also significant differences in intima/media ratio and percentage luminal stenoses were observed between mice on the three different diets (Fig. 5). Correlation between plasma cholesterol levels and intimal thickening of each mouse is depicted in Fig 6.

Figure 5. Total intimal area, intima/media ratio and percentage luminal stenosis, of ApoE*3-Leiden mice fed HFC 0.05% diet (n=9) and HFC 0.5% diet (n=9) versus chow diet (n=9) 14 days after cuff placement. Total intimal area was quantified by image analysis using 10 serial sections in each cuffed artery and expressed in μm^2 (mean ± SEM). ***$p<0.001$

Figure 6. A. Representative cross-sections of murine femoral arteries of mice with different plasma cholesterol levels (HPS staining).
B. Correlation of total intimal area and serum cholesterol level in ApoE*3-Leiden mice fed a chow diet (n=18), HFC 0.05% diet (n=9) and HFC 0.5% diet (n=9). Total intimal area was calculated by image analysis using 10 serial sections in each cuffed artery.

Discussion

In this report, we describe the effect of hypercholesterolemia on intimal lesion formation after placement of a non-occlusive cuff around the femoral artery in ApoE*3-Leiden mice. In accordance with the results previously described by Moroi *et al.* in C57BL/6 and SV129 mice, placement of a non-constrictive polyethylene cuff

around the femoral artery of ApoE*3-Leiden mice on a chow diet resulted in intimal lesions predominantly consisting of alpha smooth muscle actin positive cells only. In the lesions of these normocholesterolemic animals, no foam cell macrophages were registered. Placing a cuff on ApoE*3-Leiden mice on a high cholesterol diet, however, resulted in an extremely fast and profound induction of atherosclerotic-like lesions. These lesions predominantly consisted of lipid-loaded foam cells and to a lesser extent of alpha smooth muscle cell actin positive cells. The accumulation of spindle-like lipid-filled macrophage-derived foam cells on the luminal side of the inner elastic lamina is a typical feature of early human, post-intervention atherosclerosis[14].
Two weeks after the placement of the cuff, the atherosclerotic-like lesions that developed in mice on a high cholesterol diet were abundant and resulted in a near-total occlusion of the lumen. The total area of these lesions was significantly larger (7.8-fold) than lesions developed in mice on a chow diet. Quantification of the total intimal area of mice on different diets (chow, mild, high cholesterol rich) revealed that there is a correlation between plasma cholesterol and total intimal growth. This correlation has been described earlier in transgenic mice developing atherosclerosis spontaneously on a high cholesterol diet after several months[15].
The development of the atherosclerotic-like lesions started directly after the placement of the cuff as demonstrated by monocyte adhesion, which was already present after one day. The first deposition of lipid-filled foam cells on the luminal side of the inner elastic lamina was observed as early as 3 days after the placement of the cuff. Both phenomena illustrate the extremely fast initiation of the atherosclerotic process. Also, the total area of these lesions after 14 days is far more extensive than lesions developed spontaneously in transgenic mice after a prolonged period on a high cholesterol HFC 0.5% diet without the placement of a cuff[16].
The exact mechanism by which collar-induced intimal thickening is triggered is still unclear. The disturbance of the laminar blood flow, vascular damage, production of cytokines by the granulation tissue and elimination of transmural flow by the cuff may account for the intimal thickening[19]. In the cuffed artery, shear and wall stress is altered resulting in endothelial damage and activation[7]. This leads to intimal hyperplasia predominantly consisting of smooth muscle cells in normocholesterolemic animals. In hypercholesterolemic animals, however, endothelial activation is accompanied by the accumulation of lipids in the infiltrating monocytes resulting in excessive foam cell formation. Consistent with this

observation is the fact that the predilection sites of spontaneous atherosclerosis in transgenic mice (aortic valve, aortic branch, on bifurcation sites) are sites with disturbance in laminar flow and subsequent damage to the endothelial layer.

Human-like atherosclerotic lesions develop spontaneously in ApoE*3-Leiden mice when fed an atherogenic diet for several months. However, this process of atherosclerotic lesion development is rather slow and it takes about 3-6 months on a severe cholesterol diet before type IV and type V lesions (nomenclature of AHA classification used by Stary *et al.*[14]) can be observed[15,16]. Furthermore, the exact lesion location and onset of atherosclerosis in time is unknown. These aspects imply that the analysis of the mechanisms of atherosclerosis and the evaluation of anti-atherosclerotic pharmacological and non-pharmacological strategies in this mouse model is a time-consuming process. Also, because atherosclerotic lesions, which develop spontaneously in transgenic mice, are frequently located in the central part of the arterial tree, local anti-atherosclerotic strategies are difficult to apply. Therefore, the induction of atherosclerotic-like lesions within several weeks, at a predefined, easy accessible site in the arterial tree, with a known onset in time, would be preferable for studying factors causing the early onset of atherosclerotic-like lesions and for assessing the effect of systemic and local anti-atherosclerotic therapies. As shown, the cuff model for accelerated atherosclerotic-like lesions is suitable for studying the early steps in atherosclerotic plaque formation. Moreover, the accelerated atherosclerosis observed in human vein grafts, with less organized structures lacking typical features such as the fibrous cap and necrotic core, shows high morphological resemblance to the atherosclerotic lesions observed in this model[20].

The approach of induction of intimal thickening by placement of a nonocclusive perivascular cuff in mice as described above, has several characteristics in common with collar-induced intimal thickening in rabbits. Like in rabbits, in mice intima formation occurs without endothelial cell denudation. However, the intimal lesions in ApoE*3-Leiden mice on a high cholesterol diet predominantly consist of lipid loaded foam cells whereas collar-induced lesions in Watanabe rabbits mainly consist of alpha smooth muscle cell actin positive cells[8].

In conclusion, in the present study we demonstrated that, by the placement of a non-constrictive polyethylene cuff around a femoral artery of ApoE*3-Leiden mice, highly reproducible, accelerated, atherosclerotic-like lesions can be induced. These lesions

display human characteristics especially in the early phase and are dependent on plasma cholesterol level. This accelerated, diet dependent, induction of atherosclerotic-like lesions is of value in studying the mechanisms of accelerated atherosclerosis.

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Reference list

1. Wilens, SL. The nature of diffuse intimal thickening of arteries. Am J Pathol 27, 825-839. 1951.
2. Davies MG, Hagen PO. Pathophysiology of vein graft failure: a review. *Eur J Vasc Endovasc.Surg* 1995;9:7-18.
3. Clowes AW, Reidy MA, Clowes MM. Mechanisms of stenosis after arterial injury. *Lab Invest* 1983;49:208-215.
4. Lindner V, Fingerle J, Reidy MA. Mouse model of arterial injury. *Circ Res* 1993;73:792-796.
5. Hagihara H, Nomoto A, Mutoh S, Yamaguchi I, Ono T. Role of inflammatory responses in initiation of atherosclerosis: effects of anti-inflammatory drugs on cuff-induced leukocyte accumulation and intimal thickening of rabbit carotid artery. *Atherosclerosis* 1991;91:107-116.
6. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, Huang PL. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest* 1998;101:1225-1232.
7. Booth RF, Martin JF, Honey AC, Hassall DG, Beesley JE, Moncada S. Rapid development of atherosclerotic lesions in the rabbit carotid artery induced by perivascular manipulation. *Atherosclerosis* 1989;76:257-268.
8. Kockx MM, De Meyer GR, Jacob WA, Bult H, Herman AG. Triphasic sequence of neointimal formation in the cuffed carotid artery of the rabbit. *Arterioscler Thromb* 1992;12:1447-1457.
9. Carmeliet P, Moons L, Stassen JM, De Mol M, Bouche A, van den Oord JJ, Kockx M, Collen D. Vascular wound healing and neointima formation induced by perivascular electric injury in mice. *Am J Pathol* 1997;150:761-776.
10. Reidy MA, Schwartz SM. Endothelial regeneration. III. Time course of intimal changes after small defined injury to rat aortic endothelium. *Lab Invest* 1981;44:301-308.
11. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Nerem RM. The pathogenesis of atherosclerosis: an overview. *Clin Cardiol* 1991;14:I1-16.

12. Soma MR, Donetti E, Parolini C, Sirtori CR, Fumagalli R, Franceschini G. Recombinant apolipoprotein A-IMilano dimer inhibits carotid intimal thickening induced by perivascular manipulation in rabbits. *Circ Res* 1995;76:405-411.

13. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der Boom H, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol Chem* 1993;268:10540-10545.

14. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull WJ, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 1995;92:1355-1374.

15. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, Havekes LM. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arterioscler Thromb Vasc Biol* 1996;16:926-933.

16. Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, de Muinck ED. Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276-283.

17. van Vlijmen BJ, van 't H, Mol MJ, van der Boom H, van der Zee A, Frants RR, Hofker MH, Havekes LM. Modulation of very low density lipoprotein production and clearance contributes to age- and gender- dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1996;97:1184-1192.

18. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410.

19. De Meyer GR, Van Put DJ, Kockx MM, Van Schil P, Bosmans R, Bult H, Buyssens N, Vanmaele R, Herman AG. Possible mechanisms of collar-induced intimal thickening. *Arterioscler Thromb Vasc Biol* 1997;17:1924-1930.

20. Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* 1998;97:916-931.

Chapter 9

General discussion and future perspectives

Cardiovascular disease is one of the main causes of death in Western society. The major factor contributing to the occurrence of cardiovascular events, such as myocardial infarction and stroke, is the development of atherosclerosis. This process is characterised by the build-up of lipid-accumulating macrophages in the vessel wall. This first step in atherogenesis leads to an inflammatory-proliferative response, including smooth muscle cell migration and proliferation, formation of a lipid core, necrosis, cholesterol cleft formation and calcification. Destabilising factors in the plaque, such as loss of smooth muscle cells or breakdown of matrix material, can cause rupture of an atherosclerotic plaque, ultimately leading to thrombus formation and resulting in cessation of blood flow and ischaemia in e.g. the heart or brain.

Elevated plasma levels of cholesterol and triglycerides are generally considered to be the main risk factor for the development of atherosclerosis. Therefore, prevention of cardiovascular disease focuses largely on the reduction of plasma lipid levels. Lately, the possibility of inhibiting atherogenesis by compounds that exert anti-atherosclerotic effects directly on the vessel wall is also being considered. The studies described in this thesis investigate the underlying mechanism of lipid-lowering of a newly developed cholesterol-lowering drug; the anti-atherosclerotic properties of combinations of drugs and possible new approaches to reducing atherogenesis.

In **chapter 2** we investigated the lipid-lowering effects of the new HMG-CoA-reductase inhibitor rosuvastatin in ApoE*3-Leiden transgenic mice. HMG-CoA-reductase is the rate-limiting enzyme in cholesterol synthesis in the body and is therefore an important target enzyme for the reduction of plasma cholesterol concentrations. Other HMG-CoA-reductase inhibitors, also called statins, have been shown to be potent cholesterol-lowering drugs and reduce cardiovascular mortality in several clinical trials[1]. In the search for statins that potently reduce plasma lipid levels at low dosages, the synthetic HMG-CoA-reductase inhibitor rosuvastatin was developed. We used ApoE*3-Leiden transgenic mice to study the lipid-lowering mechanisms of rosuvastatin, since these mice have been shown to have a lipoprotein profile resembling the human situation[2,3] and to respond well to other lipid-lowering therapies[4-6]. We found that rosuvastatin potently reduces both plasma cholesterol and triglyceride levels in these transgenic mice. In humans, the main action of statins is the inhibition of cholesterol synthesis, resulting in a decrease in hepatic cholesterol, which causes an upregulation of the LDL-receptor[7].

Consequently, lipoprotein clearance from plasma is increased, contributing to the reduction of plasma cholesterol levels. In ApoE*3-Leiden transgenic mice, we did not find a decrease in total body cholesterol synthesis. However, hepatic VLDL-triglyceride and VLDL-ApoB production were reduced. After gall bladder cannulation, we saw that biliary excretion of cholesterol, phospholipids and bile acids was increased as a result of rosuvastatin treatment. However, the excretion of lipids via the faeces was not altered by rosuvastatin treatment. Altogether this indicates that the lipid-lowering effect of rosuvastatin treatment in ApoE*3-Leiden mice is due to a redistribution of cholesterol and triglycerides over the various fluxes in the body. Thus, treatment of ApoE*3-Leiden transgenic mice with rosuvastatin exhibits effects on plasma lipid levels comparable to those found in the human situation with other statins, but other mechanisms of action underlie these lipid-lowering properties. Consequently, care must be taken to extrapolate findings with regard to lipid-lowering effects in ApoE*3-Leiden transgenic mice to the human situation.

Synergism between drugs in their ability to reduce development of atherosclerosis is a possible way to improve anti-atherosclerotic effects of separate drugs. In a retrospective analysis of data from the REGRESS study, investigating the effect of pravastatin on progression and regression of coronary artery disease[8], it was found that the combination of pravastatin and a calcium antagonist, which is originally prescribed as a anti-hypertensive drug, was more successful in retarding the progression of atherosclerosis than pravastatin alone[9]. *In vitro* studies had suggested many anti-atherosclerotic properties of calcium antagonists, but these effects remained under debate because of contradictory results from animal studies and clinical trials. We wondered whether calcium antagonists were able to exert anti-atherosclerotic effects only when administered together with a cholesterol-lowering drug (**Chapter 3**). It was found that atorvastatin potently reduced plasma cholesterol levels, mainly by a reduction in hepatic VLDL-production, whereas amlodipine had no effect on plasma lipid parameters. Atorvastatin also markedly reduced atherosclerosis in the aortic root, while amlodipine alone exerted no significant effect on atherosclerotic lesion size. The combination of both drugs tended to further reduce atherosclerosis as compared to treatment with atorvastatin alone, but this effect did not reach statistical significance. An interesting finding in this study was the fact that amlodipine alone was able to inhibit lesion calcification, whereas atorvastatin had no such effect. It has been suggested that the amount of calcification in

atherosclerotic lesions is predictive of the risk of coronary artery disease[10,11], but other studies dispute this, arguing that calcification is a stabilising factor in atherosclerosis, which prevents plaque rupture[12-14]. Therefore, it remains difficult to draw conclusions on whether or not amlodipine has plaque-stabilising properties.

Since atherosclerosis development in the aortic root in this study was at an advanced stage, we further investigated whether the possible additional anti-atherosclerotic effect of amlodipine during atorvastatin treatment could be discerned from the effects of atorvastatin alone when determined in an area of the vasculature where atherogenesis had not progressed so far. Therefore, we determined atheroprotective potential of amlodipine, atorvastatin and the combination of both in the aortic arch of treated ApoE*3-Leiden mice. To examine the chemical composition of plaques in the aortic arch, Raman spectroscopic imaging was used. This technique has been shown to be a useful tool for quantitatively studying the progression of atherosclerosis, both in *ex vivo* tissue[15] as well as intravascularly *in vivo*[16]. The results in **chapter 4** corroborate findings from **chapter 3**, in that again strong atherosclerotic lesion size reductions by atorvastatin were demonstrated and no effect of amlodipine alone was found on the lesion area. The combination of both drugs showed no synergism in atheroprotection. As was also found for the aortic root, amlodipine reduced lesion calcification in the aortic arch, but in this case this effect was also seen in mice treated with atorvastatin or the combination of amlodipine and atorvastatin. Putting together the results from **chapter 3 and 4**, we suggest that atorvastatin is so potent in reducing atherogenesis as a result of dramatic lipid-lowering that it cannot be surpassed by a combination with amlodipine. In clinical practice it is nowadays customary to lower plasma lipid levels very extensively by statin therapy, so it is unlikely that calcium antagonist co-therapy can be of further benefit in reducing lesion development.

The main difference between the studies presented in **chapter 3 and 4** and the REGRESS study[9], is the fact that we investigated the effects of amlodipine and atorvastatin on plaque development from the onset of atherogenesis, whereas REGRESS studied the effects in patients with existing lesions. Given that it is also of more clinical relevance to investigate effects on already developed lesions, in **chapter 5** we set up an additional study with ApoE*3-Leiden transgenic mice in which atherosclerotic lesions were present at the onset of therapy with either amlodipine, atorvastatin or the combination of both. All different therapies resulted in

a reduction of progression of exisiting lesions. No differences in plaque area were found between the different treatments, indicating that again amlodipine and atorvastatin co-therapy did not exceed the effects of atorvastatin monotherapy. In addition, none of the different therapies reduced plaque size compared to the extent of atherosclerosis present at the onset of treatment, indicating that regression of atherosclerosis had not occurred. Contrary to the abovementioned findings that amlodipine and atorvastatin were able to reduce lesion calcification when administered from the start of atherogenesis, we found no effect of amlodipine, atorvastatin or their combination on the development of calcium deposits in existing lesions. This suggests that calcification is a process, which cannot be inhibited once it has started. In addition, collagen content, as a measure of plaque stability, as well as lesion severity was not changed by the different treatments compared to the group that remained on high fat/high cholesterol diet for the whole period. This underlines the fact that amlodipine, atorvastatin and their combination were able to reduce the progression of existing plaques, but they did not change the composition of existing lesions. Taking together the results from **chapters 3 to 5**, we conclude that the addition of amlodipine therapy to statin treatment has no extra beneficial effects to atorvastatin therapy alone with respect to the development of atherosclerosis and the progression of existing plaques in ApoE*3-Leiden transgenic mice.

Although it is hard to extrapolate these effects to the human situation, it is unlikely that calcium antagonist co-therapy can exceed anti-atherosclerotic effects of statins in patients, since statin treatment already lowers lipid levels very potently and has also been shown to have additional direct anti-atherosclerotic properties in the vessel wall. Whether calcium antagonist treatment can contribute to enhanced plaque stability and thereby reduce the risk of acute cardiovascular events, remains to be further investigated.

In **chapter 6**, we investigated the anti-atherosclerotic effects of the ACAT inhibitor avasimibe in ApoE*3-Leiden transgenic mice. ACAT is the enzyme responsible for the esterification of cholesterol in the cell and it exists in the isoforms acat1, predominantly expressed in macrophages, and acat2, mainly present in the liver and intestine in murine models. ACAT plays an important role in the uptake of cholesterol in the intestine and the secretion of VLDL from the liver. Consequently, inhibition of ACAT has been proposed as a new approach to the reduction of plasma cholesterol levels. However, apart from these lipid-lowering effects, inhibition of ACAT may also

have direct anti-atherosclerotic effects, since the reduction of cholesterol esterification in macrophages present in the vessel wall can inhibit the formation of foam cells characterising an atherosclerotic lesion. We studied whether the ACAT-inhibitor avasimibe could reduce atherosclerosis development independently from its lipid-lowering effects in ApoE*3-Leiden mice. We found that, in addition to its cholesterol-lowering effect, avasimibe also exerted direct anti-atherosclerotic effects on the vessel wall. Not only the atherosclerotic lesion area but also the severity of the lesions was reduced by avasimibe treatment. Recent studies have shown that total deficiency of the acat1 isoform, normally present in macrophages, in mouse models for atherosclerosis leads to xanthomatosis and an aggravation of atherogenesis[17-19]. Since macrophages in these acat1-deficient mice are not able to esterify cholesterol that is taken up, toxic levels of intracellular free cholesterol are built up. Ultimately, this leads to cell death and the extracellular formation of free cholesterol crystals. The presence of such crystals in an atherosclerotic plaque induces inflammatory responses, thereby exacerbating atherogenesis. To investigate whether inhibition of the enzyme ACAT by avasimibe, which inhibits both acat1 and acat2, had similar effects such as acat1-deficiency on free cholesterol accumulation in atherosclerotic lesions, the amount of cholesterol crystals in plaques was measured. In contrast to the findings in acat1-knockout mice, we found that the inhibition of ACAT by avasimibe reduced free cholesterol accumulation in atherosclerotic lesions. Hence, it seems that the subtle shift in balance between cholesterol esterification and cholesteryl ester hydrolysis brought about by ACAT-inhibition, can result in a favourable effect on cellular lipid metabolism and, consequently, atherogenesis. In addition, the fact that avasimibe treatment results in a reduction in atherosclerotic lesion severity and free cholesterol accumulation in the plaque, suggests that avasimibe therapy may result in higher plaque stability and therefore a reduced risk of plaque rupture. In contrast to studies in rodents, human trials have so far shown that ACAT-inhibition is not a very potent approach to reduce plasma cholesterol levels in patients. Therefore, possible direct anti-atherosclerotic or plaque-stabilising features of ACAT-inhibitors can be valuable additions to existing lipid-lowering therapies, such as statins.

In the search for new targets for anti-atherosclerotic therapy, we investigated the role of apolipoprotein C1 in macrophage lipid homeostasis in **chapter 7**. ApoC1 is known to play a role in lipid metabolism by inhibiting lipoprotein binding to receptors[20].

However, it has also been found recently that mice overexpressing ApoC1 are protected from obesity and insulin resistance. Their adipocytes were found to be smaller than in wild-type controls, either via the prevention of uptake of fatty acids or the enhanced efflux of fatty acids by ApoC1[21,22]. These findings indicated that ApoC1 also plays an important role in cellular lipid metabolism. The recent finding that ApoC1 expression is upregulated during differentiation of monocytes into macrophages[23] as one of the first important steps in atherogenesis, prompted us to investigate the role of ApoC1 in macrophage lipid metabolism. Cholesterol efflux from macrophages is considered to be an important process, as it protects against foam cell formation as one of the first steps in atherogenesis. We found that ApoC1 facilitates the efflux of cholesterol from lipid-laden macrophages, most probably by reducing the intracellular availability of free fatty acids used for cholesterol esterification and triglyceride synthesis. Such a mechanism fits the aforementioned action of ApoC1 in adipocytes.

These proposed mechanisms of ApoC1 in lipid homeostasis in the macrophage suggest that expression of ApoC1 in the macrophage can potentially protect against the formation of foam cells and, as a consequence, the development of atherosclerosis.

The conventional way to study atherogenesis in ApoE*3-Leiden transgenic mice is by putting the mice on a high cholesterol/high fat diet for 3 to 6 months. In this time period moderate to severe atherosclerotic plaques are formed[24,25]. Although mostly first atherosclerotic lesions form in the aortic root, the exact location of lesion development is not well defined. In **chapter 8** we investigated the effect of hyperlipidaemia in ApoE*3-Leiden mice on the induction of intimal thickening in the femoral artery after the placement of a nonconstrictive polyethylene cuff. When these mice were fed a high cholesterol/high fat diet, a rapid induction of atherosclerotic lesions occurred exactly in the cuffed arteries. The total intimal area was highly correlated to plasma cholesterol levels and near occlusion of the lumen was found after 14 days. These atherosclerotic lesions show a high resemblance to accelerated atherosclerosis observed in human vein grafts, in that they mainly consist of lipid-filled foam cells without features such as a necrotic core or a fibrous cap[26]. This model is therefore less suitable for investigating processes involved in late atherogenesis. However, the placement of a cuff around the femoral artery of a hyperlipidaemic mouse does provide a model for the investigation of monocyte

adherance to the endothelium, foam cell formation and early atherogenesis within a short time period and at a predefined location in the arterial tree. It is expected that in the future, this model will be useful for exploring anti-atherosclerotic treatment strategies, such as drug treatment and local gene delivery at an early stage of atherogenesis.

Reference List

1. Bucher HC, Griffith LE, Guyatt GH: Systematic review on the risk and benefit of different cholesterol-lowering interventions. *Arterioscler Thromb Vasc Biol* 1999;19:187-195
2. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van Vlijmen B, van der Boom H, Havekes LM, Frants RR : Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J Biol.Chem.* 1993;268:10540-10545
3. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM : Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest* 1994;93:1403-1410
4. van Vlijmen BJ, Pearce NJ, Bergö M, Staels B, Yates JW, Gribble AD, Bond BC, Hofker MH, Havekes LM, Groot PH: Apolipoprotein E*3-Leiden transgenic mice as a test model for hypolipidaemic drugs. *Arzneimittelforschung.* 1998;48 :396-402
5. van Vlijmen BJ, Mensink RP, van, Offermans RF, Hofker MH, Havekes LM: Effects of dietary fish oil on serum lipids and VLDL kinetics in hyperlipidemic apolipoprotein E*3-Leiden transgenic mice. *J Lipid Res* 1998;39:1181-1188
6. Volger OL, van der Boom H, de Wit EC, van Duyvenvoorde W, Hornstra G, Plat J, Havekes LM, Mensink RP, Princen HM: Dietary plant stanol esters reduce vldl cholesterol secretion and bile saturation in apolipoprotein e*3-leiden transgenic mice. *Arterioscler Thromb Vasc Biol* 2001;21:1046-1052
7. Brown MS, Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986;232:34-47
8. Jukema JW, Bruschke AV, van Boven AJ, Reiber JH, Bal ET, Zwinderman AH, Jansen H, Boerma GJ, van Rappard FM, Lie KI: Effects of lipid lowering by pravastatin on progression and regression of coronary artery disease in symptomatic men with normal to moderately elevated serum cholesterol levels. The Regression Growth Evaluation Statin Study (REGRESS). *Circulation* 1995;91:2528-2540
9. Jukema JW, Zwinderman AH, van Boven AJ, Reiber JH, Van der Laarse A, Lie KI, Bruschke AV: Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler Thromb Vasc Biol* 1996;16:425-430
10. Mintz GS, Pichard AD, Popma JJ, Kent KM, Satler LF, Bucher TA, Leon MB: Determinants and correlates of target lesion calcium in coronary artery disease: a clinical, angiographic and intravascular ultrasound study. *J Am Coll Cardiol* 1997;29:268-274

11. Iribarren C, Sidney S, Sternfeld B, Browner WS: Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke, and peripheral vascular disease. *JAMA* 2000;283:2810-2815

12. Doherty TM, Detrano RC, Mautner SL, Mautner GC, Shavelle RM: Coronary calcium: the good, the bad, and the uncertain. *Am Heart J* 1999;137:806-814

13. Detrano RC, Wong ND, Doherty TM, Shavelle RM, Tang W, Ginzton LE, Budoff MJ, Narahara KA: Coronary calcium does not accurately predict near-term future coronary events in high-risk adults. *Circulation* 1999;99:2633-2638

14. Huang H, Virmani R , Younis H, Burke AP, Kamm RD, Lee RT: The impact of calcification on the biomechanical stability of atherosclerotic plaques. *Circulation* 2001;103:1051-1056

15. van de Poll SWE, Romer TJ, Volger OL, Delsing DJM, Bakker Schut TC, Princen HMG, Jukema JW, Havekes LM, Van der Laarse A, Puppels GJ: Raman spectroscopic evaluation of the effects of diet and lipid-lowering therapy on atherosclerotic plaque development in mice. *Arterioscler Thromb Vasc Biol* 2001;in press

16. Buschman HP, Marple ET, Wach ML, Bennett B, Schut TC, Bruining HA, Bruschke AV, van Der LA, Puppels GJ: In vivo determination of the molecular composition of artery wall by intravascular Raman spectroscopy. *Anal Chem* 2000;72:3771-3775

17. Accad M, Smith SJ, Newland DL, Sanan DA, King LEJ, Linton MF, Fazio S, Farese RVJ: Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. *J Clin Invest* 2000;105:711-719

18. Yagyu H, Kitamine T, Osuga J, Tozawa R, Chen Z, Kaji Y, Oka T, Perrey S, Tamura Y, Ohashi K, Okazaki H, Yahagi N, Shionoiri F, Iizuka Y, Harada K, Shimano H, Yamashita H, Gotoda T, Yamada N, Ishibashi S: Absence of ACAT-1 attenuates atherosclerosis but causes dry eye and cutaneous xanthomatosis in mice with congenital hyperlipidemia. *J Biol Chem* 2000;275:21324-21330

19. Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RVJ: Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. *J Clin Invest* 2001;107:163-171

20. Jong MC, Dahlmans VE, van Gorp PJ, Van Dijk KW, Breuer ML, Hofker MH, Havekes LM: In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor-associated protein-sensitive pathway. *J Clin Invest* 1996;98:2259-2267

21. Koopmans SJ, Jong MC, Que I, Dahlmans VE, Pijl H, Radder JK, Frolich M, Havekes LM: Hyperlipidaemia is associated with increased insulin-mediated glucose metabolism, reduced fatty acid metabolism and normal blood pressure in transgenic mice overexpressing human apolipoprotein C1. *Diabetologia* 2001;44:437-443

22. Jong MC, Voshol PJ , Muurling M, Dahlmans VEH, Romijn JA, Pijl H, Havekes LM: Protection from obesity and insulin resistance in mice overexpressing human apolipoprotein C1. *Diabetes* 2001;in press:

23. Sugiyama A, Ishii M, Kohro T, Tsutsumi S, Hamakubo T, Kodama T, Hashimoto S, Matsushima K, Aburatani H: Human genes induced during monocyte/macrophage differentiation. *Atherosclerosis* 2000;151:190-190(Abstract)

24. Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon HM, Havekes LM: Quantitative assessment of aortic atherosclerosis in APOE* 3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arterioscler Thromb Vasc Biol* 1996;16:926-933

25. Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, de Muinck ED: Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276-283

26. Motwani JG, Topol EJ: Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* 1998;97:916-931

Summary & samenvatting

Summary

Elevated levels of plasma cholesterol are the main risk factor for the development of cardiovascular disease, which is still the leading cause of death in Western society. The development of cardiovascular disease is primarily caused by atherosclerosis, which is characterised by an accumulation of lipids, fibrotic tissue and cellular material in the vessel wall. Prevention of the development of atherosclerosis focusses largely on the use of lipid-lowering drugs.

In **chapter 2** we investigated the mechanism of cholesterol- and triglyceride lowering by rosuvastatin in ApoE*3-Leiden transgenic mice. Rosuvastatin is a newly developed and very potent inhibitor of the enzyme HMG-CoA-reductase, which catalyses the rate-limiting step in cholesterol biosynthesis. Rosuvastatin treatment in high fat/high cholesterol-fed mice resulted in decreased hepatic VLDL-triglyceride and VLDL-ApoB production. VLDL lipid composition remained unchanged, indicating that the plasma-lowering of cholesterol and triglycerides was caused by a reduction in the number of VLDL-particles secreted from the liver. Cholesterol and triglyceride synthesis in the liver were not found to be reduced by rosuvastatin treatment, while hepatic lipid content also did not change as a result of rosuvastatin therapy. We found that rosuvastatin treatment resulted in an increased biliary excretion of cholesterol, phospholipids and bile acids. However, excretion of lipids via the faeces was not changed in treated mice. Thus, rosuvastatin treatment in ApoE*3-Leiden mice leads to a redistribution of cholesterol and triglycerides over the body, both by reduced hepatic VLDL-production and enhanced enterohepatic circulation of cholesterol and phospholipids, leading to potent reductions in plasma cholesterol and triglyceride levels.

Development of new therapies for the treatment of atherosclerosis has lately shifted from focussing merely on lipid-lowering to finding targets for direct anti-atherosclerotic effects on the vessel wall. Calcium antagonists, originally used as antihypertensive drugs, have been implicated to have direct anti-atherosclerotic properties. So far, however, results from animal studies and clinical trials have not been able to unequivocally establish these anti-atherosclerotic effects by calcium antagonists. Retrospective analysis of clinical data revealed that statin treatment together with calcium antagonist therapy reduced the progression of atherosclerosis more that statin treatment alone[1]. We, therefore, investigated in **chapter 3** whether

calcium antagonists are only able to exert their anti-atherosclerotic properties when administered together with a statin as cholesterol-lowering drug. Treatment of ApoE*3-Leiden transgenic mice with atorvastatin resulted in a potent reduction of plasma cholesterol levels, mainly caused by a reduction in hepatic VLDL-production. The calcium antagonist amlodipine had no effect on plasma lipid levels. A group receiving both amlodipine and atorvastatin was added to the experiment to investigate possible synergistic effects in the reduction of atherosclerosis. Amlodipine had no effect on the atherosclerotic lesion area, whereas atorvastatin alone markedly reduced atherosclerosis. The combination of amlodipine and atorvastatin tended to reduce atherosclerosis even more, but this effect was not significant. Amlodipine alone did reduce atherosclerotic lesion calcification, while atorvastatin had no significant effect. The combination of both resulted in a near absence of calcium deposits in the lesions.

To further investigate the extent of atherosclerosis in the aortic arch of the mice treated with amlodipine, atorvastatin or their combination, the protein-, lipid- and calcium content of atherosclerotic lesions was determined by a new Raman-spectroscopy technique (**chapter 4**). Cholesterol accumulation in the aortic arch was not affected by amlodipine, whereas it was potently reduced by atorvastatin and the combination of amlodipine and atorvastatin. Both amlodipine and atorvastatin were able to reduce lesion calcification, while the combination of both was as effective as atorvastatin alone. These results corroborate the findings of **chapter 3**, showing that the combination of amlodipine and atorvastatin shows no synergistic effect on atherosclerosis development.

Since it is more clinically relevant to investigate the effects of anti-atherosclerotic therapy on existing lesions, treatment with amlodipine, atorvastatin and their combination was applied in ApoE*3-Leiden transgenic mice which had already developed plaques (**chapter 5**). Both in the aortic root as well as in the aortic arch, we found that amlodipine, atorvastatin and their combination were equally able to reduce the progression of existing lesions. Plaque size was not reduced as compared to the lesions present at the onset of treatment in any of the groups treated, indicating that regression of atherosclerosis had not occurred. Calcification and collagen content in the plaque were not changed by any treatment regimen, suggesting that the different treatments only reduced progression of plaque size and not lesion composition. In view of the results from **chapters 3, 4 and 5**, we can

conclude that amlodipine has no additional anti-atherosclerotic effect when co-administered with atorvastatin.

A possible new approach to reducing plasma cholesterol levels is to inhibit the enzyme ACAT, which is responsible for the intracellular esterification of cholesterol into cholesteryl esters. However, apart from their lipid-lowering properties, ACAT-inhibitors may also have direct anti-atherosclerotic effects, since they can also inhibit cholesterol esterification and -accumulation in macrophages present in the vessel wall. We studied in **chapter 6** whether the ACAT-inhibitor avasimibe has anti-atherosclerotic effects independently from its lipid-lowering properties. Treatment of ApoE*3-Leiden mice fed a high fat/high cholesterol (HFC) diet with avasimibe resulted in marked reductions in plasma cholesterol levels as compared to a group receiving only the HFC diet. Compared to the latter group, avasimibe treatment potently reduced atherosclerotic lesion area. Moreover, compared to a group with equally low plasma cholesterol levels without receiving avasimibe, treatment with avasimibe also resulted in a major reduction in atherosclerosis. This indicates that avasimibe, in addition to its lipid-lowering effects, shows direct anti-atherosclerotic properties in ApoE*3-Leiden mice.

In the search for new targets for anti-atherosclerotic therapy, we investigated the role of apolipoprotein C1 on macrophage lipid homeostasis (**chapter 7**). Uptake of cholesterol in peritoneal macrophages was hardly affected by ApoC1 deficiency, however, the ratio of cholesteryl ester (CE) to free cholesterol (FC) tended to be increased in ApoC1-deficient macrophages compared to wild type cells. ApoC1-deficient macrophages exhibited less efflux of cholesterol from the cells as compared to wild type cells. This reduced efflux of cholesterol in ApoC1-deficient macrophages was in accordance with the increased CE/FC ratio in these cells. Activity of the enzyme ACAT, on the other hand, was increased by ApoC1 deficiency. Concomitantly, triglyceride synthesis was also strongly increased in ApoC1-deficient macrophages. ApoC1-deficiency might thus lead to an enhanced availability of fatty acids for intracellular use, like triglyceride synthesis and cholesterol esterification. As a consequence, the presence of ApoC1 in a macrophage leads to reduced cholesterol esterification and enhanced cholesterol efflux. Thus, the expression of ApoC1 in the macrophage can potentially protect against the formation of foam cells and therefore the development of atherosclerosis.

In **chapter 8**, we investigated the effect of hyperlipidaemia in ApoE*3-Leiden transgenic mice on the induction of intimal thickening in the femoral artery after the placement of a non-occlusive cuff. Within a few days after cuff placement, foam cell accumulations in the intima of the vessel wall occurred. After 14 days, the process of accumulation of lipid-filled foam cells in the intima resulted in the near occlusion of the lumen. Lesion size in the cuffed femoral artery was highly dependent on plasma cholesterol levels. Conventional studies of atherogenesis in ApoE*3-Leiden mice are very time-consuming and the location where atherosclerotic lesions develop is not very well standardised. On the contrary, placement of a cuff around the femoral artery of a hyperlipidaemic ApoE*3-Leiden mouse provides a good alternative for studying foam cell formation and early atherogenesis. With this approach lesions develop rapidly within the cuff, thus in a well-defined area of the vasculature.

Reference

1. Jukema JW, Zwinderman AH, van Boven AJ, Reiber JH, Van der Laarse A, Lie KI, Bruschke AV: Evidence for a synergistic effect of calcium channel blockers with lipid-lowering therapy in retarding progression of coronary atherosclerosis in symptomatic patients with normal to moderately raised cholesterol levels. The REGRESS Study Group. *Arterioscler Thromb Vasc Biol* 1996;16:425-430

Samenvatting

Hart- en vaatziekten zijn de meest voorkomende doodsoorzaak in de Westerse wereld. Het belangrijkste proces wat leidt tot acute problemen in de bloedvaten, zoals een hartinfarct of een beroerte, is de ontwikkeling van atherosclerose. In de volksmond wordt dit proces ook wel aderverkalking genoemd. Atherosclerose wordt gekarakteriseerd door de stapeling van macrofagen in de vaatwand die veranderen in schuimcellen wanneer ze vetten en cholesterol gaan accumuleren. Deze eerste stap in de vorming van atherosclerose veroorzaakt een ontstekingsreactie in de vaatwand, gevolgd door de migratie van gladde spiercellen, celdood, vorming van cholesterol kristallen en verkalking. Een plaque, zoals een atherosclerotische laesie in de vaatwand wordt genoemd, kan losscheuren en de vorming van een stolsel veroorzaken. Dit kan uiteindelijk resulteren in het blokkeren van de bloedstroom in bijvoorbeeld de richting van het hart of de hersenen.

De belangrijkste risicofactoren voor de ontwikkeling van atherosclerose zijn verhoogde concentraties van cholesterol en triglyceriden in het bloed. Omdat cholesterol en triglyceriden niet oplosbaar zijn in water, worden ze in het bloed getransporteerd in deeltjes die lipoproteïnen worden genoemd. Lipoproteinen worden opgedeeld in verschillende subklassen: chylomicronen bevatten met name triglyceriden afkomstig uit voedingsvet, en worden geproduceerd in de darmen; VLDL (very low density lipoprotein) wordt geproduceerd in de lever en bevat ook relatief meer triglyceriden dan cholesterol; wanneer triglyceriden uit VLDL worden afgebroken tot vetzuren voor de levering van energie, ontstaat LDL (low density lipoprotein) wat relatief meer cholesterol bevat dan triglyceriden; en tenslotte het HDL (high density lipoprotein) zorgt voor het transport van cholesterol vanuit perifere weefsels richting de lever, waar het afgebroken kan worden. Een hoog VLDL en LDL gehalte in het bloed is geassocieerd met een verhoogd risico op hart- en vaatziekten, terwijl het HDL ook wel het "goede cholesterol" wordt genoemd en bescherming biedt tegen de ontwikkeling van atherosclerose.

Omdat plasma cholesterol en triglyceriden gehalten algemeen beschouwd worden als de belangrijkste risicofactor voor de ontwikkeling van atherosclerose, richt de preventie van hart- en vaatziekten zich met name op het verlagen van plasma lipiden. De laatste tijd komt er ook steeds meer interesse voor stoffen die mogelijk atherogenese remmen door directe effecten op de vaatwand. In dit proefschrift

hebben we het lipid-verlagende mechanisme van een nieuw cholesterol verlagend medicijn onderzocht; gekeken naar mogelijke anti-atherosclerotische effecten van combinaties van medicijnen; en nieuwe benaderingen voor de remming van atherosclerose onderzocht.

In **hoofdstuk 2** hebben we het mechanisme van cholesterol- en triglyceriden verlaging van het medicijn rosuvastatine onderzocht in ApoE*3-Leiden transgene muizen. Statines zijn remmers van het enzym HMG-CoA-reductase, wat de belangrijkste stap in de synthese van cholesterol in het lichaam katalyseert. We hebben ApoE*3-Leiden transgene muizen gebruikt, omdat de verdeling van de verschillende lipoproteïnen in het plasma van deze muizen vergelijkbaar is met die bij de mens. Tevens ontwikkelen deze muizen atherosclerotische plaques in de loop van de tijd, als ze op een hoog vet/hoog cholesterol (HFC) dieet worden gezet. Behandeling van HFC-gevoede muizen met rosuvastatine resulteerde in een verlaging van de VLDL-productie in de lever. De samenstelling van het VLDL bleef gelijk, zodat het verminderde aantal VLDL-deeltjes geproduceerd door de lever ervoor zorgde dat plasma concentraties van zowel cholesterol als triglyceriden aanzienlijk daalden. De productie van cholesterol en triglyceriden in de lever werd niet geremd door rosuvastatine behandeling, terwijl ook de lipidensamenstelling van de lever niet veranderde. De excretie van cholesterol uit de lever via de gal in de vorm van galzuren en vrij cholesterol was verhoogd na rosuvastatine behandeling. Triglyceriden werden ook via de gal verwijderd uit de lever in de vorm van fosfolipiden. Echter, de secretie van lipiden via de faeces was niet verhoogd in deze behandelde muizen. Dus behandeling van ApoE*3-Leiden muizen met rosuvastatine veroorzaakt een herverdeling van cholesterol en triglyceriden in het lichaam als gevolg van zowel verlaagde VLDL-productie in de lever en verhoogde enterohepatische circulatie (d.w.z. het verblijf van lipiden in het lever-darm compartiment), wat leidt tot een duidelijke verlaging van plasma concentraties van cholesterol en triglyceriden.

Tegenwoordig wordt bij de ontwikkeling van nieuwe behandelingsmethoden voor atherosclerose niet alleen meer gekeken naar lipiden verlaging, maar ook naar mogelijkheden om direct aan te grijpen in de processen die in de vaatwand atherogenese veroorzaken. Van calcium antagonisten, oorspronkelijk voorgeschreven als bloeddruk-verlagende middelen, is in verschillende studies aangetoond dat ze directe anti-atherosclerotische eigenschappen kunnen hebben.

Echter, de resultaten van verschillende dier- en klinische studies spreken elkaar vaak tegen met betrekking tot deze anti-atherosclerotische karakteristieken. Recentelijk is gebleken uit een klinische studie naar de effecten van behandeling met pravastatine op de progressie en regressie van atherosclerose, dat behandeling van patiënten met een combinatie van pravastatine en een calcium antagonist beter in staat was het atherosclerotische proces te remmen dan pravastatine alleen. Daarom hebben wij in **hoofdstuk 3** onderzocht of de calcium antagonist amlodipine alleen zijn anti-atherosclerotische werking kan uitoefenen wanneer het in combinatie gegeven wordt met een cholesterol-verlagend medicijn, in ons geval atorvastatine, in ApoE*3-Leiden transgene muizen. Behandeling met atorvastatine resulteerde in een duidelijke verlaging van het plasma cholesterol gehalte, met name als gevolg van een verlaagde VLDL-productie in de lever. Amlodipine had geen effect op plasma lipiden. Aan het experiment werd een groep toegevoegd die zowel amlodipine als atorvastatine ontving om mogelijk een elkaar versterkend effect te vinden op de remming van atherosclerose. Amlodipine had geen effect op het atherosclerotische laesie oppervlak in het kleppengebied van de aorta, terwijl atorvastatine behandeling atherosclerose aanzienlijk verminderde. De combinatie van amlodipine en atorvastatine leek atherosclerose nog verder te remmen, maar dit effect was niet statistisch significant. Amlodipine behandeling remde wel de verkalking van de atherosclerotische plaques, terwijl atorvastatine hier geen effect op had. Behandeling met de combinatie van beide resulteerde vrijwel in de afwezigheid van verkalking in de plaques.

Om de mate van atherosclerose in de aortaboog van de muizen behandeld met amlodipine, atorvastatine of hun combinatie verder te onderzoeken, hebben we het eiwit-, lipiden- en calcium gehalte in atherosclerotische laesies bepaald met behulp van een nieuwe Raman spectroscopie techniek in **hoofdstuk 4**. Cholesterol stapeling in de aortaboog werd niet beïnvloed door behandeling met amlodipine, terwijl het aanzienlijk geremd werd door atorvastatine en de combinatie van amlodipine en atorvastatine. Zowel amlodipine als atorvastatine reduceerden verkalking van de plaque, maar de combinatie van beide was even effectief als atorvastatine alleen. Deze resultaten onderschrijven de bevindingen van hoofdstuk 3, omdat ze laten zien dat de combinatie van amlodipine en atorvastatine geen additioneel effect heeft op atherosclerose ontwikkeling.

Voor een eventuele vergelijking met de humane situatie is het relevanter om te onderzoeken of medicijnen een effect hebben op atherosclerotische plaques die al aanwezig zijn bij aanvang van behandeling. Daarom hebben we in **hoofdstuk 5** ApoE*3-leiden muizen behandeld met amlodipine, atorvastatine of hun combinatie, nadat deze muizen al atherosclerose hadden ontwikkeld. Zowel in het kleppengebied van de aorta als in de aortaboog vonden we dat amlodipine, atorvastatine en de combinatie van beide even effectief waren in het remmen van de progressie van bestaande plaques. De plaque grootte werd niet verkleind in vergelijking met de laesies die aanwezig waren op het moment van de start van behandeling, wat aangeeft dat regressie van atherosclerose niet is opgetreden. Verkalking en de hoeveelheid collageen, wat de plaque stabiliseert, waren niet veranderd in de plaque na de verschillende behandelingen. Dit duidt aan dat de verschillende behandelingen alleen de progressie van de plaque-grootte en niet de plaque samenstelling hebben veranderd. Uit de samengenomen resultaten van de **hoofdstukken 3, 4 en 5** kunnen we concluderen dat amlodipine behandeling geen additioneel anti-atherosclerotisch effect heeft wanneer het toegevoegd wordt aan behandeling met atorvastatine.

Een mogelijke nieuwe benadering voor het verlagen van plasma cholesterol waarden is het remmen van het enzym acyl-CoA:cholesterol acyltransferase (ACAT), wat verantwoordelijk is voor de verestering van cholesterol in de cel. ACAT speelt namelijk een belangrijke rol in de opname van cholesterol in de darm en in de secretie van VLDL uit de lever. Echter, naast lipiden verlaging kan remming van ACAT ook directe anti-atherosclerotische effecten hebben, omdat remming van cholesterol verestering in macrofagen in de vaatwand de vorming van schuimcellen kan verminderen. Wij hebben in **hoofdstuk 6** onderzocht of de ACAT-remmer avasimibe in ApoE*3-Leiden transgene muizen anti-atherosclerotische effecten heeft onafhankelijk van zijn lipid-verlagende eigenschappen. Behandeling van HFC-gevoede muizen met avasimibe resulteerde in sterke verlaging van plasma cholesterol concentraties in vergelijking met muizen die alleen het HFC dieet kregen. Vergeleken met deze groep verminderde avasimibe behandeling duidelijk het oppervlak van atherosclerotische laesies. Bovendien resulteerde behandeling met avasimibe in vergelijking met een groep met dezelfde plasma cholesterol waarden die geen avasimibe kreeg, ook in een sterke vermindering van atherosclerotisch plaque oppervlak. Dit duidt aan dat avasimibe naast lipiden-verlagende eigenschappen ook directe anti-atherosclerotische effecten op de vaatwand heeft.

In de zoektocht naar nieuwe aanknopingspunten voor de ontwikkeling van anti-atherosclerotische behandelingsmethoden, hebben we in **hoofdstuk 7** de rol van het eiwit apolipoproteïne C1 (ApoC1) op het lipidenmetabolisme in de macrofaag onderzocht. Wanneer ApoC1 afwezig was in macrofagen was de opname van cholesterol vrijwel niet veranderd t.o.v. macrofagen met normale ApoC1 expressie (wildtype macrofagen). Echter de ratio van cholesteryl ester t.o.v. vrij cholesterol leek verhoogd te zijn in ApoC1-deficiënte macrofagen in vergelijking met wildtype macrofagen. ApoC1-deficiënte macrofagen vertoonden minder uitstroom (efflux) van cholesterol uit de cel dan wildtype macrofagen. Deze verminderde efflux van vrij cholesterol uit ApoC1-deficiënte macrofagen is in overeenstemming met de bevinding dat er relatief meer cholesteryl ester in ApoC1-deficiënte macrofagen zit. De hoeveelheid vrij cholesterol in een cel wordt namelijk boven een bepaald niveau toxisch, zodat de cel vrij cholesterol gaat veresteren tot cholesteryl esters als vorm van opslag. Activiteit van het enzym ACAT, wat deze verestering katalyseert, was hoger in ApoC1-deficiënte macrofagen. Tegelijkertijd was triglyceriden synthese in ApoC1-deficiënte macrofagen ook sterk verhoogd. Afwezigheid van ApoC1 in macrofagen zou dus kunnen leiden tot een verhoogde beschikbaarheid van vetzuren voor gebruik binnen de cel, zoals cholesterol verestering en triglyceriden synthese. In tegenstelling tot ApoC1 deficiëntie leidt de aanwezigheid van ApoC1 in macrofagen tot verminderde cholesterol verestering en verhoogde cholesterol efflux uit de cel. Dit suggereert dat expressie van ApoC1 in de macrofaag mogelijk kan beschermen tegen de vorming van schuimcellen en daardoor tegen de ontwikkeling van atherosclerose.

De gebruikelijke manier voor het onderzoeken van atherogenese in ApoE*3-Leiden transgene muizen bestaat uit het voeden van de muizen met een HFC dieet gedurende 3 tot 6 maanden. In deze periode ontwikkelen de muizen atherosclerotische plaques in verschillende mate van ernst. Hoewel de eerste plaques met name in het kleppengebied van de aorta ontwikkelen, is de exacte locatie van atherogenese niet goed gedefinieerd. In **hoofdstuk 8** hebben we het effect van verhoogde plasma cholesterol concentraties in ApoE*3-Leiden transgene muizen op de ontwikkeling van verdikking van de vaatwand na plaatsing van een polyethyleen cuff om de femoralis arterie onderzocht. Binnen een paar dagen na plaatsing van de cuff zagen we schuimcel stapeling in de binnenste cellaag van de arterie. Na 14 dagen resulteerde het proces van schuimcel accumulatie vrijwel in de

afsluiting van het bloedvat. De grootte van de plaques in de femoralis arterie na plaatsing van een cuff was afhankelijk van het plasma cholesterol gehalte. Deze methode verschaft daarom een goed alternatief model voor het onderzoeken van de vorming van schuimcellen en vroege atherogenese, omdat de plaques snel ontwikkelen in een goed gedefinieerd deel van het bloedvatenstelsel.

List of publications

Full papers

Nijsen, M.J., Croiset, G., Diamant, M., Stam, R., Delsing, D.J.M., de Wied, D., Wiegant, V.M. Conditioned fear-induced tachycardia in the rat: vagal involvement. *European Journal of Pharmacology* 1998; 350:2-3, 211-222

Rijnkels, J.M., Delsing, D.J.M., van der Reijden, A.C., Alink, G.M. Effects of vegetables-fruit extracts and indole-3-carbinol on stearic acid-modulated intracellular communication and cytochrome P450-IA activity. *Environmental Toxicology and Pharmacology* 1998; 6, 103-109

Lardenoye, J.H.P., Delsing, D.J.M., de Vries, M.R., Deckers, M.M.L., Princen, H.M.G., Havekes, L.M., van Hinsbergh, V.W.M., van Bockel, J.H., Quax, P.H.A. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3-Leiden transgenic mice. *Circulation Research* 2000; 87, 248-253

Delsing, D.J.M., Offerman, E.H., van Duyvenvoorde, W., van der Boom, H., de Wit, E.C.M., Gijbels, M.J.J., van der Laarse, A., Jukema, J.W., Havekes, L.M., Princen, H.M.G. The acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in ApoE*3-Leiden mice. *Circulation* 2001; 103, 1778-1786

van de Poll, S.W.E., Romer, T.J., Volger, O.L., Delsing, D.J.M., Bakker Schut, T.C., Princen, H.M.G., Jukema, J.W., Havekes, L.M., van der Laarse, A., Puppels, G.J. Raman spectroscopic evaluation of the effects of diet and lipid-lowering therapy on atherosclerotic plaque development in mice. *Arterioscler Thromb Vasc Biol* 2001; 21, 1630-1635

Tacken, P.J., Delsing, D.J.M., Gijbels, M.J.J., Quax, P.H.A, Havekes, L.M., Hofker, M.H., Willems van Dijk, K. VLDL receptor deficiency enhances intimal thickening after vascular injury but does not affect atherosclerotic lesion area. *Atherosclerosis, in press*

Delsing, D.J.M., Post, S.M., van der Boom, H., van Duyvenvoorde, W., de Wit, E.C.M., Bloks, V.W., Kuipers, F., Havekes, L.M., Princen, H.M.G. Rosuvastatin potently reduces plasma total cholesterol and triglyceride levels by inhibition of VLDL-production in ApoE*3-Leiden transgenic mice. *Submitted for publication*

Delsing, D.J.M., Jukema, J.W., Emeis, J.J., van der Laarse, A., Havekes, L.M., Princen, H.M.G. Differential effects of amlodipine and atorvastatin treatment and their combination on atherosclerosis in ApoE*3-Leiden transgenic mice. *Submitted for publication*

Van de Poll, S.W.E., Delsing, D.J.M., Jukema, J.W., Princen, H.M.G., Havekes, L.M., Puppels, G.W., van der Laarse, A. Raman spectroscopic investigation of atorvastatin, amlodipine and both on atherosclerotic plaque development in ApoE*3-Leiden mice. *Submitted for publication*

Van de Poll, S.W.E., Delsing, D.J.M., Jukema, J.W., Princen, H.M.G., Havekes, L.M., Puppels, G.W., van der Laarse, A. Effects of amlodipine, atorvastatin and the combination of both on advanced atherosclerotic plaque in ApoE*3-Leiden transgenic mice. A combined histological and Raman spectroscopic approach. *Submitted for publication*

Abstracts

Delsing, D.J.M., Croiset, G., Nijsen, M.J.M.A., Broekhoven, M.H., Frankhuijzen-Sierevogel, J.C., Spierenburg, H.A., Wiegant, V.M. Neuroendocrine and autonomic response to conditioned-fear in rats selected for high and low locomotor activity. *Neuroscience Research Communications* 1997; 20:3, 182

Delsing, D.J.M., Offerman, E.H., van Duyvenvoorde, W., van der Boom, H., Havekes, L.M., Princen, H.M.G. The acyl-CoA:cholesterol acyltransferase (ACAT)-inhibitor avasimibe reduces atherosclerosis independently of its lipid-lowering effect in ApoE*3-Leiden mice. *Circulation* 1999; 100:18, 613

Delsing, D.J.M., Jukema, J.W., van der Boom, H., Offerman, E.H., van der Laarse, A., Havekes, L.M., Princen, H.M.G. Effects of amlodipine and atorvastatin on the development of atherosclerosis in ApoE*3-Leiden transgenic mice. *Atherosclerosis* 2000;151:1, 218

Van de Poll, S.W.E., Delsing, D.J.M., Jukema, J.W., Puppels, G.J., van der Laarse, A. Quantitative evaluation of the effects of amlodipine and atorvastatin on atherosclerotic plaque formation in mice by Raman spectroscopy. *Atherosclerosis* 2001;2:2, 89

Van de Poll, S.W.E., Delsing, D.J.M., Jukema, J.W., Puppels, G.J., van der Laarse, A. Raman spectroscopy provides quantitative evaluation of the effects of anti-atherosclerotic drugs on plaque formation. *Journal of the American College of Cardiology* 2001;37:2, 250A

Jukema, J.W., Delsing, D.J.M., van der Laarse, A., van der Boom, H., Offerman, E.H., Havekes, L.M., Princen, H.M.G. Calcium antagonist (amlodipine) and atorvastatin synergy in inhibiting atherosclerosis in ApoE*3-Leiden mice: The CASSIA study. *Journal of the American College of Cardiology* 2001;37:2, 253A

Van de Poll, S.W.E., Delsing, D.J.M., Puppels, G.J., Jukema, J.W., Princen, H.M.G., Havekes, L.M., van der Laarse, A. Quantitative evaluation of the effects of amlodipine and atorvastatin on atherosclerotic plaque formation in mice by Raman spectroscopic imaging. *European Heart Journal* 2001;22,274

Jukema, J.W., Delsing, D.J.M., van der Laarse, A., van der Boom, H., Offerman, E.H., Havekes, L.M., Princen, H.M.G. Calcium antagonist (amlodipine) and atorvastatin synergy in inhibiting atherosclerosis in ApoE*3-Leiden mice: The CASSIA study. *European Heart Journal* 2001;22,162

Curriculum Vitae

Dianne Delsing werd geboren op 5 april 1974 in Goirle. In 1992 behaalde zij haar Gymnasium ß diploma aan het Pauluslyceum te Tilburg. In datzelfde jaar begon zij met de studie Biologie aan de Landbouwuniversiteit Wageningen. Hiervoor werd in 1993 de propedeuse behaald. In 1997 behaalde zij haar doctoraal examen met als specialisatie Organisme in de afstudeerrichtingen Fysiologie van Mens en Dier en Toxicologie. In het kader van de richting Fysiologie van Mens en Dier volgde zij een afstudeervak bij het Rudolf Magnus Instituut voor Neurowetenschappen in Utrecht onder begeleiding van Drs. M.J.M.A. Nijsen, Dr. G. Croiset en Prof. dr. V.M. Wiegant. Bij de vakgroep Toxicologie van de Landbouwuniversiteit Wageningen verrichtte zij een afstudeervak onder begeleiding van Drs. J.M. Rijnkels en Prof. dr. J.H. Koeman. Tevens doorliep zij een stage bij de afdeling Endocrinologie van N.V. Organon, die werd begeleid door Dr. A.J. Grootenhuis.

In november 1997 werd zij voor 4 jaar aangesteld als promovendus binnen de divisie Vaat- en Bindweefsel Onderzoek van TNO Preventie en Gezondheid in Leiden. Het in dit proefschrift beschreven onderzoek werd uitgevoerd onder begeleiding van Prof. dr. ir. L.M. Havekes en Dr. H.M.G. Princen in nauwe samenwerking met Dr. J.W. Jukema en Prof. dr. A. van der Laarse van de afdeling Cardiologie van het Leids Universitair Medisch Centrum.

Vanaf oktober 2001 werkt zij als onderzoeker dierfysiologie bij de afdeling Condition and Disease Specific Research van Numico Research B.V. te Wageningen.