CETP and Inflammation in Lipid Metabolism and Atherosclerosis

CETP and Inflammation in Lipid Metabolism and Atherosclerosis

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 3 februari 2010
klokke 15.00 uur

door

Annette Jessica van der Weij

geboren te Enkhuizen

in 1982

Promotiecomissie

Promotores:	Prof. Dr. L.M. Havekes Prof. Dr. R.R. Frants
Co-Promotores:	Dr. T. Kooistra Dr. P.C.N. Rensen
Overige leden:	Dr. E.S.G. Stroes (AMC, Amsterdam) Dr. R. Kleemann (TNO, Leiden) Prof. Dr. G.J. van Ommen Prof. Dr. J.A. Romijn

The research described in this thesis was supported by a grant of the Center for Medical Systems Biology (CMSB 115).

The studies presented in this thesis were performed at the Gaubius Laboratory, TNO Quality of Life, Leiden, The Netherlands and at the Department of Endocrinology, Leiden University Medical Center, Leiden, The Netherlands.

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Colophon

© A.J. de Vries-van der Weij, 2010. All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any mean, without the prior written permission of the author.

ISBN :
9789490122959

Cover illustration:
© iStockphoto/Jan Pietruszka

Printed by
Gildeprint Drukkerijen, Enschede

The printing of this thesis was kindly supported by:

AstraZeneca B.V.
Center for Medical Systems Biology (CMSB)
Dutch Atherosclerosis Society
J.E. Juriaanse Stichting
Roche Nederland B.V.
Servier Nederland Farma B.V.
Biotronik Nederland B.V.
Daan Traas Fund
GlaxoSmithKline B.V.
Merck Sharp & Dohme B.V.
Sanofi-Aventis Nederland B.V.
Tebu-Bio

Table of contents

Chapter 1 **9**
General Introduction

Chapter 2 **33**
High cholesterol feeding induces hepatic inflammation through disturbed cholesterol homeostasis in APOE*3-Leiden mice
Submitted

Chapter 3 **49**
Combined suppression of NF-κB activity and cholesterol lowering by salicylate induces regression of pre-existing atherosclerotic lesions beyond cholesterol lowering alone
Submitted

Chapter 4 **63**
LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in ApoE*3-Leiden mice: time course and potential mechanisms
Journal of Lipid Research 2009; 50: 301-311

Chapter 5 **85**
Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more pro-inflammatory lesions than atorvastatin
Circulation 2008; 117: 2515-2522

Chapter 6 **101**
Human CETP aggravates atherosclerosis by increasing VLDL-cholesterol rather than by decreasing HDL-cholesterol in APOE*3-Leiden mice
Atherosclerosis 2009; 206: 153-158

Chapter 7 **115**
Bexarotene induces dyslipidemia by increased VLDL production and cholesteryl ester transfer protein (CETP)-mediated reduction of HDL
Endocrinology 2009; 150: 2368-2375

Chapter 8 **133**
General Discussion

Summary **147**
Nederlandse samenvatting voor niet-ingewijden **153**
List of publications **161**
Curriculum Vitae **165**

Chapter 1

General Introduction

1.1 Introduction

According to the World Health Organization, cardiovascular diseases (CVDs) were the number one cause of death globally in 2005 and will remain so at least until 2015. CVDs include coronary heart disease (CHD), cerebrovascular disease, peripheral artery disease, rheumatic heart disease, congenital heart disease, and thrombosis and embolisms. The most important contributor to the growing burden of CVD is atherosclerosis. The World Health Organization estimated that in 2005, about 30% of all global deaths could be attributed to CVD. There are a number of factors that are well known to increase the risk for CVD. These risk factors can be divided into non-modifiable and modifiable risk factors. Non-modifiable risk factors include gene polymorphisms, gender, and age, whereas modifiable risk factors include smoking, increased blood pressure, dietary factors, obesity, lack of exercise, thrombogenic factors, dyslipidemia and inflammation.[1] Over 80% of the premature deaths caused by CVDs could be prevented by adjusting life style: keeping a healthy diet, practicing regular physical activity, and avoiding tobacco smoke.

Unfortunately, changing lifestyle proves to be very difficult. Furthermore, this may not be sufficient for each individual to prevent CVD, as non-modifiable risk factors also play a role in the development of CVD. Also, people that suffered from CVD often need secondary prevention to decrease the chance for recurrence of disease. Pharmaceutical intervention is therefore important to reduce the risk for CVD. The most widely used class of drugs to lower CHD risk are statins, which act through lowering low density lipoprotein-cholesterol (LDL-C). However, statins reduce the number of cardiovascular events with only about 30%.[2] This indicates that other factors, besides LDL-C, also contribute to CHD. In this thesis we mainly focus on two other factors involved in CHD development, namely cholesteryl ester transfer protein (CETP) and inflammation. CETP transfers cholesteryl esters from HDL to (V)LDL in exchange for triglycerides, thereby lowering HDL-C and increasing (V)LDL-C. HDL-C is inversely correlated with CHD prevalence, and is thus thought to have a protective role in CHD development. During the past years, a lot of research was aimed at developing strategies to increase HDL-C. One of the experimental strategies to achieve this goal is inhibition of CETP. The other factor studied in this thesis, inflammation, is increasingly recognized as a factor that increases CHD risk, and therefore strategies to reduce inflammation are also under development. In this introduction, some background information about lipoprotein metabolism, metabolic inflammation, atherosclerosis development and some selected targets modulating lipoprotein metabolism and/or inflammation is given.

1.2 Lipids and lipoprotein metabolism

Triglycerides (TG) and cholesterol, the main dietary lipid constituents, are lipophilic molecules that are insoluble in a hydrophilic environment such as blood. Therefore, cholesterol and TG are packed into water-soluble particles called lipoproteins.

Lipoproteins have a lipid-rich inner core containing TG and cholesteryl esters (CE) and an outer core containing hydrophilic phospholipids (PL), unesterified cholesterol and proteins to solubilize these lipoproteins. Lipoproteins are subdivided into different classes according to density, namely (from lowest to highest density) chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Lipoprotein metabolism will be discussed in more detail in the following sections and a schematic overview is depicted in Figure 1.

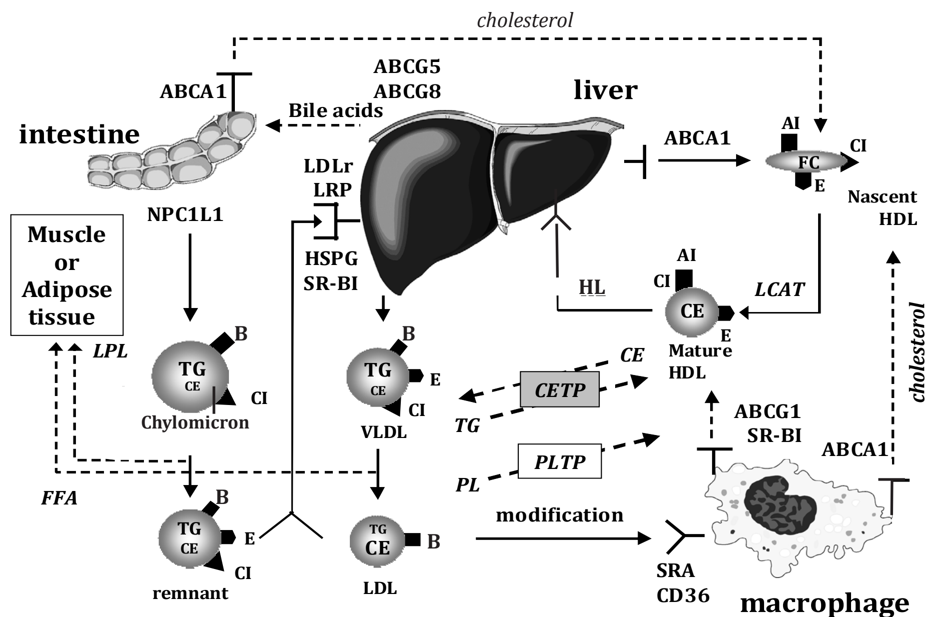


Figure 1. Schematic overview of lipoprotein metabolism. See text for explanation.

1.2.1 Chylomicrons

In the intestine, dietary lipids are absorbed and packed into chylomicrons that are transported through the lymphatic system to the blood.[3] Chylomicrons mainly consist of TG, and also contain fat-soluble vitamins. Apolipoproteins on chylomicrons include apoAI, apoAIV, apoB48, apoCI, apoCII, apoCIII and apoE.[3,4] In the circulation, TG of the chylomicrons are lipolyzed by lipoprotein lipase (LPL) into glycerol and fatty acids (FAs), which are taken up by skeletal muscle and heart to serve as an energy source, or by adipocytes for storage. The resulting chylomicron remnants that are relatively enriched in cholesterol and apoE are then taken up by the liver through the LDL receptor (LDLr),[5,6] LDLr-related protein (LRP),[5,6] heparan-sulphate proteoglycans (HSPGs)[7] or scavenger receptor-class B type I (SR-BI).[8]

1.2.2 VLDL, IDL and LDL

The liver produces apoB100 (and in some species, including mice, apoB48), a very long protein (about 512 kDa) that transports lipids out of the liver in the form of VLDL particles. During its translation apoB associates with the microsomal triglyceride transfer protein (MTP) in the endoplasmic reticulum (ER), where MTP transfers lipids onto apoB, thus forming a pre-VLDL particle. Subsequent fusion with a lipid droplet creates a mature VLDL particle that is secreted into the blood.[5,9] The lipids loaded onto apoB can originate from the diet or can be synthesized de novo by the liver itself. The most important genes involved in the de novo synthesis of TG are fatty acid synthase (FAS) and stearyl-CoA desaturase-1 (SCD1), while the rate-limiting enzyme for the production of cholesterol is 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR).

In the bloodstream VLDL is enriched with apoCI, apoCII, apoCIII and apoE.[5,10,11] In the fasted state, when there is no chylomicron production by the intestine, VLDL is the main donor of TG for extrahepatic tissues. To this end, TG are hydrolyzed by LPL that is particularly expressed by skeletal muscle as compared to adipose tissue under fasting conditions.[12] As a result of lipolysis, VLDL becomes depleted of TG and is thereby converted into an IDL particle and eventually an LDL particle that is relatively enriched in cholesteryl esters as compared to TG, and contains apoB as its most characteristic apolipoprotein.[5] LDL is mainly taken up from the plasma through interaction of apoB with the LDLr. Up to 50% of LDL is taken up by the liver, while the remainder is taken up by extrahepatic tissues that use the cholesterol from LDL to maintain membrane integrity or to produce steroid hormones.[5,13] High levels of apoB-containing lipoproteins (VLDL, IDL, LDL) can lead to deposition of these lipoproteins in the vessel wall, where they are modified and taken up by macrophages, initiating the process of atherosclerosis development.

1.2.3 HDL

The smallest of lipoproteins is HDL, which is produced by the liver and by the intestine.[14] These tissues synthesize apoAI, which is secreted into the plasma. ApoAI is subsequently lipidated with PL to form nascent, disc-shaped HDL through the involvement of the hepatic or intestinal ATP-binding cassette transporter A1 (ABCA1), a protein that is essential for HDL biosynthesis.[15-18] This HDL particle can take up cholesterol from various tissues via ABCA1. The acquired cholesterol is then esterified by lecitin:cholesterol acyl transferase (LCAT), and the resulting CE are stored in the core of the HDL particle. Through the action of LCAT the HDL particle expands, accumulates even more cholesterol and becomes a mature, spherical HDL particle[19] that, besides apoAI, can acquire other apolipoproteins including apoAII, apoAIV, apoAV, apoCI, apoCII, apoCIII and apoE. Additional loading with cholesterol may occur via the ATP-binding cassette transporter G1 (ABCG1) and/or SR-BI.[20,21] HDL can exchange lipids with other plasma lipoproteins through interaction with the phospholipid transfer protein (PLTP), which facilitates the transport of PL from chylomicrons and VLDL to HDL during remodeling of those TG-rich lipoproteins by LPL. Furthermore, the cholesteryl

ester transfer protein (CETP) can exchange CE from HDL with TG from apoB-containing lipoproteins, resulting in TG-enriched HDL particles. HDL is then remodeled by the lipolytic enzymes hepatic lipase (HL) and endothelial lipase (EL) that lipolyze HDL-TG and HDL-PL, processes that enhance HDL catabolism.[22-26] HDL-derived cholesteryl esters can be directly taken up by the liver via SR-BI,[27] where they can be stored in the form of CE, incorporated into newly assembled lipoproteins or excreted into bile in the form of bile acids or neutral sterols.

1.3 Inflammation

Classically, inflammation is defined as a response to injury and is characterized by redness, swelling, pain and fever. This classical response is a strong and acute response with a short duration to fight infection, after which the inflammatory reaction fades. In contrast to this acute inflammatory response, it has been found in the past decades that many subjects have only slightly elevated levels of inflammatory markers in plasma, which remain elevated over a longer period of time. This kind of inflammation is sometimes referred to as low-grade or chronic inflammation.[28]

1.3.1 Metabolic inflammation

Obesity, one of the risk factors for atherosclerosis development, is often associated with low-grade inflammation in the absence of infection or diseases such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE).[29,30] It has recently been hypothesized that this type of inflammation is triggered by a surplus of nutrients, and therefore this type of inflammation is referred to as metabolically triggered inflammation.[28] Metabolic inflammation can be induced by several types of nutrients: dietary supplementation with FA has been shown to mildly increase inflammatory markers in plasma[31] and it has been shown that hyperglycemia induces low-grade hepatic inflammation.[32] Some recent studies indicate that cholesterol also induces low-grade hepatic inflammation in humans and in mice,[33,34] and that this is dependent on the amount of cholesterol in the diet.[35] Although it becomes increasingly clear that cholesterol metabolism and inflammation are strongly intertwined processes, it is not known how an excess of dietary cholesterol leads to the induction of metabolic inflammation.

1.3.2 The role of chronic inflammation in atherosclerosis development

Chronic inflammation, either originating from a chronic infection, from inflammatory diseases such as RA or SLE or from metabolic dysregulation, is increasingly recognized as a risk factor for the development of CVD.[36-38] Numerous population studies have found a relation between serum levels of inflammatory markers, such as C-reactive protein (CRP), fibrinogen, serum amyloid A (SAA) and soluble adhesion molecules and the prevalence of CVD.[36] It is not yet clear whether or not these markers have a causal role in the development of CVD, although some studies suggest that these markers

have a direct effect on certain processes involved in atherosclerosis development. For example, SAA was shown to affect proteoglycan synthesis in a way that leads to increased LDL binding.[39] Furthermore, in vitro experiments showed that SAA stimulates the production of the chemokine monocyte chemoattractant protein (MCP)-1, thus increasing monocyte recruitment to the site of atherosclerotic lesions.[40] CRP may negatively affect blood pressure by causing endothelial dysfunction.[41,42] The role of adhesion molecules and immune cells in the development of atherosclerosis is better understood, and is described in the next section.

1.4 Atherosclerosis

Atherosclerosis is a multifactorial disease affecting the arteries, in which lipids, connective tissue elements, SMC and immune cells accumulate inside the vessel wall and cause a narrowing of the blood vessel.[43] The pathogenesis of atherosclerosis is complex, and over the recent year a lot of insight has been gained in the processes involved in its development.

1.4.1 Mechanisms of atherosclerosis development

The vessel wall consists of three layers: the innermost layer is called the intima and consists of a mono-layer of endothelial cells (EC). A thin matrix, the internal elastic lamina consisting of elastic fibers, separates the intima from the second layer, the media. The media consists of smooth muscle cells (SMC) that have a transverse arrangement with regard to the intima. The external elastic lamina separates the media from the third and outermost layer, the adventitia. The adventitia consists of SMC, fibroblasts and connective tissue, the latter serving to stabilize the vessels and anchor it to its surroundings.

Atherosclerosis development starts when LDL enters the vessel wall and gets modified, for example by oxidation. This induces an inflammatory response in the vessel wall, leading to activation of endothelial cells (EC). Activation of EC involves the NF-κB pathway and leads to expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and E-selectin, to which leukocytes (e.g. monocytes and T-cells) from the circulation can adhere,[44] followed by trans-migration into the vessel wall. There, monocytes differentiate into macrophages that proliferate upon stimulation by macrophage-colony stimulating factor (M-CSF), and that take up the modified LDL via scavenger receptor (SR)-A and cluster designation (CD)36. Inside the macrophage, cholesterol is esterified by acyl CoA:cholesterol acyltransferase (ACAT)-1 and is stored in lipid droplets, thus turning the macrophage into a lipid-laden foam cell. These foam cells excrete chemokines, such as MCP-1, and cytokines that amplify the inflammatory response in the plaque by attracting and activating more immune cells.[44] Lesions consisting only of macrophage foam cells and other immune cells such as T-cells and neutrophils are called fatty streaks or mild lesions that have no clinical symptoms.[1,43-46]

However, macrophage foam cells can exert various effects inside a lesion that lead to development of a more complex, severe lesion that may cause cardiovascular complications. First, macrophage foam cells produce inflammatory stimuli that activate SMC. This leads to proliferation and migration of the SMC to the intimal side of the lesion. When activated, SMC produce extracellular matrix (ECM) proteins such as collagen, and form a fibrous cap that protects the content of the lesion from exposure to the blood. Second, macrophage foam cells produce matrix metalloproteinases that break down the ECM. Third, upon accumulation of large amounts of cholesterol, macrophage foam cells will undergo apoptosis and/or necrosis, forming a necrotic core that consists of extracellular lipid and cellular debris, and contains factors that can activate the coagulation cascade. The stability of a lesion depends on its composition. Stable plaques usually have a thick fibrous cap and a relatively low macrophage and lipid content, whereas unstable plaques have a thin fibrous cap, less stabilizing ECM and a relatively high content of lipid and macrophages. Thus, depending on the balance between ECM production and degradation, a plaque can be less or more vulnerable to rupture. Exposure of pro-thrombotic material to the blood triggers the formation of a thrombus that can occlude the blood vessel, thus causing an infarction.[1,43-46] Figure 2 illustrates the different steps involved in atherosclerosis development.

1.4.2 The role of lipoproteins in atherosclerosis development

The apoB-containing lipoproteins, chylomicrons, VLDL, their remnants, and LDL, are considered to be atherogenic because they can enter the vessel wall, thereby triggering the onset of atherosclerosis development as described in the previous section. HDL, on the other hand, is considered to be atheroprotective by a number of different mechanisms. Firstly, the most important anti-atherosclerotic function of HDL is thought to be in reverse cholesterol transport (RCT). It is thought that the CE stored in foam cells, after hydrolysis by cholesteryl ester hydrolase (CEH), can be transported out of the macrophage foam cells, both passively via aqueous diffusion and actively via ABCA1, ABCG1 and possibly also SR-BI, where lipid-poor apolipoproteins (e.g. apoAI) or HDL can act as cholesterol acceptors.[20,47,48] Through this mechanism HDL may deliver cholesterol derived from atherosclerotic lesions to the liver, thus reducing the atherosclerotic burden. Secondly, HDL may have anti-inflammatory capacities. HDL has been shown to be able to inhibit the expression of adhesion molecules on endothelial cells and (thereby) the transmigration of monocytes across an endothelial cell layer.[49] Thirdly, HDL could act as an anti-oxidant and thereby prevent the oxidation of LDL. This anti-oxidative capacity could be exerted through apolipoproteins such as apoAI, or through enzymes that are present on HDL, including platelet-activating factor acetyl hydrolase (PAF-AH), paraoxonase-1 (PON1) or glytathione phospholipid peroxidase.[49,50] Lastly, HDL has been shown to be able to stimulate the release of nitric oxide (NO), thereby promoting vasorelaxation and improving endothelial function.[50] Because of these potentially beneficial properties of HDL-C, new drugs are being developed that aim at increasing HDL-C levels as a new strategy to reduce CVD. It should be noted,

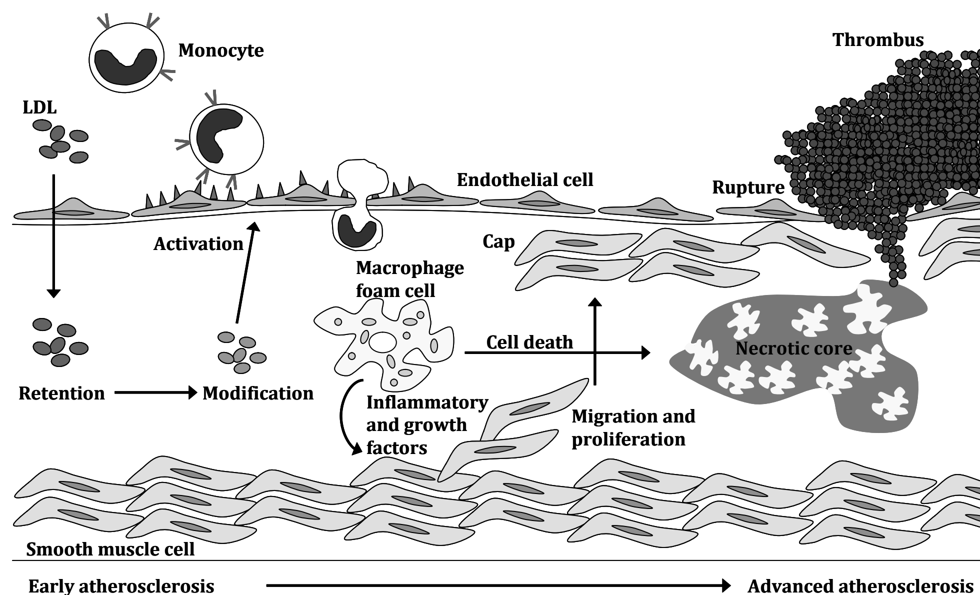


Figure 2. Schematic overview of the different steps in atherosclerosis development. See text for explanation.

however, that the atheroprotective role of HDL still has to be confirmed in both animal and human studies.

1.4.3 Mouse models for studying lipid metabolism and atherosclerosis

Epidemiologic studies have largely increased our knowledge about biomarkers and risk factors for the development of CVD. However, these studies do not provide mechanistic insight in the specific role of certain factors and processes in the development of atherosclerosis. Therefore, animal models allowing a detailed analysis of the various stages in atherosclerosis are a useful tool to gain insight in processes involved the development of atherosclerosis. Since wild type mice show large differences in lipid metabolism and inflammation (two major risk factors for atherosclerosis development) as compared to humans, genetically modified mouse strains have been developed, in which these processes are more similar as compared to humans. There are three mouse models that are extensively used for studying atherosclerosis: the apoE$^{-/-}$ model, the LDLr$^{-/-}$ model and the apoE*3-Leiden (E3L) model. These three models develop atherosclerotic lesions starting in the aortic root and progressing along the arterial tree in a time dependent fashion. Furthermore, these lesions are similar in pathology to human atherosclerotic lesions, varying from fatty streaks and mild lesions to severe and more complex lesions.

ApoE$^{-/-}$ mice lack expression of apoE, the apolipoprotein that has an important role in the clearance of lipids via LDLr, LRP and HSPGs. As a consequence, these mice have increased cholesterol levels compared to wild type mice, ranging from 9 mM on

a chow diet to about 80 mM on a western type diet containing fat and cholesterol. Taken together with the fact that macrophage-produced apoE has been shown to be an important factor in the cholesterol efflux from macrophages,[51] these mice develop atherosclerosis already on a chow diet, and this process is accelerated on a western type diet.[52-55]

LDLr$^{-/-}$ mice lack expression of the LDLr. In humans, mutations in the gene coding for the LDLr can cause familial hypercholesterolemia, a disease that leads to CVD at a very young age. As in LDLr$^{-/-}$ mice only particle clearance via the LDLr route is abrogated, leaving clearance of apoE-containing lipoproteins via e.g. LRP and HSPG unaffected, these mice have only mildly elevated plasma cholesterol levels on a chow diet (about 6 mM). When fed a western type diet, cholesterol levels strongly increase (upto about 50 mM) leading to rapid atherosclerosis development.[56,57]

E3L mice carry a construct containing the human apoE*3-Leiden gene, a dominant negative mutant form of the human apoE3 gene that is characterized by a tandem duplication of codons 120-126 and that causes hyperlipidemia in humans, together with the gene encoding for human apoCI. Expression of the E3L transgene leads to impaired hepatic clearance of apoE-containing lipoproteins, albeit less dramatically than in apoE$^{-/-}$ mice. Expression of the human apoCI furthermore leads to an increase in plasma TG levels by inhibition of LPL[58] and by disturbance of the interaction of lipoproteins with the LDLr and LRP.[59,60] On a chow diet, E3L mice have cholesterol levels of about 2-3 mM and show moderately elevated VLDL and LDL levels. On a Western type diet, VLDL and LDL levels increase strongly, leading to the development of atherosclerosis. By varying the dietary cholesterol content, plasma cholesterol levels can be modulated up to approximately 25 mM. E3L mice therefore represent a somewhat milder model for atherosclerosis development than apoE$^{-/-}$ and LDLr$^{-/-}$ mice.[61-64] In addition to that, E3L mice are more sensitive to lipid-modulating therapies, such as statins and fibrates, than the apoE$^{-/-}$ and LDLr$^{-/-}$ mouse models.[65] Recently, E3L mice have been crossbred with mice expressing human CETP under control of its own promoter, generating E3L.CETP mice.[66] These mice have higher VLDL and LDL levels and lower HDL levels as compared to E3L mice and are more responsive to HDL-modulating drugs than the other mouse models discussed above. Because of their advantageous response to lipid-modifying drugs E3L and E3L.CETP mice were used in the studies described in this thesis.

1.5 Selected targets modulating lipid metabolism and/or inflammation

In order to develop new treatment strategies to reduce CVD prevalence, the search for new drug targets is ongoing. In the following sections five selected factors, which can modulate lipid metabolism and/or inflammation through different mechanisms, are described. Because of their role in lipid metabolism and/or inflammation, these factors are potentially interesting targets for the development of new anti-atherosclerotic drugs.

1.5.1 NF-κB

NF-κB is the collective name for a family of transcription factors that consists of 5 members, namely p65, p50, p52, c-Rel and RelB. These members can form different combinations of homodimers or heterodimers that bind to an NF-κB consensus sequence in target genes to regulate gene transcription. The complex most often referred to as NF-κB is the p65/p50 heterodimer. Under unstimulated conditions, the NF-κB complex is present in the cytoplasm of the cell where it is bound to its inhibitory protein: inhibitor of κB (IκB). Upon an inflammatory stimulus, the IκB kinase (IKK) complex phosphorylates IκB, which is then released from the NF-κB complex and is subsequently degraded. NF-κB is then free to translocate to the nucleus to activate gene transcription. NF-κB regulates the expression of a diverse set of genes, including cytokines, chemokines, acute phase proteins, adhesion molecules and genes involved in apoptosis.[67] Studies addressing the role of NF-κB in atherosclerosis development have shown that reduced NF-κB signaling can either reduce or aggravate the development of atherosclerosis, apparently depending on the cell type or the stage of lesion development studied.[68-70] Salicylate, an anti-inflammatory drug, has been shown to inhibit the IKK complex, thus preventing the phosphorylation of IκB and the subsequent activation of NF-κB.[71] Unpublished observations by Zadelaar et al. have shown that salicylate lowers inflammatory parameters and plasma lipid levels in E3L mice, concomitant with strongly reduced atherosclerosis development. However, in a clinical setting people already have some extent of atherosclerosis development at the onset of treatment. Several studies have shown that plasma lipid lowering can induce regression of pre-existing atherosclerotic lesions in mice.[72-74] It is however not known if suppression of inflammation can have an additional beneficial effect on top of plasma lipid lowering in the process of atherosclerosis regression.

1.5.2 LXR

Nuclear receptors form a large family of transcription factors that regulate various cellular processes, such as reproduction, development, inflammation and metabolism.[75] A number of nuclear receptors are involved in the regulation of lipid metabolism. Among these receptors are the liver-X-receptors (LXRs) α and β. LXRα is expressed mainly in liver, but also in macrophages, adipose tissue, kidney, intestine, lung and adrenals, whereas LXRβ is ubiquitously expressed. The endogenous ligands for LXRα and β are oxysterols, derivatives of cholesterol, and LXRs thus function as cholesterol sensors. As yet, no specific ligands for either of the LXR isoforms have been identified. Upon activation, LXR heterodimerizes with the retinoid X receptor (RXR) and binds to LXR responsive elements to regulate target gene expression.[75-77] Genes that are regulated by LXR include ABCA1 and ABCG1 that are involved in cholesterol efflux from macrophages;[78] ABCG5 and ABCG8 that mediate the transport of intestinally absorbed cholesterol back into the intestinal lumen, thus leading to a decrease in net cholesterol absorption;[79,80] CYP7a1 that converts cholesterol in the liver into bile acids for elimination,[81] and FAS and SCD-1 that are involved in FA biosynthesis. Furthermore, LXR has been suggested

to have anti-inflammatory capacities by inhibiting the NF-κB pathway.[77] Despite the fact that activation of LXR leads to increased plasma cholesterol and TG levels by enhancing VLDL production,[82] LXR agonists are able to inhibit the progression of atherosclerosis and even to induce regression of pre-existing atherosclerotic lesions.[83-86] However, the mechanisms underlying these beneficial effects have not been fully revealed as yet.

1.5.3 RXR

The nuclear receptor RXR appears in three isoforms (RXRα, β and γ), of which no functional characterization has been made. All three RXR isoforms can form homodimers and they are also partners for heterodimerization with other members of the nuclear receptor family, such as LXR, farnesoid X receptor (FXR), pregnane X receptor (PXR), retinoic acid receptor (RAR) and peroxisome proliferator-activated receptors (PPARs). Activation of RXR can therefore affect a diverse set of processes.[75,87] RXR agonists are used in the clinic as a therapeutic approach to treat cancers and dermatologic diseases. However, adverse effects of RXR agonists have been reported regarding lipid metabolism, as exemplified by the chemotherapeutic agent bexarotene, which induces hypertriglyceridemia and hypercholesterolemia in humans.[88] A few studies have addressed the effects of bexarotene on lipid metabolism,[89-91] but these studies gave conflicting results. Therefore, no definite conclusions can be drawn regarding the effect of bexarotene on lipid metabolism and the underlying processes that are affected by bexarotene.

1.5.4 HMG CoA reductase

HMGCR is the rate-limiting enzyme in the cholesterol biosynthesis pathway, and statins, inhibitors of HMGCR, are widely used to lower plasma VLDL-C and LDL-C levels in patients at risk for CVD. Statins are structural analogs of HMG CoA, the substrate for HMGCR, and therefore block the binding of HMG CoA to HMGCR and thereby the formation of mevalonate, a precursor of newly synthesized cholesterol.[92,93] As a consequence, less VLDL is secreted by the liver, resulting in the formation of less LDL.[94] Furthermore, blocking cholesterol synthesis leads to a lowering of the cholesterol content of the liver and a subsequent induction of LDLr expression.[95-97] These two combined mechanisms result in a lowering of plasma (V)LDL levels of up to -40% and a reduction of CHD of up to -30%.[2,98] Other mechanisms that are potentially involved in the cardioprotective effect of statins are their HDL-C increasing effect (up to approximately 10%), stimulation of blood vessel growth, protection against oxidative modification of LDL and anti-inflammatory effects (i.e. reduction of CRP levels).[93,99,100]

1.5.5 CETP

CETP is a 74 kDa glycoprotein that is expressed in liver, adipose tissue and macrophages. Expression of CETP is regulated by various factors, among which SREBP and LXR.[101-105] CETP protein is secreted into the plasma, where it is mainly bound to HDL and facilitates the transfer of neutral lipids (CE and TG) between lipoproteins. This leads to the net

transfer of CE from HDL to apoB-containing lipoproteins in exchange for TG and thereby to reduced HDL-C levels and increased (V)LDL-C levels.[106] Mutations that cause CETP deficiency (e.g. Intron 14+1 G>A) or reduce CETP mass and/or activity (e.g. D442G and TaqIB) lead to increased HDL-C levels. On the other hand, the effect of CETP reduction on TG and LDL-C levels is less manifest: some studies show no effect, while others show a mild decrease in TG and/or LDL-C levels.[107-112]

The fact that CETP decreases HDL-C levels raised the idea that CETP is an atherogenic protein, and that inhibition of CETP might reduce CVD risk. This led to the development of CETP inhibitors. Two compounds, dalcetrapib (JTT-705) and torcetrapib, have been extensively studied in both animal models and humans. In rabbits, both dalcetrapib and torcetrapib strongly increased HDL-C and reduced atherosclerosis development.[113,114] In humans, both compounds also increased HDL-C in short-term studies.[115-118] Torcetrapib was the first CETP inhibitor tested in large clinical trials to evaluate its effect on atherosclerosis progression as determined by intima media thickness (IMT) and intravascular ultrasound (IVUS) measurements. In those studies, all patients were treated with atorvastatin, and either with or without torcetrapib. Combination treatment led to an increase in HDL-C of about 60%. However, the combination of torcetrapib and atorvastatin did not reduce atherosclerosis progression as compared to patients that were treated with atorvastatin alone,[119-121] and even increased mortality, which was attributed largely to cardiovascular death.[122] Unfortunately, the effect of CETP inhibition alone on atherosclerosis development has not been evaluated in humans. Furthermore, it is not clear from the clinical trials if the detrimental effects observed in the torcetrapib and atorvastatin-treated subjects are a general effect of CETP inhibition, or a compound specific effect of torcetrapib.

Because the studies on the effect of CETP or CETP inhibition on atherosclerosis development showed contradicting results, the precise role of CETP in atherosclerosis development is still not clear. Studies in established experimental mouse models for atherosclerosis that have been crossbred with CETP transgenic mice showed that CETP expression increases atherosclerosis development.[66,123] However, human studies present less unequivocal results. Some studies indicate that CETP is atherogenic,[108,109,111,124-128] whereas other studies indicate that CETP has no effect on atherosclerosis development[129-131] or is even atheroprotective.[132-136] As CETP can affect both (V)LDL-C and HDL-C levels, the relative contribution of either of these changes to atherosclerosis development can not be determined easily and is therefore not known yet. Furthermore, most studies focus on the role of CETP in plasma, and a possible local effect of CETP in atherosclerotic lesions has not been studied.

1.6 Outline of this thesis

Although statins efficiently lower LDL-C levels in plasma, they do not reduce the prevalence of CVD sufficiently. Therefore, other strategies to treat patients at risk for CVD are needed. The research described in this thesis focuses on two important factors

in CVD development that may be candidate targets to reduce CVD risk, i.e. inflammation and CETP.

In the first part of this thesis, studies addressing the role of inflammation in atherosclerosis are described. The aim of **Chapter 2** was to study how high cholesterol (HC), but not low cholesterol (LC) diet feeding can lead to metabolic inflammation. To this end, E3L mice were fed a control diet, an LC diet or an HC diet and the effect of these diets on cholesterol homeostasis and on inflammatory parameters was studied.

In **Chapter 3** we studied the effect of salicylate, a drug that has both anti-inflammatory and cholesterol-lowering capacities, on pre-existing atherosclerotic lesions in E3L mice. To determine if suppressing NF-κB activity with salicylate has an additional effect beyond cholesterol lowering alone, we compared salicylate treated mice to a group of mice that were matched for plasma cholesterol levels by reducing the dietary cholesterol content.

A drug that modulates inflammation without lowering plasma cholesterol levels is the LXR agonist T0901317. Treating E3L mice with T0901317 therefore allowed us to analyze the effect of modulating inflammation independent of cholesterol lowering on local inflammatory processes in the vessel wall. The effect of T0901317 on both atherosclerosis development and on pre-existing atherosclerotic lesions was studied in **Chapter 4**.

The second part of this thesis describes studies involving the role of CETP. Clinical trials with the CETP inhibitor torcetrapib showed that the combination of torcetrapib and atorvastatin led to an increase in HDL-C of about 60% compared to atorvastatin only. Nonetheless, subjects treated with a combination of torcetrapib and atorvastatin had a higher incidence of cardiovascular events and an increased death rate. However, the effect of torcetrapib treatment alone on atherosclerosis development has not been studied in humans. Therefore, in **Chapter 5** the effect of torcetrapib with or without atorvastatin treatment on atherosclerosis development was studied in E3L. CETP mice. In this study we also sought to gain insight in the mechanisms underlying the adverse effects of torcetrapib on atherosclerosis development.

CETP affects both (V)LDL-C and HDL-C levels, but the relative contribution of these changes in (V)LDL-C and HDL-C levels to atherosclerosis development is not known. To address this question, in **Chapter 6** the relative contribution of the increase in VLDL-C in E3L.CETP mice compared to E3L was determined by comparing atherosclerosis development between E3L mice and E3L.CETP mice that were matched for VLDL-C levels. Furthermore, this study investigated if there is a possible local effect of CETP in atherosclerotic lesions.

The aim of the study described in **Chapter 7** was to specifically determine the adverse effects of the chemotherapeutic agent bexarotene on plasma lipids in humans and to unravel the underlying mechanism of these effects in mice. To study the potential involvement of CETP, the effect of bexarotene was studied in both E3L and E3L.CETP mice.

The results obtained from these studies and its implications are discussed in **Chapter 8**.

References

1. Lusis AJ. Atherosclerosis. *Nature*. 2000; 407: 233-241.
2. Libby P and Aikawa M. Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat. Med.* 2002; 8: 1257-1262.
3. Mu H and Hoy CE. The digestion of dietary triacylglycerols. *Prog. Lipid Res.* 2004; 43: 105-133.
4. Hussain MM. A proposed model for the assembly of chylomicrons. *Atherosclerosis*. 2000; 148: 1-15.
5. Ginsberg HN. Lipoprotein physiology. *Endocrinol. Metab Clin. North Am.* 1998; 27: 503-519.
6. Mahley RW and Innerarity TL. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta*. 1983; 737: 197-222.
7. Ji ZS, Fazio S, Lee YL, Mahley RW. Secretion-capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 1994; 269: 2764-2772.
8. Out R, Kruijt JK, Rensen PC, Hildebrand RB, de VP, Van EM, Van Berkel TJ. Scavenger receptor BI plays a role in facilitating chylomicron metabolism. *J. Biol. Chem.* 2004; 279: 18401-18406.
9. Olofsson SO, Asp L, Boren J. The assembly and secretion of apolipoprotein B-containing lipoproteins. *Curr. Opin. Lipidol.* 1999; 10: 341-346.
10. Gotto AM, Jr., Pownall HJ, Havel RJ. Introduction to the plasma lipoproteins. *Methods Enzymol.* 1986; 128: 3-41.
11. Shelness GS and Sellers JA. Very-low-density lipoprotein assembly and secretion. *Curr. Opin. Lipidol.* 2001; 12: 151-157.
12. Sugden MC, Holness MJ, Howard RM. Changes in lipoprotein lipase activities in adipose tissue, heart and skeletal muscle during continuous or interrupted feeding. *Biochem. J.* 1993; 292 (Pt 1): 113-119.
13. Brown MS, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. *Science*. 1981; 212: 628-635.
14. Rigotti A, Miettinen HE, Krieger M. The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. *Endocr. Rev.* 2003; 24: 357-387.
15. Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* 1999; 22: 347-351.
16. Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van DM, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, shbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest J, Jr., Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* 1999; 22: 336-345.
17. McNeish J, Aiello RJ, Guyot D, Turi T, Gabel C, Aldinger C, Hoppe KL, Roach ML, Royer LJ, de WJ, 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.

18. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* 1999; 22: 352-355.
19. Jonas A. Lecithin-cholesterol acyltransferase in the metabolism of high-density lipoproteins. *Biochim. Biophys. Acta*. 1991; 1084: 205-220.
20. Ji Y, Jian B, Wang N, Sun Y, Moya ML, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* 1997; 272: 20982-20985.
21. Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A*. 2004; 101: 9774-9779.
22. Ishida T, Choi S, Kundu RK, Hirata K, Rubin EM, Cooper AD, Quertermous T. Endothelial lipase is a major determinant of HDL level. *J. Clin. Invest*. 2003; 111: 347-355.
23. Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, South V, Amin D, Perrone M, Rader DJ. A novel endothelial-derived lipase that modulates HDL metabolism. *Nat. Genet.* 1999; 21: 424-428.
24. Jin W, Millar JS, Broedl U, Glick JM, Rader DJ. Inhibition of endothelial lipase causes increased HDL cholesterol levels in vivo. *J. Clin. Invest*. 2003; 111: 357-362.
25. Murase T and Itakura H. Accumulation of intermediate density lipoprotein in plasma after intravenous administration of hepatic triglyceride lipase antibody in rats. *Atherosclerosis*. 1981; 39: 293-300.
26. Shirai K, Barnhart RL, Jackson RL. Hydrolysis of human plasma high density lipoprotein 2- phospholipids and triglycerides by hepatic lipase. *Biochem. Biophys. Res. Commun.* 1981; 100: 591-599.
27. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. 1996; 271: 518-520.
28. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006; 444: 860-867.
29. Frohlich M, Imhof A, Berg G, Hutchinson WL, Pepys MB, Boeing H, Muche R, Brenner H, Koenig W. Association between C-reactive protein and features of the metabolic syndrome: a population-based study. *Diabetes Care*. 2000; 23: 1835-1839.
30. Rasouli N and Kern PA. Adipocytokines and the metabolic complications of obesity. *J. Clin. Endocrinol. Metab*. 2008; 93: S64-S73.
31. Baer DJ, Judd JT, Clevidence BA, Tracy RP. Dietary fatty acids affect plasma markers of inflammation in healthy men fed controlled diets: a randomized crossover study. *Am. J. Clin. Nutr.* 2004; 79: 969-973.
32. Iwasaki Y, Kambayashi M, Asai M, Yoshida M, Nigawara T, Hashimoto K. High glucose alone, as well as in combination with proinflammatory cytokines, stimulates nuclear factor kappa-B-mediated transcription in hepatocytes in vitro. *J. Diabetes Complications*. 2007; 21: 56-62.
33. Tannock LR, O'Brien KD, Knopp RH, Retzlaff B, Fish B, Wener MH, Kahn SE, Chait A. Cholesterol feeding increases C-reactive protein and serum amyloid A levels in lean insulin-sensitive subjects. *Circulation*. 2005; 111: 3058-3062.
34. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, Kerksiek A, van KR, Maeda N, Staels B, van BM, Shiri-Sverdlov R, Hofker MH. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. *Hepatology*. 2008; 48: 474-486.

35. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der GJ, van OB, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol.* 2007; 8: R200.
36. Lind L. Circulating markers of inflammation and atherosclerosis. *Atherosclerosis*. 2003; 169: 203-214.
37. Salmon JE and Roman MJ. Subclinical atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus. *Am. J. Med.* 2008; 121: S3-S8.
38. Valtonen VV. Role of infections in atherosclerosis. *Am. Heart J.* 1999; 138: S431-S433.
39. Wilson PG, Thompson JC, Webb NR, de Beer FC, King VL, Tannock LR. Serum amyloid A, but not C-reactive protein, stimulates vascular proteoglycan synthesis in a pro-atherogenic manner. *Am. J. Pathol.* 2008; 173: 1902-1910.
40. Lee HY, Kim SD, Shim JW, Lee SY, Lee H, Cho KH, Yun J, Bae YS. Serum amyloid A induces CCL2 production via formyl peptide receptor-like 1-mediated signaling in human monocytes. *J. Immunol.* 2008; 181: 4332-4339.
41. Hein TW, Singh U, Vasquez-Vivar J, Devaraj S, Kuo L, Jialal I. Human C-reactive protein induces endothelial dysfunction and uncoupling of eNOS in vivo. *Atherosclerosis*. 2009;
42. Virdis A, Ghiadoni L, Plantinga Y, Taddei S, Salvetti A. C-reactive protein and hypertension: is there a causal relationship? *Curr. Pharm. Des*. 2007; 13: 1693-1698.
43. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 2005; 352: 1685-1695.
44. Libby P. Inflammation and cardiovascular disease mechanisms. *Am. J. Clin. Nutr.* 2006; 83: 456S-460S.
45. Glass CK and Witztum JL. Atherosclerosis. the road ahead. *Cell*. 2001; 104: 503-516.
46. Ross R. Atherosclerosis--an inflammatory disease. *N. Engl. J. Med.* 1999; 340: 115-126.
47. Klucken J, Buchler C, Orso E, Kaminski WE, Porsch-Ozcurumez M, Liebisch G, Kapinsky M, Diederich W, Drobnik W, Dean M, Allikmets R, Schmitz G. ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proc. Natl. Acad. Sci. U. S. A*. 2000; 97: 817-822.
48. Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem.* 2000; 275: 34508-34511.
49. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. *Circ. Res.* 2004; 95: 764-772.
50. Assmann G and Gotto AM, Jr. HDL cholesterol and protective factors in atherosclerosis. *Circulation*. 2004; 109: III8-14.
51. Van Eck M, Herijgers N, Vidgeon-Hart M, Pearce NJ, Hoogerbrugge PM, Groot PH, Van Berkel TJ. Accelerated atherosclerosis in C57Bl/6 mice transplanted with ApoE-deficient bone marrow. *Atherosclerosis*. 2000; 150: 71-80.
52. 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.
53. Plump AS, Smith JD, Hayek T, alto-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.

54. Reddick RL, Zhang SH, Maeda N. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. *Arterioscler. Thromb.* 1994; 14: 141-147.
55. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science*. 1992; 258: 468-471.
56. 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.
57. 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.
58. Berbee JF, van der Hoogt CC, Sundararaman D, Havekes LM, Rensen PC. Severe hypertriglyceridemia in human APOC1 transgenic mice is caused by apoC-I-induced inhibition of LPL. *J. Lipid Res.* 2005; 46: 297-306.
59. 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.
60. Jong MC, van Dijk KW, Dahlmans VE, van der BH, Kobayashi K, Oka K, Siest G, Chan L, Hofker MH, Havekes LM. Reversal of hyperlipidaemia in apolipoprotein C1 transgenic mice by adenovirus-mediated gene delivery of the low-density-lipoprotein receptor, but not by the very-low-density-lipoprotein receptor. *Biochem. J.* 1999; 338 (Pt 2): 281-287.
61. 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.
62. van den Maagdenberg AM, de KP, Stalenhoef AF, Gevers Leuven JA, 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.
63. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van VB, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 1993; 268: 10540-10545.
64. van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der BH, 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.
65. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1706-1721.
66. Westerterp M, van der Hoogt CC, de HW, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2552-2559.
67. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25: 904-914.
68. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP.

Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest*. 2003; 112: 1176-1185.

69. Kanters E, Gijbels MJ, van dM, I, Vergouwe MN, Heeringa P, Kraal G, Hofker MH, de Winther MP. Hematopoietic NF-kappaB1 deficiency results in small atherosclerotic lesions with an inflammatory phenotype. *Blood*. 2004; 103: 934-940.
70. Wolfrum S, Teupser D, Tan M, Chen KY, Breslow JL. The protective effect of A20 on atherosclerosis in apolipoprotein E-deficient mice is associated with reduced expression of NF-kappaB target genes. *Proc. Natl. Acad. Sci. U. S. A*. 2007; 104: 18601-18606.
71. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature*. 1998; 396: 77-80.
72. Gijbels MJ, van der CM, 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.
73. Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc. Natl. Acad. Sci. U. S. A*. 2006; 103: 3781-3786.
74. Tsukamoto K, Tangirala R, Chun SH, Pure E, Rader DJ. Rapid regression of atherosclerosis induced by liver-directed gene transfer of ApoE in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 1999; 19: 2162-2170.
75. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. *Science*. 2001; 294: 1866-1870.
76. Baranowski M. Biological role of liver X receptors. *J. Physiol Pharmacol.* 2008; 59 Suppl 7: 31-55.
77. Zelcer N and Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin. Invest*. 2006; 116: 607-614.
78. Naik SU, Wang X, Da Silva JS, Jaye M, Macphee CH, Reilly MP, Billheimer JT, Rothblat GH, Rader DJ. Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. *Circulation*. 2006; 113: 90-97.
79. Plosch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK, Kuipers F. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J. Biol. Chem.* 2002; 277: 33870-33877.
80. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J. Biol. Chem.* 2002; 277: 18793-18800.
81. Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE, Mangelsdorf DJ. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell*. 1998; 93: 693-704.
82. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J. Biol. Chem.* 2002; 277: 34182-34190.
83. Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc. Natl. Acad. Sci. U. S. A*. 2002; 99: 7604-7609.

84. Levin N, Bischoff ED, Daige CL, Thomas D, Vu CT, Heyman RA, Tangirala RK, Schulman IG. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25: 135-142.
85. Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc. Natl. Acad. Sci. U. S. A*. 2002; 99: 11896-11901.
86. Terasaka N, Hiroshima A, Koieyama T, Ubukata N, Morikawa Y, Nakai D, Inaba T. T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS Lett.* 2003; 536: 6-11.
87. Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H. International Union of Pharmacology. LXIII. Retinoid X receptors. *Pharmacol. Rev.* 2006; 58: 760-772.
88. Farol LT and Hymes KB. Bexarotene: a clinical review. *Expert. Rev. Anticancer Ther.* 2004; 4: 180-188.
89. Lalloyer F, Fievet C, Lestavel S, Torpier G, van d, V, Touche V, Bultel S, Yous S, Kuipers F, Paumelle R, Fruchart JC, Staels B, Tailleux A. The RXR agonist bexarotene improves cholesterol homeostasis and inhibits atherosclerosis progression in a mouse model of mixed dyslipidemia. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2731-2737.
90. Mukherjee R, Strasser J, Jow L, Hoener P, Paterniti JR, Jr., Heyman RA. RXR agonists activate PPARalpha-inducible genes, lower triglycerides, and raise HDL levels in vivo. *Arterioscler. Thromb. Vasc. Biol.* 1998; 18: 272-276.
91. Vu-Dac N, Gervois P, Torra IP, Fruchart JC, Kosykh V, Kooistra T, Princen HM, Dallongeville J, Staels B. Retinoids increase human apo C-III expression at the transcriptional level via the retinoid X receptor. Contribution to the hypertriglyceridemic action of retinoids. *J. Clin. Invest*. 1998; 102: 625-632.
92. Endo A, Kuroda M, Tanzawa K. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. *FEBS Lett.* 1976; 72: 323-326.
93. Istvan ES and Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science*. 2001; 292: 1160-1164.
94. Arad Y, Ramakrishnan R, Ginsberg HN. Effects of lovastatin therapy on very-low-density lipoprotein triglyceride metabolism in subjects with combined hyperlipidemia: evidence for reduced assembly and secretion of triglyceride-rich lipoproteins. *Metabolism*. 1992; 41: 487-493.
95. Bilheimer DW, Grundy SM, Brown MS, Goldstein JL. Mevinolin and colestipol stimulate receptor-mediated clearance of low density lipoprotein from plasma in familial hypercholesterolemia heterozygotes. *Proc. Natl. Acad. Sci. U. S. A*. 1983; 80: 4124-4128.
96. Kovanen PT, Bilheimer DW, Goldstein JL, Jaramillo JJ, Brown MS. Regulatory role for hepatic low density lipoprotein receptors in vivo in the dog. *Proc. Natl. Acad. Sci. U. S. A*. 1981; 78: 1194-1198.
97. Ma PT, Gil G, Sudhof TC, Bilheimer DW, Goldstein JL, Brown MS. Mevinolin, an inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamsters and rabbits. *Proc. Natl. Acad. Sci. U. S. A*. 1986; 83: 8370-8374.
98. Wilt TJ, Bloomfield HE, MacDonald R, Nelson D, Rutks I, Ho M, Larsen G, McCall A, Pineros S, Sales A. Effectiveness of statin therapy in adults with coronary heart disease.

Arch. Intern. Med. 2004; 164: 1427-1436.

99. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, Macfadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ. Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial. *Lancet.* 2009;
100. Singh IM, Shishehbor MH, Ansell BJ. High-density lipoprotein as a therapeutic target: a systematic review. *JAMA*. 2007; 298: 786-798.
101. Chouinard RA, Jr., Luo Y, Osborne TF, Walsh A, Tall AR. Sterol regulatory element binding protein-1 activates the cholesteryl ester transfer protein gene in vivo but is not required for sterol up-regulation of gene expression. *J. Biol. Chem.* 1998; 273: 22409-22414.
102. Gauthier B, Robb M, Gaudet F, Ginsburg GS, McPherson R. Characterization of a cholesterol response element (CRE) in the promoter of the cholesteryl ester transfer protein gene: functional role of the transcription factors SREBP-1a, -2, and YY1. *J. Lipid Res.* 1999; 40: 1284-1293.
103. Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. *J. Clin. Invest.* 1992; 90: 1290-1295.
104. Luo Y and Tall AR. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *J. Clin. Invest*. 2000; 105: 513-520.
105. Oliveira HC, Chouinard RA, Agellon LB, Bruce C, Ma L, Walsh A, Breslow JL, Tall AR. Human cholesteryl ester transfer protein gene proximal promoter contains dietary cholesterol positive responsive elements and mediates expression in small intestine and periphery while predominant liver and spleen expression is controlled by 5'-distal sequences. Cis-acting sequences mapped in transgenic mice. *J. Biol. Chem.* 1996; 271: 31831-31838.
106. Ha YC, Calvert GD, Barter PJ. In vivo metabolism of esterified cholesterol and apoproteins in rabbit plasma low density lipoproteins. *Atherosclerosis*. 1979; 34: 451-455.
107. Arai H, Yamamoto A, Matsuzawa Y, Saito Y, Yamada N, Oikawa S, Mabuchi H, Teramoto T, Sasaki J, Nakaya N, Itakura H, Ishikawa Y, Ouchi Y, Horibe H, Egashira T, Hattori H, Shirahashi N, Kita T. Polymorphisms in four genes related to triglyceride and HDL-cholesterol levels in the general Japanese population in 2000. *J. Atheroscler. Thromb.* 2005; 12: 240-250.
108. Brousseau ME, O'Connor JJ, Jr., Ordovas JM, Collins D, Otvos JD, Massov T, McNamara JR, Rubins HB, Robins SJ, Schaefer EJ. Cholesteryl ester transfer protein TaqI B2B2 genotype is associated with higher HDL cholesterol levels and lower risk of coronary heart disease end points in men with HDL deficiency: Veterans Affairs HDL Cholesterol Intervention Trial. *Arterioscler. Thromb. Vasc. Biol.* 2002; 22: 1148-1154.
109. Hsieh MC, Chen CC, Wang JY, Chong IW, Yhe CJ, Shin SJ, Lin SR. Cholesteryl ester transfer protein B1B1 genotype is associated with a parental history of cardiovascular diseases in Taiwanese people. *Med. Princ. Pract.* 2008; 17: 143-148.
110. Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, . Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J. Clin. Invest*. 1994; 94: 1872-1882.
111. Ordovas JM, Cupples LA, Corella D, Otvos JD, Osgood D, Martinez A, Lahoz C, Coltell O, Wilson PW, Schaefer EJ. Association of cholesteryl ester transfer protein-TaqIB

polymorphism with variations in lipoprotein subclasses and coronary heart disease risk: the Framingham study. *Arterioscler. Thromb. Vasc. Biol.* 2000; 20: 1323-1329.

112. Yamashita S, Matsuzawa Y, Okazaki M, Kako H, Yasugi T, Akioka H, Hirano K, Tarui S. Small polydisperse low density lipoproteins in familial hyperalphalipoproteinemia with complete deficiency of cholesteryl ester transfer activity. *Atherosclerosis*. 1988; 70: 7-12.
113. Morehouse LA, Sugarman ED, Bourassa PA, Sand TM, Zimetti F, Gao F, Rothblat GH, Milici AJ. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *J. Lipid Res.* 2007; 48: 1263-1272.
114. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. 2000; 406: 203-207.
115. Brousseau ME, Schaefer EJ, Wolfe ML, Bloedon LT, Digenio AG, Clark RW, Mancuso JP, Rader DJ. Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. *N. Engl. J. Med.* 2004; 350: 1505-1515.
116. Brousseau ME, Diffenderfer MR, Millar JS, Nartsupha C, Asztalos BF, Welty FK, Wolfe ML, Rudling M, Bjorkhem I, Angelin B, Mancuso JP, Digenio AG, Rader DJ, Schaefer EJ. Effects of cholesteryl ester transfer protein inhibition on high-density lipoprotein subspecies, apolipoprotein A-I metabolism, and fecal sterol excretion. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25: 1057-1064.
117. Clark RW, Sutfin TA, Ruggeri RB, Willauer AT, Sugarman ED, Magnus-Aryitey G, Cosgrove PG, Sand TM, Wester RT, Williams JA, Perlman ME, Bamberger MJ. Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. *Arterioscler. Thromb. Vasc. Biol.* 2004; 24: 490-497.
118. de Grooth GJ, Kuivenhoven JA, Stalenhoef AF, de GJ, Zwinderman AH, Posma JL, van TA, Kastelein JJ. Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study. *Circulation*. 2002; 105: 2159-2165.
119. Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial. *Lancet*. 2007; 370: 153-160.
120. Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. *N. Engl. J. Med.* 2007; 356: 1620-1630.
121. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. *N. Engl. J. Med.* 2007; 356: 1304-1316.
122. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *N. Engl. J. Med.* 2007; 357: 2109-2122.
123. Plump AS, Masucci-Magoulas L, Bruce C, Bisgaier CL, Breslow JL, Tall AR. Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arterioscler. Thromb. Vasc. Biol.* 1999; 19: 1105-1110.

124. Boekholdt SM, Kuivenhoven JA, Wareham NJ, Peters RJ, Jukema JW, Luben R, Bingham SA, Day NE, Kastelein JJ, Khaw KT. Plasma levels of cholesteryl ester transfer protein and the risk of future coronary artery disease in apparently healthy men and women: the prospective EPIC (European Prospective Investigation into Cancer and nutrition)-Norfolk population study. *Circulation*. 2004; 110: 1418-1423.
125. de Grooth GJ, Smilde TJ, Van WS, Klerkx AH, Zwinderman AH, Fruchart JC, Kastelein JJ, Stalenhoef AF, Kuivenhoven JA. The relationship between cholesteryl ester transfer protein levels and risk factor profile in patients with familial hypercholesterolemia. *Atherosclerosis*. 2004; 173: 261-267.
126. Freeman DJ, Samani NJ, Wilson V, McMahon AD, Braund PS, Cheng S, Caslake MJ, Packard CJ, Gaffney D. A polymorphism of the cholesteryl ester transfer protein gene predicts cardiovascular events in non-smokers in the West of Scotland Coronary Prevention Study. *Eur. Heart J.* 2003; 24: 1833-1842.
127. Kuivenhoven JA, Jukema JW, Zwinderman AH, de KP, McPherson R, Bruschke AV, Lie KI, Kastelein JJ. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. *N. Engl. J. Med.* 1998; 338: 86-93.
128. Moriyama Y, Okamura T, Inazu A, Doi M, Iso H, Mouri Y, Ishikawa Y, Suzuki H, Iida M, Koizumi J, Mabuchi H, Komachi Y. A low prevalence of coronary heart disease among subjects with increased high-density lipoprotein cholesterol levels, including those with plasma cholesteryl ester transfer protein deficiency. *Prev. Med.* 1998; 27: 659-667.
129. Carlquist JF, Muhlestein JB, Horne BD, Hart NI, Bair TL, Molhuizen HO, Anderson JL. The cholesteryl ester transfer protein Taq1B gene polymorphism predicts clinical benefit of statin therapy in patients with significant coronary artery disease. *Am. Heart J.* 2003; 146: 1007-1014.
130. de Grooth GJ, Zerba KE, Huang SP, Tsuchihashi Z, Kirchgessner T, Belder R, Vishnupad P, Hu B, Klerkx AH, Zwinderman AH, Jukema JW, Sacks FM, Kastelein JJ, Kuivenhoven JA. The cholesteryl ester transfer protein (CETP) TaqIB polymorphism in the cholesterol and recurrent events study: no interaction with the response to pravastatin therapy and no effects on cardiovascular outcome: a prospective analysis of the CETP TaqIB polymorphism on cardiovascular outcome and interaction with cholesterol-lowering therapy. *J. Am. Coll. Cardiol.* 2004; 43: 854-857.
131. McCaskie PA, Beilby JP, Chapman CM, Hung J, McQuillan BM, Thompson PL, Palmer LJ. Cholesteryl ester transfer protein gene haplotypes, plasma high-density lipoprotein levels and the risk of coronary heart disease. *Hum. Genet.* 2007; 121: 401-411.
132. Agerholm-Larsen B, Nordestgaard BG, Steffensen R, Jensen G, Tybjaerg-Hansen A. Elevated HDL cholesterol is a risk factor for ischemic heart disease in white women when caused by a common mutation in the cholesteryl ester transfer protein gene. *Circulation*. 2000; 101: 1907-1912.
133. Borggreve SE, Hillege HL, Wolffenbuttel BH, de Jong PE, Zuurman MW, van der SG, van TA, Dullaart RP. An increased coronary risk is paradoxically associated with common cholesteryl ester transfer protein gene variations that relate to higher high-density lipoprotein cholesterol: a population-based study. *J. Clin. Endocrinol. Metab*. 2006; 91: 3382-3388.
134. Bruce C, Sharp DS, Tall AR. Relationship of HDL and coronary heart disease to a common amino acid polymorphism in the cholesteryl ester transfer protein in men with and

without hypertriglyceridemia. *J. Lipid Res.* 1998; 39: 1071-1078.

135. Mohrschladt MF, van der Sman-de Beer, Hofman MK, van der KM, Westendorp RG, Smelt AH. TaqIB polymorphism in CETP gene: the influence on incidence of cardiovascular disease in statin-treated patients with familial hypercholesterolemia. *Eur. J. Hum. Genet.* 2005; 13: 877-882.
136. Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR. Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. *J. Clin. Invest*. 1996; 97: 2917-2923.

Chapter 2

High cholesterol feeding induces hepatic inflammation through disturbed cholesterol homeostasis in ApoE*3-Leiden mice

Jitske de Vries-van der Weij [1,4], Lars Verschuren [1], Annie Jie [1], Ivana Bobeldijk [2], Wim van Duyvenvoorde [1], Karin Toet [1], Marjan van Erk [3], Robert Kleemann [1], Teake Kooistra [1]

[1] The Netherlands Organization for Applied Scientific Research - BioSciences, Gaubius Laboratory, Leiden, The Netherlands.
[2] The Netherlands Organization for Applied Scientific Research - Quality and Safety, Zeist, The Netherlands.
[3] The Netherlands Organization for Applied Scientific Research - BioSciences, Zeist, The Netherlands.
[4] Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands.

Submitted

Abstract

Objectives: This study aimed at elucidating the molecular mechanisms by which high cholesterol feeding induces hepatic inflammation.
Methods and Results: APOE*3-Leiden mice were fed increasing dietary concentrations of cholesterol (0% - CON, 0.25% - LC or 1.0% - HC), whereby the liver switches from an adaptive (CON and LC diet) to an inflammatory state (HC diet). Cholesterol feeding dose-dependently increases plasma cholesterol levels and hepatic cholesteryl ester content, and dose-dependently reduces hepatic cholesterol synthesis. In contrast, the intrahepatic free cholesterol (FC) concentration and plasma levels of the hepatic inflammation marker serum amyloid A (SAA) increased only with HC feeding and were found to be significantly correlated (R=0.675), suggesting that increased hepatic FC is the molecular stressor that induces hepatic inflammation. Microarray analysis of livers showed that HC, but not LC, compromises the endoplasmic reticulum (ER), as indicated by altered expression of ER stress-related genes. In line with this, the activity of ER stress-inducible transcription factors and positive regulators of SAA expression, NF-κB and STAT3, were found to be enhanced upon HC, but not LC feeding.
Conclusion: We propose that HC feeding induces hepatic inflammation and SAA gene expression in the liver through an FC-induced ER stress response and a concomitant increase of hepatic NF-κB and STAT3 activity.

Introduction

It is well established that atherosclerosis can be induced in virtually any animal species if the circulating cholesterol can be raised to a sufficiently high level [1]. It is equally evident that from the very beginning of lesion formation, atherogenesis requires an inflammatory component, which is thought to drive the progression of the disease [2]. Some of the variation in lesion progression rate may relate to variation in the inflammatory state, and evidence is accumulating that if an inflammatory component is superimposed on hypercholesterolemia, it can promote the atherosclerotic process [3,4].

Diet-induced inflammation is increasingly recognized as an important risk factor for the development of cardiovascular and other metabolic diseases [5,6]. We previously demonstrated that upon feeding ApoE*3-Leiden (E3L) mice (a humanized model for atherosclerosis [7]) a diet containing increasing amounts of cholesterol, the liver switches from a mainly resilient, adaptive state to a predominantly inflammatory state, which is associated with a concomitant increase in plasma levels of systemic inflammation proteins such as serum amyloid A (SAA), cytokines and chemokines [8]. This systemic inflammatory response precedes the onset of early lesion formation and significantly contributes to the atherosclerotic process [8]. Understanding the mechanisms of this inflammatory response is a critical goal in atherosclerosis research. However, the exact way in which dietary cholesterol switches the liver to an inflammatory state remained elusive so far.

Notably, the hepatic inflammatory gene response by dietary cholesterol occurs in mice with markedly different genetic backgrounds and lipoprotein profiles, including C57BL6/J mice [9], low density lipoprotein receptor-deficient (LDLR-/-) mice [10], ApoE2 knock in mice [10], and E3L mice [8]. These findings indicate that the inflammatory effect of dietary cholesterol is a common phenomenon and possibly related to influx of chylomicron remnants (i.e. the carriers of dietary cholesterol) into the liver rather than to plasma cholesterol levels per se [8,11]. The question that arises then is: what changes occur in cholesterol homeostasis in the liver as a consequence of feeding a high cholesterol diet and how do these changes lead to hepatic inflammation?

There is recent evidence that perturbation of homeostatic pathways at metabolically active sites by a surplus of nutrients, such as lipids and carbohydrates, compromises the endoplasmic reticulum (ER) and induces ER stress [12,13]. Subsequently, ER stress triggers an inflammatory response (metabolic inflammation), which results in a chronic, low-grade inflammatory state that is different from the “classic” acute phase response [14-16]. We reasoned that the induction of hepatic inflammation by high cholesterol diet feeding might be a consequence of dysregulation of hepatic cholesterol homeostasis and the ensuing ER stress response. To address the outstanding questions, E3L mice were fed increasing amounts of dietary cholesterol and effects on hepatic cholesterol homeostasis were analyzed and related to the development of liver-specific inflammation (e.g. SAA expression). Using transcriptome analysis we investigated whether HC diet feeding compromises the ER and activates downstream transcription factors that positively regulate SAA, i.e. nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3).

Methods

Animals and diets

Mice were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water unless indicated otherwise. Female E3L mice (n=11-13 per group) were treated with a cholesterol-free diet (diet T; Hope Farms, Woerden, The Netherlands) (control group, Con), the same diet supplemented with 0.25% w/w cholesterol (low cholesterol group; LC), or 1.0% w/w cholesterol (high cholesterol group; HC) for 10 weeks. For the drug intervention study, mice were fed the HC diet supplemented with rosuvastatin (HC+ROSU; 0.005% w/w; Astra Zeneca), fenofibrate (HC+FF; 0.03% w/w; Sigma), or T-0901317 (HC+T; 0.01% w/w; Sigma Aldrich) for 10 weeks. After 10 weeks of diet feeding, mice were sacrificed, livers were collected and snap-frozen in liquid nitrogen and stored at -80°C until further use. All animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Analysis of plasma cholesterol, SAA and ALAT

Total plasma cholesterol levels were measured after 4 hours of fasting, using kit No. 1489437 (Roche Diagnostics, Almere, The Netherlands). The plasma levels of SAA (Biosource) were determined by ELISA as reported [17]. Plasma ALAT levels were determined spectrophotometrically using a Reflotron system (Roche Diagnostics) [17].

Analysis of bile acid composition and concentrations in feces

Feces were collected during a period of 48 hours. Fecal samples were lyophilized and weighed. Dried feces (5 mg) were treated with 1 mL alkaline methanol (methanol : 1 M NaOH 3:1 v/v) for 2 h at 80°C in screw capped tubes. Then 9 mL of distilled water was added and the tubes were mixed and centrifuged. The supernatant was applied to a prepared Sep-Pak C18 solid phase extraction cartridge for determination of individual bile acid concentrations. After a clean up by wash procedures, bile acids were eluted with 75% methanol [18]. Nor-hyodeoxycholate was added as an internal standard. The eluate was evaporated to dryness and the bile acids were derivatized as described [18]. The bile acid derivatives were separated on CP-Sil 5B GC column (Chrompack International, Middelburg, The Netherlands) in a Varian 3800 gas chromatograph equipped with flame ionization detector (FID). The injector and the FID were kept at 300°C. Helium was used as carrier gas at a flow rate of 1.4 mL/min. The column temperature was programmed from 230 to 280°C at a rate of 40°/min. 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.

Analysis of neutral sterol composition and concentrations in feces

Dried feces (5 mg) were treated with 1 mL alkaline methanol as described for bile acid measurement to liberate neutral sterols from feces material. Prior to this treatment

5a-cholestane was added as internal standard. After treatment the tubes were cooled to room temperature and the neutral sterols extracted two times with 2 mL petroleum ether. The combined petroleum ether layers were evaporated to dryness and the neutral sterols were silylated as described [18]. Analysis of the sterol derivatives was performed by GC applying the same column as described for the bile acid derivatives. Quantitation was based on the area ratio of the individual neutral sterol to the internal standard.

Analysis of liver lipids and bile acids

To determine the cholesterol content of the liver, liver samples were homogenized and samples were taken for measurement of protein content. 2 mg of cholesterol acetate was added to each sample as an internal standard. Lipids were extracted according to Bligh and Dyer [19]. The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 pre-coated plates as described previously [20]. Quantification of the lipid amounts was performed by scanning the plates with a Hewlett Packard Scanjet 4c and by integration of the density areas with the computer program Tina version 2.09. To analyze liver histology, livers were fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. Cross-sections were stained with hematoxylin-phloxin-saffron (HPS) for histological analysis. Quantification of the hepatic bile acid content was performed as described [21].

Gene expression data analysis

For microarray analysis we used a previously published transcriptomics open-source dataset that investigated the effect of 10 weeks of 0%, 0.25% and 1% (w/w) cholesterol diet feeding in E3L mice employing comparable experimental conditions [8]. Datasets are freely accessible online at www.ebi.ac.uk/arrayexpress. Normalized signal intensities below 10 were replaced by 10. Probe sets with an absent call in all arrays were removed before further analysis of the data. Statistical analysis was performed in BRB ArrayTools (Dr. Richard Simon and Amy Peng Lam, http://linus.nci.nih.gov/BRB-ArrayTools.html). Con, LC and HC groups were tested for differentially expressed genes using class comparisons with multiple testing corrections by estimation of false discovery rate (FDR). Differentially expressed genes were identified at a threshold for significance of a<0.01 and a FDR<5%. Within the set of differentially expressed genes, a Student's *t*-test was carried out to analyze differential expression of individual genes between the cholesterol-fed groups and the Con group. For the LC and HC groups, differences of P<0.01 vs. Con were considered significant. Enrichment analysis of differentially expressed genes was performed using GenMAPP, biological processes with a Z-score >2 and PermuteP < 0.05 were considered as significantly changed.
Microarray data are available from the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/), accession number E-TABM-253.

Analysis of NF-κB and STAT 3 activity

To determine the amounts of active NF-κB and STAT3 activity in livers, liver homogenates

were prepared using the Nuclear Extract Kit (no. 40010, Active Motif, Rixensart, Belgium) and samples were taken to determine the protein content. Equal amounts of protein were used in the NF-κB and STAT3 TransAM transcription factor assay kits no. 40097 and 45196 (Active Motif, Rixensart, Belgium), respectively. The assays were performed according to the manufacturer's instructions. The amount of active transcription factor present in each sample was determined by measuring the binding of the active transcription factor to a consensus sequence in the presence of either a competitive or a mutated (non-competitive) oligonucleotide to be able to correct for aspecific binding.

Statistical analysis
In general, significance of difference was calculated by 1-way analysis of variance (ANOVA) test followed by a least significant difference post hoc analysis. For analysis of fecal sterols and bile acids and for gene expression analysis, differences were assessed using the Student's *t* test. The level of statistical significance was set at $P<0.05$ unless stated otherwise. SPSS 14.0 for Windows (SPSS, Chicago, USA) was used for statistical analysis.

Results

Dietary cholesterol intake and total fecal cholesterol excretion
Female E3L mice were fed a control diet without cholesterol (CON), or the same diet supplemented with either a low dose (0.25% w/w) of cholesterol (LC) or a high dose (1.0% w/w) of cholesterol (HC). After 10 weeks, we explored overall cholesterol homeostasis in E3L mice by examining the relationship between cholesterol input (calculated from the food intake and the percentage of cholesterol in the diet) and cholesterol output, i.e. fecal excretion of neutral sterols, which consisted for over 90% of cholesterol, and fecal excretion of bile acids.

Table 1: Dietary consumption and fecal excretion of cholesterol and cholesterol derivatives

	CON	LC	HC
Cholesterol intake (mmol/day)	0.0 ± 0.0	18.5 ± 0.7 ***	66.1 ± 2.2 ***
Fecal neutral sterol excretion (mmol/day)	2.2 ± 0.1	6.5 ± 0.0 ***	54.5 ± 4.1 **
Fecal bile acid excretion (mmol/day)	1.9 ± 0.3	2.9 ± 0.6	4.3 ± 0.6 *
Intake - Excretion (mmol/day)	-4.1 ± 0.4 ‡	9.1 ± 1.2 ‡	7.3 ± 5.6 ‡

** P<0.05 compared to CON*
*** P<0.01 compared to CON*
**** P<0.001 compared to CON*
‡ significant difference between intake and excretion, P<0.05

The CON group (with no cholesterol in the diet) excreted 4.1±0.4 mmol/day cholesterol and cholesterol derivatives in feces, indicating net cholesterol synthesis. In the LC group, calculation of the difference between cholesterol intake and fecal excretion showed that there was net uptake of 9.1 mmol cholesterol per day (P<0.01). In the HC group, there was net uptake of 7.3 mmol cholesterol per day (P<0.05). Notably, the net amount of cholesterol uptake is not significantly different between the LC and HC groups.

Qualitative analysis of the cholesterol products in feces showed that the amount of neutral sterols increases significantly in a dose-dependent way from 2.2±0.1 mmol/day in the CON group to 6.5±0.0 mmol/day in the LC group (P<0.001) and 54.5±4.1 mmol/day in the HC group (P<0.01). Similarly, the amount of bile acids in feces increases in a dose-dependent way from 1.9±0.3 mmol/day in the CON group to 2.9±0.6 mmol/day in the LC group and 4.3±0.6 mmol/day in the HC group (P<0.05) (Table 1). This indicates that with increasing dietary cholesterol intake, adaptations in hepatic cholesterol homeostasis take place that lead to an increase in the excretion of cholesterol and liver-derived bile acids.

Effect of dietary cholesterol on plasma cholesterol and inflammation in E3L mice

To evaluate the effect of increasing amounts of dietary cholesterol on plasma cholesterol and inflammation markers, steady state levels of plasma cholesterol were determined. Plasma cholesterol was 6.1±1.3 mM in the CON group, increased to 11.9±3.7 mM in

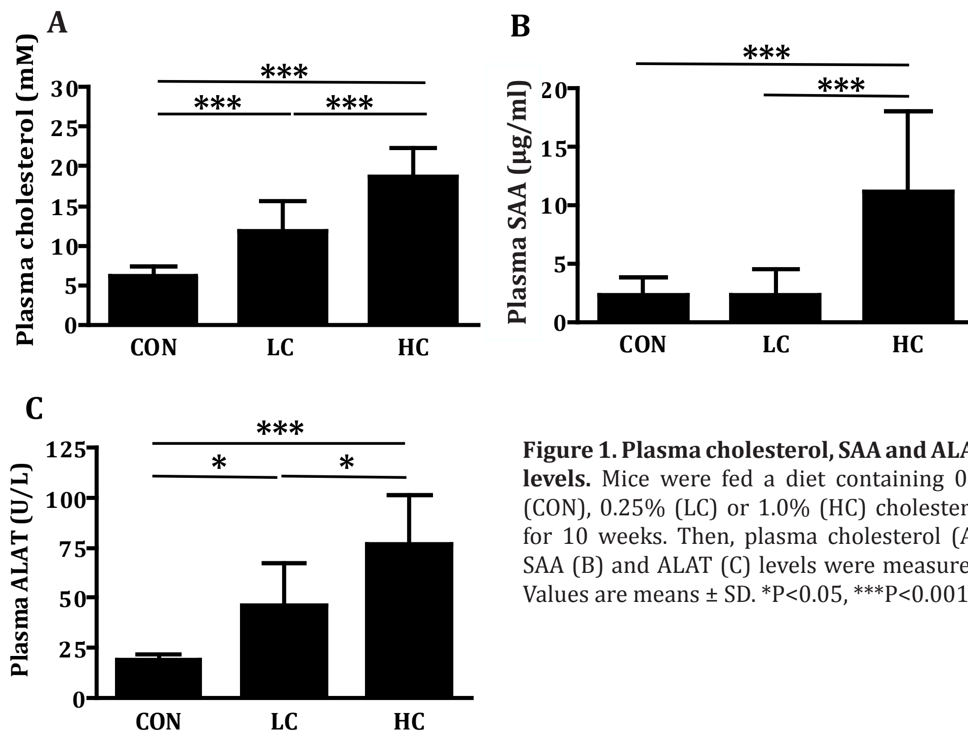


Figure 1. Plasma cholesterol, SAA and ALAT levels. Mice were fed a diet containing 0% (CON), 0.25% (LC) or 1.0% (HC) cholesterol for 10 weeks. Then, plasma cholesterol (A), SAA (B) and ALAT (C) levels were measured. Values are means ± SD. *P<0.05, ***P<0.001.

LC (P<0.001), and was further elevated to 18.7±3.6 mM in HC (P<0.001) (Figure 1A). Corresponding plasma levels of the liver-derived inflammation marker SAA were 2.3±1.5 mg/ml (CON), 2.4±2.2 mg/ml (LC), and 11.2±6.9 mg/ml (HC) (Figure 1B), respectively, indicating that with increasing dietary cholesterol, plasma cholesterol levels increase, while plasma SAA levels increase with 1.0% cholesterol (P<0.001), but not 0.25% cholesterol. Levels of the liver activation marker ALAT were 19±3 U/L in the CON group and increased dose dependently upon cholesterol feeding to 46±21 U/L in the LC (P<0.05) and 77±24 U/L in the HC group (P<0.001).

Hepatic cholesterol homeostasis

To get further insight into how the liver handles increasing amounts of cholesterol, the contents of hepatic cholesteryl esters (CE), free cholesterol (FC) and bile acids were measured. Under CON conditions, the liver contained 15.9±4.7 mg CE/mg protein (Figure 2A). This dose-dependently increased to 35.9±8.6 mg/mg protein (P<0.01) and 58.4±9.6 mg/mg protein (P<0.001) with the LC and HC diets, respectively. The amount of FC was 14.1±2.5 mg/mg protein in the CON group and remained unchanged (14.4±0.7 mg/mg protein) in the LC group, whereas it increased significantly to 19.8±2.8 mg/mg protein (P<0.01) in the HC group (Figure 2B). Fat accumulation in the liver was also markedly increased in livers of HC, but not of LC diet fed mice compared to the CON group, as is shown in representative pictures of each group in Figure 2C. Oil-red-O staining confirmed the accumulation of lipids in livers of HC diet fed mice (Figure 2D). Hepatic bile acid levels were not significantly different between the CON group (20.4±6.3 ng/mg liver) and the LC group (27.2±7.1 ng/mg liver), but were markedly lower in the HC group (11.1±3.0 ng/mg liver, P<0.05) (Figure 2E).

Next, the expression of genes involved in regulating cholesterol homeostasis was analyzed (Table 2) by microarray analysis. Upon cholesterol feeding, cholesterol biosynthesis, as reflected by 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) mRNA expression, decreased in a dose-dependent manner (P<0.01). Furthermore, the expression of the LDL receptor (LDLr), which mediates cholesterol uptake from the plasma decreased dose-dependently (P<0.01), thus explaining the dose-dependent

Table 2: Hepatic gene expression levels

		Fold change compared to CON	
	CON	**LC**	**HC**
Bsep	1.0	1.0	1.5 *
Hmgcr	1.0	-2.8 *	-10.6 *
Ldlr	1.0	-1.4 *	-2.1 *
Saa1	1.0	1.5	3.5 *
Saa2	1.0	1.4	3.2 *
Saa3	1.0	1.4	3.2 *
Saa4	1.0	1.5	4.1 *

* *significantly different from CON, P<0.01*

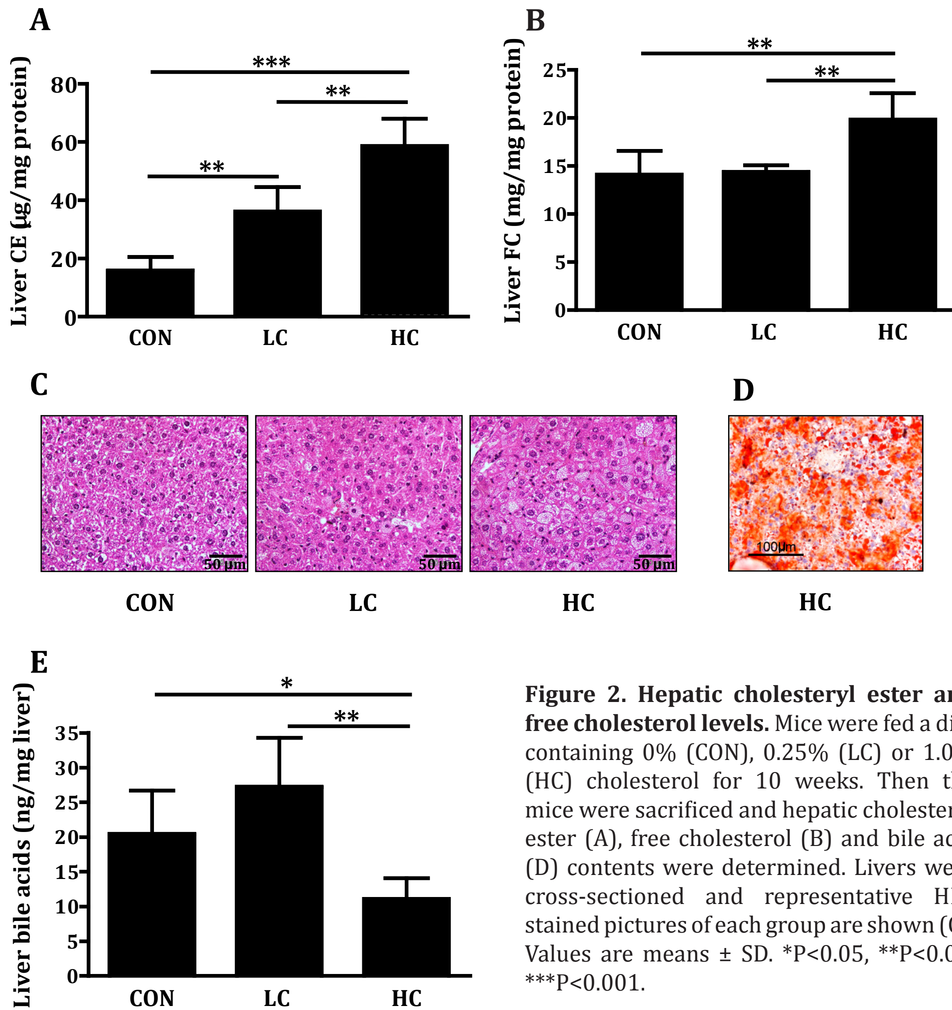


Figure 2. Hepatic cholesteryl ester and free cholesterol levels. Mice were fed a diet containing 0% (CON), 0.25% (LC) or 1.0% (HC) cholesterol for 10 weeks. Then the mice were sacrificed and hepatic cholesteryl ester (A), free cholesterol (B) and bile acid (D) contents were determined. Livers were cross-sectioned and representative HPS stained pictures of each group are shown (C). Values are means ± SD. *P<0.05, **P<0.01, ***P<0.001.

increase in plasma cholesterol levels (Figure 1A). The expression of genes involved in cholesterol excretion (ABCG5, ABCG8) was not significantly changed upon cholesterol feeding (data not shown), while the expression of the bile salt export pump (BSEP) was significantly increased in the HC group compared to the CON and LC groups (P<0.01), explaining the decrease in hepatic bile acid levels in the HC group.

Hepatic FC content correlates with plasma SAA levels

The above data show that cholesterol feeding induces several adaptations in cholesterol homeostasis that are dose-dependent, such as the reduction of endogenous cholesterol synthesis and cholesterol uptake from the plasma, which are two processes regulated via SREBP-1c, and the increase in the levels of plasma cholesterol, hepatic CE and ALAT. In contrast, hepatic FC and plasma SAA levels increased with HC but not with LC

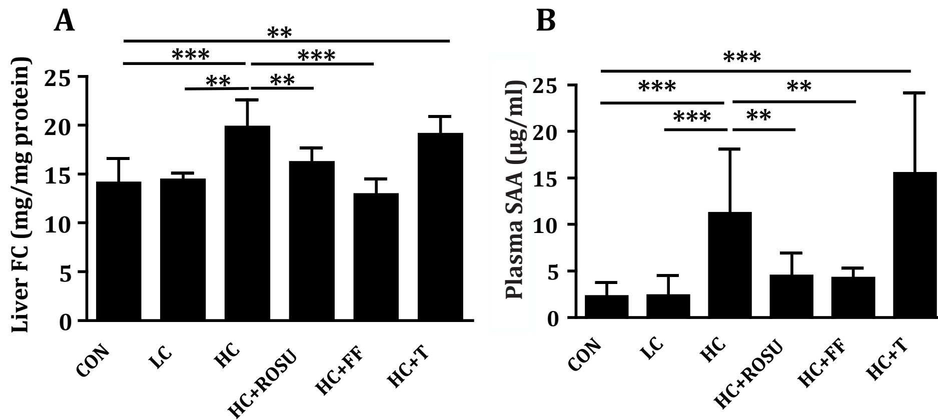


Figure 3. Hepatic FC levels and plasma SAA after drug intervention. Mice were fed a diet containing 0% (CON), 0.25% (LC) or 1.0% (HC) cholesterol or the HC diet supplemented with ROSU, FF, or T) for 10 weeks. Then the mice were sacrificed and hepatic free cholesterol (FC) and plasma SAA levels were determined. Values are means ± SD. **P<0.01, ***P<0.001.

diet feeding. In all, of the measured parameters only the hepatic FC content changed concomitantly with plasma SAA levels, suggesting that the hepatic FC content is the molecular trigger involved in inducing hepatic inflammation. Indeed, linear regression analysis showed a significant correlation between hepatic FC and plasma SAA (R=0.675; P<0.01).

Intervention with drugs modulating cholesterol metabolism underlines the relation between hepatic FC and plasma SAA

To further explore the relationship between hepatic FC and inflammation, we have evaluated how drugs that modulate cholesterol metabolism affect hepatic FC levels and inflammation. To that end, mice were fed the HC diet supplemented with either the HMGCR inhibitor rosuvastatin (ROSU), the PPARα agonist fenofibrate (FF) or the LXR agonist T-0901317 (T) for 10 weeks. Intervention with ROSU or FF normalized hepatic FC levels, and concommitantly SAA levels were reduced to a level that was comparable to that in CON fed mice. On the other hand, hepatic FC levels remained high in mice treated with HC+T (Figure 3A), and plasma SAA levels also remained elevated (Figure 3B). These data show that lowering of hepatic FC levels by drug intervention is paralleled by a reduction in plasma SAA levels, thus supporting our finding of that hepatic FC levels are linked to the development of hepatic inflammation.

HC diet but not LC diet feeding induces ER stress and increases the activity of the inflammatory mediators NF-κB and STAT3

FC has been shown to induce ER stress and subsequently an inflammatory response in macrophages *in vitro* [15]. We hypothesized that ER stress may also underlie the metabolic inflammation in liver *in vivo* as observed in the present study. Functional biological

Table 3: Hepatic gene expression levels of ER stress responsive genes			
	Fold change compared to CON		
	CON	LC	HC
Hspa4	1.0	1.1	1.2 *
Hspa8	1.0	-1.1	-1.5 *
Hspb1	1.0	1.0	2.2 *
Vcp	1.0	1.1	1.3 *
Herpud1	1.0	-1.1	-1.6 *
Hspa14	1.0	1.1	1.3 *
Edem1	1.0	1.1	1.5 *

Hsp is heat shock protein; Vcp is valosin containing protein; Herpud1 is homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1; Edem1 is ER degradation enhancer, mannosidase alpha-like 1
** significantly different from CON, P<0.01*

process analysis on microarray data of livers from CON, LC, and HC mice revealed that the expression of ER stress responsive genes was significantly changed upon HC (Z>2; P<0.01), but not LC feeding, identifying HC feeding as an inducer of ER stress (Table 3).

One of the reported consequences of ER stress is the induction of an inflammatory response. To further evaluate whether or not the dietary cholesterol-induced ER stress observed in the present study also is paralleled by an inflammatory response, and to gain insight into the nature of this inflammation we examined the activity of NF-κB, STAT3 and CCAAT-enhancer-binding protein (C/EBP)a/b, which are important transcription factors involved in the regulation of SAA gene expression [8]. Notably, NF-κB (Figure 4A) and STAT3 (Figure 4B) activity did not change upon LC diet feeding, but did increase upon HC diet feeding. In contrast, the LC and HC groups did

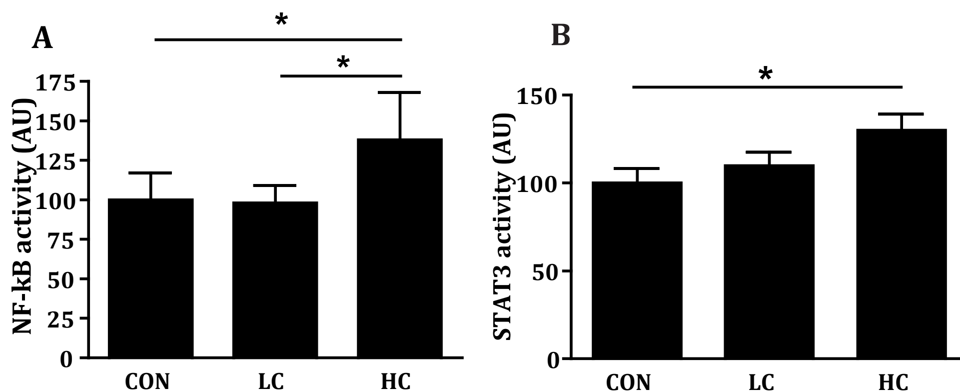


Figure 4. Hepatic NF-κB and STAT3 activity. Mice were fed a diet containing 0% (CON), 0.25% (LC) or 1.0% (HC) cholesterol for 10 weeks. Then the mice were sacrificed and hepatic NF-κB (A) and STAT3 (B) activity were determined. Values are means ± SD. *P<0.05.

not differ in C/EBPa or C/EBPb activity (data not shown). Analysis of hepatic SAA gene expression showed increased mRNA levels of all four SAA isoforms, i.e. SAA1, SAA2, SAA3 and SAA4 in the HC but not the LC group, thus reflecting the observed increase in STAT3 and NF-κB activity and explaining the increased plasma levels of SAA upon HC, but not LC diet feeding (Table 2).

Discussion

Nutrient excess-induced inflammation is increasingly recognized as an important risk factor for the development of cardiovascular and other metabolic diseases [5,6], but the mechanistic basis for the connection between metabolic overload and inflammation is poorly understood. In a previous study we demonstrated that upon feeding E3L mice a diet containing increasing amounts of cholesterol, the liver switches from a mainly resilient state to an inflammatory state, characterized by increased expression of genes coding for inflammatory factors such as cytokines and chemokines, which significantly contributed to atherogenesis [8]. The present study was directed at understanding the mechanisms underlying the link between high cholesterol feeding and the inflammatory responses in liver by feeding E3L mice a CON, LC, or HC diet. We demonstrated that the HC, but not the LC or CON diet induces a hepatic inflammatory response in E3L mice, as exemplified by increased gene expression of the NF-κB/STAT3-regulated inflammation marker SAA, and accompanied by an increase in cellular FC levels in HC only. Elevated FC is a known inducer of ER stress and inflammation [15]. In line with this we showed that an increase in hepatic FC levels was paralleled by changes in the expression of ER stress-related genes. Furthermore, we found increased activity of downstream targets of ER stress, the transcription factors NF-κB and STAT3, which positively regulate SAA gene expression. Taken together, our data suggest that a disturbed cholesterol homeostasis caused by feeding a HC diet results in elevated intrahepatic FC concentrations, which evoke an ER stress-type response and a concomitant increase in hepatic NF-κB and STAT3 activity, thereby inducing enhanced SAA gene expression.

Our data indicate that increased hepatic FC levels due to high dietary cholesterol intake, but not plasma cholesterol levels, correlate with the occurrence of hepatic inflammation. The lack of a relationship between plasma cholesterol and hepatic inflammation in our study is in line with reports stating that chylomicron remnants, which transport the dietary cholesterol to the liver, rather than plasma cholesterol levels are related to dietary cholesterol-induced inflammation [8,11]. This conclusion is deviant from a recent report suggesting that increased plasma lipoprotein levels trigger hepatic inflammation [10]. This latter conclusion was based on the observation that a high fat/high cholesterol (HFC) diet led to an increase in Mac1 positive cells in livers of LDLR$^{-/-}$ and ApoE2 knock-in mice, but not of C57BL6/J wild type (wt) mice. However, the authors also reported increased expression of CD68, tumor necrosis factor-α, and monocyte chemoattractant protein-1 in all three mouse-models, indicating that hepatic inflammation was also present in wt mice fed an HFC diet, despite low plasma lipoprotein

levels. Unfortunately hepatic FC levels were not reported in this study.

This study addresses the important question why disturbance of hepatic cholesterol homeostasis leads to an inflammatory response. In vitro studies showed previously that FC loading of macrophages leads to ER stress, and subsequently to NF-κB mediated cytokine expression [15]. In line with this notion, we now show that FC-induced ER stress may also be relevant for HC diet-induced hepatic inflammation in vivo. An increase in hepatic FC was paralleled by changes in the expression of ER stress-related genes. Furthermore, we found increased activity of downstream targets of ER stress, the transcription factors NF-κB and STAT3 that regulate SAA gene expression and increased mRNA expression of all SAA isoforms. Also, a positive and statistically significant correlation was found between hepatic FC levels and plasma SAA levels. Strikingly, this relationship between hepatic FC levels and plasma SAA was maintained in the presence of drugs that interfere in cholesterol metabolism in different ways (ROSU, FF and T). ROSU and FF lowered hepatic FC levels to control levels and correspondingly reduced plasma SAA concentrations, while with T increased hepatic FC and plasma SAA levels were maintained. Reduction of hepatic FC therefore seems an interesting therapeutic target to reduce cholesterol-induced metabolic inflammation.

In addition to cholesterol, other nutrients have also been shown to induce metabolic inflammation via increased NF-κB activity, and there is increasing evidence that the ER is the central site for the sensing of metabolic stress and the translation of that stress into chronic inflammatory signals and responses [14]. For example, high fat diet feeding induces a 2-fold increase in NF-κB activity in livers of wild type mice [22]. Furthermore, infusion of free fatty acids in rats increases hepatic NF-κB activity about 1.7 fold [23]. Beside lipids, hyperglycemia has also been shown to mildly induce NF-κB activity in vitro in hepatocytes [24]. The mild increase in NF-κB activity we observe during metabolic inflammation is thus comparable to other types of metabolic inflammation that have been described. In the studies mentioned, STAT3 activity was either not determined or not changed. However, it is known that STAT3 activity is part of the insulin signaling cascade and that impaired STAT3 signaling leads to an insulin resistant state [25]. The increase in STAT3 activity that we observed as a consequence of HC diet feeding may thus well be typical for cholesterol-induced metabolic inflammation.

Although many of the same mediators and signaling pathways are involved, metabolic inflammation (sometimes referred to as “low-grade” or “chronic” inflammation) and classic or acute phase inflammation clearly differ in cause, intensity, duration, and long-term consequences. Classically, inflammation is defined as a response to injury and is characterized by redness, swelling, pain and fever [14]. This response is usually short and is a crucial and beneficial component of tissue repair. Metabolic inflammation is principally triggered by nutrients and metabolic surplus and its induction is mild compared to an acute phase response [14]. During the acute phase response, NF-κB and STAT3 activity have been shown to be increased manifold [26,27], whereas we only find mild increases in NF-κB and STAT3 activity upon cholesterol-induced metabolic inflammation. Although the induction of inflammation that we

observe upon HC diet feeding is very mild compared to an acute phase response, it is not less important: low grade, systemic inflammation has increasingly been recognized as a risk factor for the development of cardiovascular disease, insulin resistance and diabetes over the past decades [28-31].

The current lifestyle of people in our Western society is increasingly associated with metabolic overload. Although there are acute compensatory mechanisms to maintain metabolic homeostasis healthy, these mechanisms are not sufficient in the long term: 4 weeks of high dietary cholesterol intake in the form of eggs induces an increase in plasma CRP levels in humans [32]. As our western life style favors long term exposure to nutrient excess, slightly shifting the metabolic balance towards enhanced clearance of nutrients may have a significant beneficial impact on health. However, before such manipulation becomes feasible, much more needs to be understood about the pathways that lead to accumulation of an excess of hepatic FC and about the reason that the liver is not capable of handling the additional amount of cholesterol to which it is exposed upon HC diet feeding compared to LC diet feeding. Future studies are needed to elucidate how the liver changes from an adaptive state (under conditions of LC diet feeding) to a non-adaptive state (under conditions of HC diet feeding) that leads to accumulation of FC in the liver. This may then lead to new treatment strategies that improve the capacity of the liver to handle increasing amounts of cholesterol, thus preventing the accumulation of FC and the resulting inflammatory response.

In conclusion, our data show that a high dietary intake of cholesterol induces a chronic, low-grade inflammatory state in liver, as reflected by increased plasma SAA levels. We propose that as a result of disturbed cholesterol homeostasis, intrahepatic FC levels increase, which is accompanied by induction of ER stress (as suggested by altered expression of ER stress-related genes) and increased activity of the SAA expression-inducing transcription factors NF-κB and STAT3.

Acknowledgements

This work was supported by the Centre for Medical Systems Biology (CMSB project 115) within the framework of the Netherlands Genomics Initiative (NGI) and the TNO research program Personalized Health VP9.

References

1. Steinberg D. Hypercholesterolemia and inflammation in atherogenesis: two sides of the same coin. *Mol. Nutr. Food Res.* 2005; 49: 995-998.
2. Steinberg D. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat. Med.* 2002; 8: 1211-1217.
3. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002; 105: 1135-1143.
4. Verschuren L, Kleemann R, Offerman EH, Szalai AJ, Emeis SJ, Princen HM, Kooistra T. Effect of low dose atorvastatin versus diet-induced cholesterol lowering on atherosclerotic lesion progression and inflammation in apolipoprotein E*3-Leiden transgenic mice. *Arterioscler.*

Thromb. Vasc. Biol. 2005; 25: 161-167.
5. Wellen KE and Hotamisligil GS. Inflammation, stress, and diabetes. *J. Clin. Invest***.** 2005; 115: 1111-1119.
6. Wymann MP and Schneiter R. Lipid signalling in disease. *Nat. Rev. Mol. Cell Biol.* 2008; 9: 162-176.
7. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1706-1721.
8. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der GJ, van OB, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis 1. *Genome Biol.* 2007; 8: R200.
9. Vergnes L, Phan J, Strauss M, Tafuri S, Reue K. Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression. *J. Biol. Chem.* 2003; 278: 42774-42784.
10. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, Kerksiek A, van KR, Maeda N, Staels B, van BM, Shiri-Sverdlov R, Hofker MH. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. *Hepatology***.** 2008; 48: 474-486.
11. Subramanian S and Chait A. The effect of dietary cholesterol on macrophage accumulation in adipose tissue: implications for systemic inflammation and atherosclerosis. *Curr. Opin. Lipidol.* 2009; 20: 39-44.
12. Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. *PLoS. ONE.* 2009; 4: e4954.
13. Wang H, Kouri G, Wollheim CB. ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. *J. Cell Sci.* 2005; 118: 3905-3915.
14. Hotamisligil GS. Inflammation and metabolic disorders. *Nature***.** 2006; 444: 860-867.
15. Li Y, Schwabe RF, Vries-Seimon T, Yao PM, Gerbod-Giannone MC, Tall AR, Davis RJ, Flavell R, Brenner DA, Tabas I. Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis. *J. Biol. Chem.* 2005; 280: 21763-21772.
16. Zhang K and Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature***.** 2008; 454: 455-462.
17. Rein D, Schijlen E, Kooistra T, Herbers K, Verschuren L, Hall R, Sonnewald U, Bovy A, Kleemann R. Transgenic flavonoid tomato intake reduces C-reactive protein in human C-reactive protein transgenic mice more than wild-type tomato 2. *J. Nutr.* 2006; 136: 2331-2337.
18. Post SM, de CR, van HR, van TA, Princen HM. Increased fecal bile acid excretion in transgenic mice with elevated expression of human phospholipid transfer protein. *Arterioscler. Thromb. Vasc. Biol.* 2003; 23: 892-897.
19. BLIGH EG and DYER WJ. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol***.** 1959; 37: 911-917.
20. 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.

21. Bobeldijk I, Hekman M, de Vries-Van der Weij, Coulier L, Ramaker R, Kleemann R, Kooistra T, Rubingh C, Freidig A, Verheij E. Quantitative profiling of bile acids in biofluids and tissues based on accurate mass high resolution LC-FT-MS: compound class targeting in a metabolomics workflow. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2008; 871: 306-313.
22. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat. Med.* 2005; 11: 183-190.
23. Boden G, She P, Mozzoli M, Cheung P, Gumireddy K, Reddy P, Xiang X, Luo Z, Ruderman N. Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. *Diabetes***.** 2005; 54: 3458-3465.
24. Iwasaki Y, Kambayashi M, Asai M, Yoshida M, Nigawara T, Hashimoto K. High glucose alone, as well as in combination with proinflammatory cytokines, stimulates nuclear factor kappa-B-mediated transcription in hepatocytes in vitro. *J. Diabetes Complications***.** 2007; 21: 56-62.
25. Inoue H, Ogawa W, Ozaki M, Haga S, Matsumoto M, Furukawa K, Hashimoto N, Kido Y, Mori T, Sakaue H, Teshigawara K, Jin S, Iguchi H, Hiramatsu R, LeRoith D, Takeda K, Akira S, Kasuga M. Role of STAT-3 in regulation of hepatic gluconeogenic genes and carbohydrate metabolism in vivo. *Nat. Med.* 2004; 10: 168-174.
26. Alonzi T, Maritano D, Gorgoni B, Rizzuto G, Libert C, Poli V. Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. *Mol. Cell Biol.* 2001; 21: 1621-1632.
27. Notebaert S, Carlsen H, Janssen D, Vandenabeele P, Blomhoff R, Meyer E. In vivo imaging of NF-kappaB activity during Escherichia coli-induced mammary gland infection. *Cell Microbiol.* 2008; 10: 1249-1258.
28. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol.* 2004; 25: 4-7.
29. Lind L. Circulating markers of inflammation and atherosclerosis. *Atherosclerosis***.** 2003; 169: 203-214.
30. Paoletti R, Bolego C, Poli A, Cignarella A. Metabolic syndrome, inflammation and atherosclerosis. *Vasc. Health Risk Manag.* 2006; 2: 145-152.
31. Verschuren L, Kooistra T, Bernhagen J, Voshol PJ, Ouwens DM, van EM, de Vries-Van der Weij J, Leng L, van Bockel JH, van Dijk KW, Fingerle-Rowson G, Bucala R, Kleemann R. MIF Deficiency Reduces Chronic Inflammation in White Adipose Tissue and Impairs the Development of Insulin Resistance, Glucose Intolerance, and Associated Atherosclerotic Disease. *Circ. Res.* 2009;
32. Tannock LR, O'Brien KD, Knopp RH, Retzlaff B, Fish B, Wener MH, Kahn SE, Chait A. Cholesterol feeding increases C-reactive protein and serum amyloid A levels in lean insulin-sensitive subjects. *Circulation***.** 2005; 111: 3058-3062.

Chapter 3

Combined suppression of NF-κB activity and lowering of cholesterol by salicylate induces regression of pre-existing atherosclerotic lesions beyond cholesterol lowering alone

Jitske de Vries-van der Weij [1,2], Karin Toet [1], Susanne Zadelaar [1], Robert Kleemann [1], Patrick C.N. Rensen [3], Teake Kooistra [1]

[1] The Netherlands Organization for Applied Scientific Research (TNO) - Biosciences, Gaubius Laboratory, Leiden, The Netherlands
[2] Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
[3] Dept. of General Internal Medicine, Endocrinology, and Metabolic Disease, Leiden University Medical Center, Leiden, The Netherlands

Submitted

Abstract

Aims: This study aimed at determining if suppressing inflammation using the NF-κB inhibitor salicylate contributes to atherosclerosis regression on top of cholesterol-lowering.
Methods and Results: ApoE*3-Leiden mice were fed a high cholesterol diet to induce the formation of mild atherosclerotic lesions. Subsequently, one group of mice was sacrificed to determine lesion area and lesion severity at the start of different regression treatments (reference group). A second group of mice was then fed the same high cholesterol diet supplemented with salicylate (HC+SAL) to suppress NF-κB activity. As salicylate not only quenches inflammation but also reduces plasma cholesterol levels (- ~50%), a third group of mice was fed a low cholesterol (LC) diet to establish similar plasma cholesterol levels as obtained by salicylate treatment. The effects of these treatments on lesion area and severity were assessed at 8 and 16 weeks. Compared to the reference group, HC+SAL suppressed hepatic NF-κB activity, tended to suppress hepatic STAT3 activity and reduced plasma levels of the hepatic inflammation marker SAA. Compared to HC+SAL, LC diet feeding suppressed hepatic NF-κB activity to a lesser extent, similarly suppressed STAT3 activity, and more strongly reduced plasma SAA levels; HC+SAL and LC feeding similarly suppressed aortic NF-κB activity. At 16 weeks after starting the regression treatments, neither HC+SAL nor LC treatment showed an effect on lesion area (compared to the reference group), but HC+SAL had reduced the macrophage area of lesions and increased the plaque stability index (ratio of collagen to macrophage area) more strongly than LC diet feeding.
Conclusions: The combined effect of suppressing NF-κB activity and reducing plasma cholesterol with SAL promotes lesion regression by reducing macrophage area and induces lesion stability more efficiently than cholesterol lowering alone.

Introduction

Hypercholesterolemia is widely recognized as an important risk factor for atherosclerosis development.[1] In addition, it is well-established that inflammation plays a role in all stages of atherosclerosis development, from initiation of lesion formation to progression into a more complex lesion and eventually to plaque rupture.[2,3] Targeting either of these factors can reduce the formation of new atherosclerotic lesions, which is of obvious importance. However, in a clinical setting, subjects at risk for cardiovascular events often already have established atherosclerotic lesions. For these subjects, treatment strategies aimed at regression of pre-existing lesions are important to reduce the prevalence of cardiovascular events. It is therefore crucial to gain insight in the processes involved in lesion regression. As a first approach to tackle this research question we chose to evaluate the effect of lowering plasma cholesterol and inflammation on plaque regression, because of their key role in atherosclerotic lesion development.

Over the past years, a number of animal studies have addressed the effect of cholesterol lowering on pre-existing lesions. These studies have shown that reducing plasma cholesterol induces a strong decrease in the number of lesional macrophage foam cells[4-6] after a few days.[7] Later in time, a reduction of plaque size was reported.[4,5,7] In humans, non-invasive imaging confirmed that plasma cholesterol-lowering leads to atherosclerosis regression.[8] Thus, various studies have shown that cholesterol lowering can induce atherosclerosis regression. However, the role of inflammation in regression of pre-existing plaques has been poorly studied.

Salicylate (SAL) is an anti-inflammatory drug that inhibits the activity of nuclear factor-κB (NF-κB), a key mediator of the inflammatory response. SAL acts by suppressing the activity of the inhibitor of κB (IκB) kinase (IKK) complex,[9] thereby preventing the phosphorylation of IκB, and the subsequent release and nuclear translocation of NF-κB. We found that SAL also lowered plasma cholesterol levels in APOE*3-Leiden (E3L) mice (Zadelaar et al., unpublished results).

In the present study we aimed at determining whether or not suppressing NF-κB activity with SAL can induce regression of pre-existing atherosclerotic lesions on top of its cholesterol-lowering effect. To study this, we used E3L mice, a mouse model that responds in a human-like manner to anti-atherosclerotic drugs and that has been shown to be a useful model for atherosclerosis regression.[4,6,10] After inducing atherosclerosis formation by feeding E3L mice a high cholesterol (HC) diet for 19 weeks, we treated these mice with the same diet, supplemented with SAL for 8 or 16 weeks to study the effect of SAL on pre-existing plaques. As SAL treatment resulted in a lowering of plasma cholesterol, we used a control group that was fed a diet with a lower cholesterol content (LC diet) to match for the plasma cholesterol levels in the SAL treated mice. This experimental set-up enabled us to study the effect of suppressing NF-κB activity on top of cholesterol-lowering on pre-existing atherosclerotic lesions in a time-dependent manner.

Methods

Animals

Female E3L mice of 12-14 weeks old (n=14-15 per group) were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water unless indicated otherwise. All mice were fed a HC diet containing 15% (w/w) cacao butter (diet T, Hope Farms, Woerden, the Netherlands) supplemented with 1.0% (w/w) cholesterol (Sigma) for 19 weeks. After this period, mice were randomized into four groups according to their plasma cholesterol levels. One group of mice (n=15) was sacrificed by CO_2 inhalation and hearts and livers were isolated for further analysis. One group of mice (n=30) received an HC diet supplemented with 0.4% (w/w) salicylic acid (cat. no. 24,758-8, Aldrich, Steinheim, Germany) for 8 (n=15) or 16 (n=15) weeks. The other group of mice (n=30) received an LC diet containing 15% (w/w) cacao butter supplemented with 0.12% (w/w) cholesterol for an additional 8 (n=15) or 16 (n=15) weeks. After these periods the mice were sacrificed by CO_2 inhalation and hearts and livers were isolated for further analysis. During the study, blood was drawn every 4 weeks after 4 h of fasting. All animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Analysis of plasma cholesterol, cholesterol distribution over lipoproteins and inflammatory markers

Plasma total cholesterol was measured by an enzymatic procedure using kit no. 1489437, Roche Diagnostics, Mannheim, Germany. For lipoprotein profiles, plasma was pooled per group and fractionated using an ÄKTA fast protein liquid chromatography (FPLC) system (Pharmacia, Roosendaal, The Netherlands) as described.[11] Fractions of 50 µL were collected and assayed for cholesterol as described above. Plasma levels of serum amyloid A (SAA) (Tridelta, Ireland) were determined by ELISA, according to manufacturer's instructions.

Analysis of NF-κB and STAT 3 activities in liver and aorta

To determine the amounts of active NF-κB and STAT3 activity in livers and/or aortas, homogenates of each tissue were prepared using the Nuclear Extract Kit (no. 40010, Active Motif, Rixensart, Belgium) and samples were taken to determine the protein content. Equal amounts of protein were used in the NF-κB TransAM and STAT3 TransAM transcription factor assay kits no. 40097 and 45196 (Active Motif, Rixensart, Belgium), respectively. The assays were performed according to the manufacturer's instructions. The amount of active transcription factor present in each sample was determined by measuring the binding of the active transcription factor to a consensus sequence in the presence of either a competitive or a mutated (non-competitive) oligonucleotide to be able to correct for non-specific binding.

Atherosclerosis quantification
After isolation, hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin, and cross-sectioned throughout the aortic root area. For each mouse, 4 sections with 50 μm intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxin-saffron (HPS) for histological analysis. Lesion area was determined using Cell D imaging software (Olympus Soft Imaging Solutions). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific) was used to quantify the macrophage area and Sirius Red was used to quantify the collagen area. The plaque stability index was determined by dividing the collagen area by the macrophage area for each mouse.

Statistical analysis
Significance of difference was calculated by one-way analysis of variance (ANOVA) test followed by a least significant difference post hoc analysis. SPSS 16.0 for Windows (SPSS, Chicago, USA) was used for statistical analysis.

Results

Salicylate treatment and LC diet feeding reduce HC diet-induced plasma VLDL-cholesterol to a similar extent
To study the effect of suppressing inflammation on top of cholesterol lowering on pre-existing atherosclerotic lesions, 75 E3L mice were fed a high cholesterol (HC) diet containing 1.0% (w/w) cholesterol for 19 weeks to induce the formation of mild atherosclerotic lesions. HC diet feeding increased plasma cholesterol levels from approximately 2 mM to 15.4±2.7 mM (Figure 1A). After 19 weeks, the mice were randomized into 3 different groups according to plasma cholesterol levels. One group of mice was sacrificed at this time point as a reference group (HC/REF, n=15) for atherosclerotic lesion development. A second group of mice was then fed the HC diet supplemented with 0.4% (w/w) SAL to suppress NF-κB activity for an additional 8 (n=15) or 16 weeks (n=15). As HC+SAL treatment reduced plasma cholesterol levels (to 7.9±1.5 mM), a third group of mice was fed a low cholesterol (LC) diet for an additional 8 (n=15) or 16 weeks (n=15) to match for the plasma cholesterol levels in the HC+SAL treated mice, resulting in an average plasma cholesterol of 7.6±1.7 mM that was not significantly different from plasma cholesterol in the HC+SAL treated group at any of the time points measured. Lipoprotein profiling showed that the observed decrease in plasma cholesterol was mainly confined to the VLDL fraction and to a lesser extent to the LDL/HDL-1 fraction and that the cholesterol distribution profile was similar in the HC+SAL and LC groups (Figure 1B). After prolonged 8 or 16 weeks of HC+SAL or LC diet feeding the mice were sacrificed and livers, hearts and thoracic aortas were isolated for further analysis.

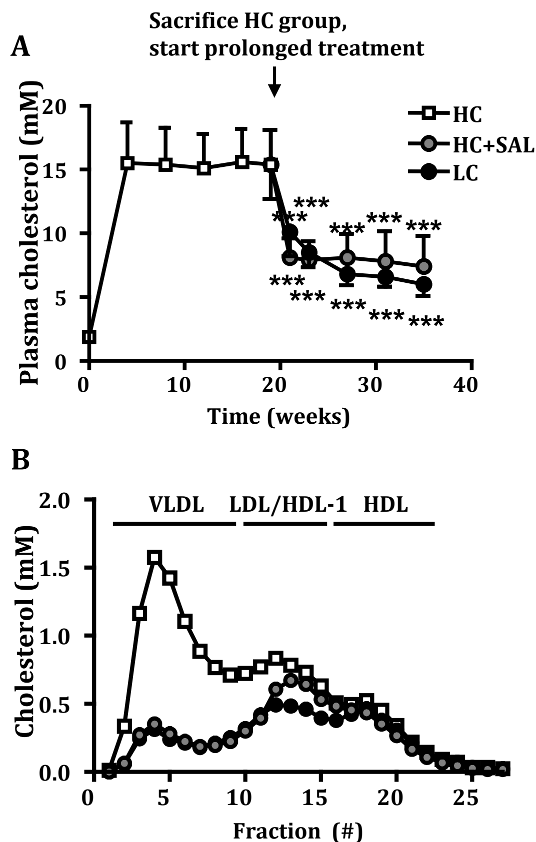


Figure 1: SAL treatment and LC diet feeding reduce HC diet-induced plasma VLDL-cholesterol to a similar extent. E3L mice were fed a HC diet for 19 weeks. After 19 weeks, mice were randomized into three different groups. One group of mice was sacrificed as a reference group. A second group of mice was then fed the HC diet supplemented with SAL for an additional 8 or 16 weeks. A third group of mice was fed an LC diet for an additional 8 or 16 weeks to match for the plasma cholesterol levels in the HC+SAL treated group. At the indicated time points, blood was drawn and plasma was assayed for total cholesterol (A). Values are means±SD. (***P<0.001 compared to the HC group at t=19 weeks) Pooled plasma samples were fractionated using FPLC and the individual fractions were assayed for TC at different time points during the course of the study. Representative pictures of one time point are shown (B) (HC group: t=19 weeks; HC+SAL group: t=27 weeks; LC group: t=27 weeks).

Salicylate suppresses hepatic NF-κB activity, but not overall hepatic inflammation, beyond LC diet feeding

To evaluate the effect of SAL treatment on hepatic inflammation, plasma levels of the hepatic inflammatory marker SAA were measured. HC diet feeding increased plasma SAA from 5.4±0.4 µg/mL at baseline (t=0) to 10.3±3.9 µg/mL after 19 weeks (Figure 2A). Subsequent treatment with HC+SAL reduced SAA to 8.0±3.9 µg/mL after 8 weeks (P<0.05) and to 7.6±3.3 µg/mL after 16 weeks (P<0.01 compared to 19 weeks of HC diet feeding). LC diet feeding reduced SAA levels to 6.2±2.0 µg/mL after 8 weeks (P<0.001) and to 5.5±2.0 µg/mL after 16 weeks (P<0.001 compared to 19 weeks of HC diet feeding). The reduction of SAA by cholesterol lowering alone was beyond that achieved with HC+SAL treatment, and became significantly different after 16 weeks (P<0.05).

To verify that SAL treatment reduced hepatic NF-κB activity, livers were homogenized and assayed for NF-κB activity. NF-κB activity in HC diet fed mice was set at 100 AU (Figure 2B). Compared to HC diet fed mice, prolonged treatment of 8 weeks with HC+SAL strongly suppressed NF-κB activity (-75%, P<0.001), which was sustained after 16 weeks of prolonged treatment (-63%, P<0.001). Compared to the HC group, prolonged feeding with the LC diet reduced NF-κB activity to a lesser extent than HC+SAL

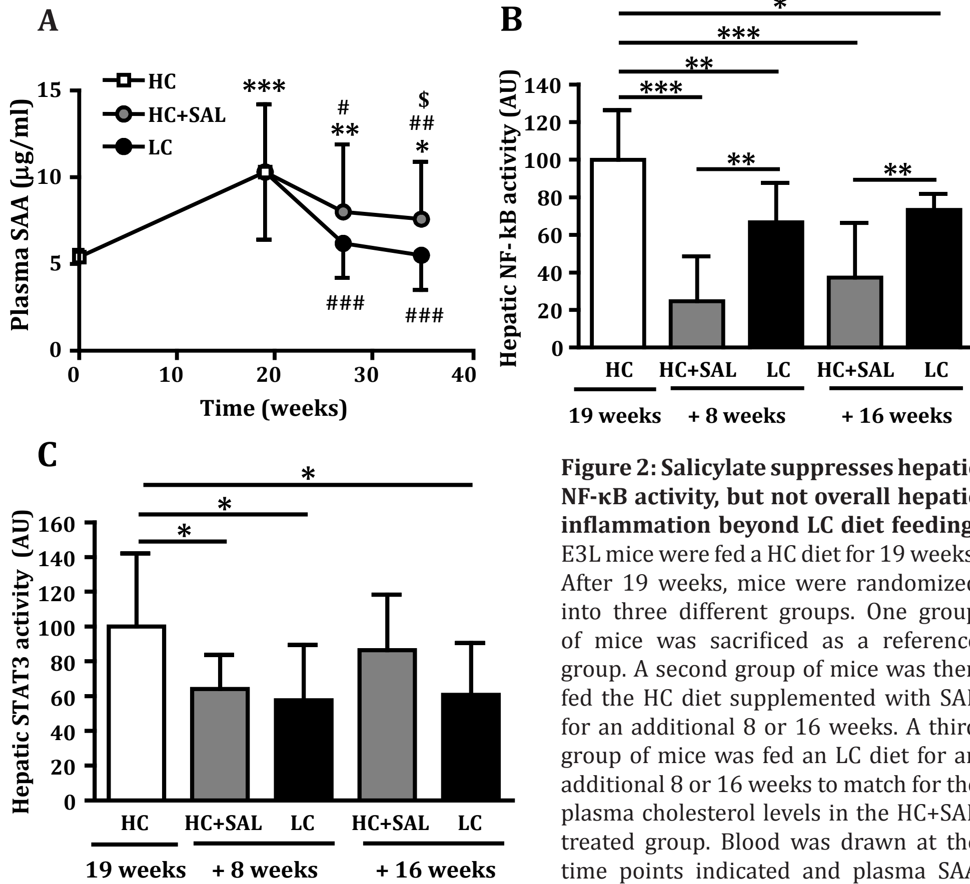


Figure 2: Salicylate suppresses hepatic NF-κB activity, but not overall hepatic inflammation beyond LC diet feeding. E3L mice were fed a HC diet for 19 weeks. After 19 weeks, mice were randomized into three different groups. One group of mice was sacrificed as a reference group. A second group of mice was then fed the HC diet supplemented with SAL for an additional 8 or 16 weeks. A third group of mice was fed an LC diet for an additional 8 or 16 weeks to match for the plasma cholesterol levels in the HC+SAL treated group. Blood was drawn at the time points indicated and plasma SAA levels were determined (A). (*P<0.05, **P<0.01, ***P<0.001 compared to t=0 weeks; #P<0.05, ##P<0.01, ###P<0.001 compared to the HC diet group at t=19 weeks; $P<0.05 compared to the LC diet fed group at the same time point) After prolonged 8 or 16 weeks of HC+SAL or LC diet feeding, the mice were sacrificed and livers were isolated and homogenized. The presence of active NF-κB (B) and STAT3 (C) in liver homogenates was determined. Values are means±SD. (*P<0.05, **P<0.01, ***P<0.001)

treatment (P<0.01), both after an additional 8 weeks (-33%, P<0.01) and 16 weeks (-27%, P<0.05) of experimental treatment. To verify that the enhanced suppression of NF-κB activity observed for HC+SAL treatment compared to LC treatment was not a general anti-inflammatory effect, we also determined the activity of signal transducer and activator of transcription 3 (STAT3), which is another transcription factor that can regulate the expression of inflammatory genes. Compared to the HC group, cholesterol lowering reduced STAT3 activity (-42%, P<0.05 at 8 weeks and -39%, P<0.05 at 16 weeks of prolonged treatment) (Figure 2C). A comparable reduction was found for HC+SAL (-36%, P<0.05 at 8 weeks and -16%, n.s. at 16 weeks), indicating that SAL treatment does not reduce STAT3 activity beyond the effect obtained upon cholesterol

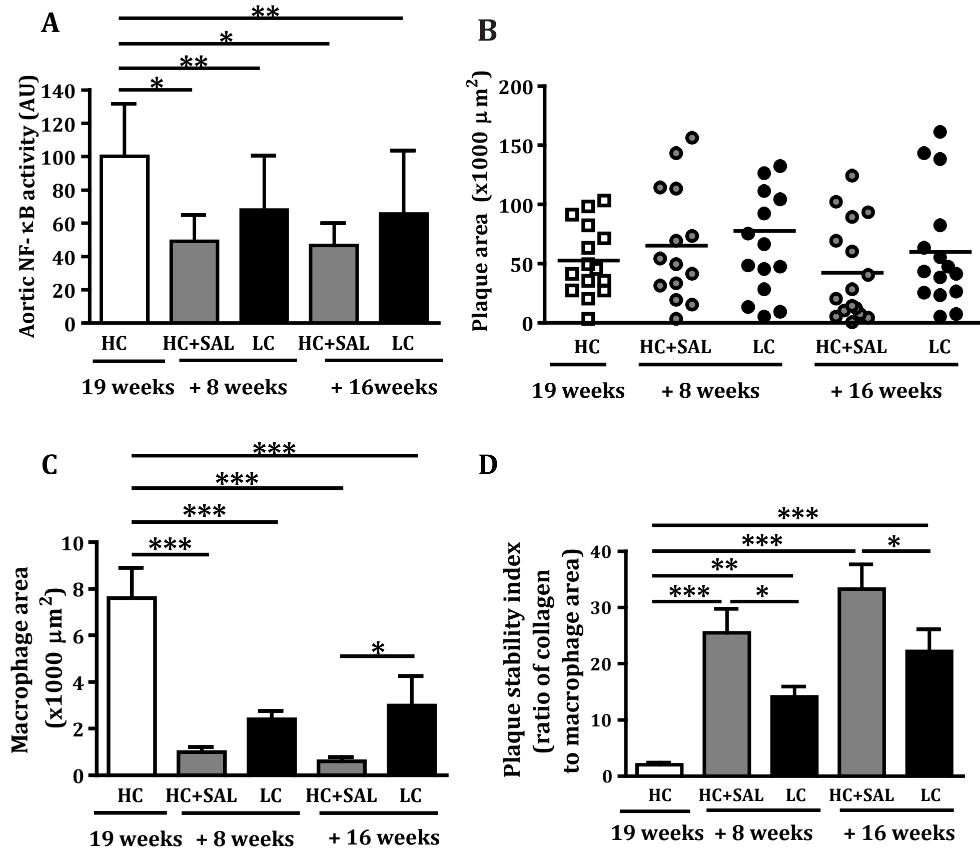


Figure 3: Salicylate reduces lesion macrophage area and increases lesion stability beyond cholesterol lowering alone. E3L mice were fed a HC diet for 19 weeks. After 19 weeks, mice were randomized into three different groups. One group of mice was sacrificed as a reference group. A second group of mice was then fed the HC diet supplemented with SAL for an additional 8 or 16 weeks. A third group of mice was fed an LC diet for an additional 8 or 16 weeks to match for the plasma cholesterol levels in the HC+SAL treated group. After prolonged 8 or 16 weeks of HC+SAL or LC diet feeding, the mice were sacrificed and hearts and thoracic aortas were isolated. Aortas were homogenized and the presence of active NF-κB in aorta homogenates was determined (A). Hearts were embedded in paraffin and cross-sectioned throughout the aortic root area. Four sections per mouse with 50 μm intervals were analyzed. Total lesion area was determined. Horizontal bars indicate mean lesion area. Each point corresponds to one mouse (B). In addition, the macrophage content of the lesions was quantified (C) and the plaque stability index (ratio of collagen-to-macrophage area of the lesions) was determined (D). Values are means±SD. (*P<0.05, **P<0.01, ***P<0.001)

lowering. These data suggest that the effect of SAL treatment on NF-κB activity is not a general phenomenon.

Salicylate induces lesion regression by reducing macrophage area and increases lesion stability beyond cholesterol lowering alone

To evaluate if SAL suppresses NF-κB activity in the vessel wall as it did in the liver, NF-κB activity was determined in homogenates of the thoracic aorta. Compared to HC diet fed mice, SAL treatment significantly reduced aortic NF-κB activity (-51%, $P<0.01$ after 8 weeks and -53%, $P<0.01$ after 16 weeks) (Figure 3A). Cholesterol lowering alone also reduced NF-κB activity, with -32% at 8 weeks ($P<0.05$) and with -34% after 16 weeks of prolonged treatment ($P<0.05$), which reduction was not significantly different from that obtained with SAL treatment. We then set out to analyze atherosclerotic lesions in the aortic root. After 19 weeks of HC diet feeding, mild atherosclerotic lesions had developed in the HC group ($52\pm31 \times 10^3$ μm^2). Total lesion area did not progress with either treatment in time: total lesion area in mice fed the LC diet was $77\pm66 \times 10^3$ μm^2 at 8 weeks, and $60\pm50 \times 10^3$ μm^2 at 16 weeks of prolonged treatment, and HC+SAL treatment resulted in a total lesion area of $65\pm49 \times 10^3$ μm^2 at 8 weeks, and $42\pm41 \times 10^3$ μm^2 at 16 weeks of prolonged treatment (Figure 3B).

Next, the effect of NF-κB suppression on the regression of macrophage area, an early process in lesion regression that improves the severity of atherosclerotic lesions, was determined. Both LC diet feeding and HC+SAL treatment markedly induced regression of the macrophage content of lesions (Figure 3C) compared to the HC group. At 8 weeks of prolonged treatment, the macrophage area in lesions of LC diet fed mice had decreased by -69% to $2.4\pm1.4 \times 10^3$ μm^2 compared to $7.6\pm5.1 \times 10^3$ μm^2 in the HC group at time point 19 weeks of the study ($P<0.001$), and remained at this level also at week 16 ($3.0\pm4.9 \times 10^3$ μm^2). HC+SAL treatment induced this regression more efficiently, by -87% ($1.0\pm0.8 \times 10^3$ μm^2) after 8 additional weeks ($P<0.001$) and by -92% ($0.6\pm0.7 \times 10^3$ μm^2) after 16 additional weeks of treatment ($P<0.001$). The difference between HC+SAL and LC became significant at t=16 weeks ($P<0.05$).

The relative collagen content of the lesions increased in both the LC and HC+SAL groups compared to the HC group. We therefore determined the ratio of collagen-to-macrophage area as a measure of plaque stability. Plaque stability increased in both LC diet fed mice ($P<0.01$) and the HC+SAL treated group ($P<0.001$) compared to the HC diet fed reference group in a time-dependent fashion (Figure 3D), and was higher in HC+SAL treated mice compared to LC diet fed mice after both 8 and 16 weeks of prolonged treatment ($P<0.05$).

Discussion

It is well recognized that hypercholesterolemia and inflammation are important factors in the development of atherosclerotic lesions. While it has been shown that plasma cholesterol-lowering can induce regression of pre-existing lesions, it is not yet clear if suppressing inflammation can lead to further regression of pre-existing atherosclerotic lesions on top of cholesterol-lowering. In this study, we showed that treatment of mice with pre-existing atherosclerotic lesions with HC+SAL strongly lowered plasma

cholesterol levels and suppressed hepatic and aortic NF-κB activity. A group of mice that was fed an LC diet to match for plasma cholesterol levels in the HC+SAL group showed a less pronounced decrease in hepatic NF-κB activity, and a similar reduction of aortic NF-κB activity. HC+SAL treatment and LC diet feeding markedly induced regression of the macrophage content of lesions and increased plaque stability as compared to the reference group. This induction of regression and increase in plaque stability were more pronounced in the HC+SAL treated mice than in the LC group. Our data indicate that suppressing NF-κB activity with SAL induces regression of pre-existing atherosclerotic lesions and increases plaque stability beyond those obtained by cholesterol-lowering alone.

We studied the effect of the NF-κB inhibitory capacity of SAL on top of its cholesterol-lowering effect on atherosclerosis regression in a time dependent manner. While we did not find a change in total lesion area during the chosen experimental treatment period of 16 weeks, lesion composition markedly changed: the macrophage content of lesions decreased, and the lesion stability index increased in time. Our data show a reduction of 69% in the macrophage content of LC diet fed mice and of 90% in HC+SAL treated mice after 8 weeks of intervention, while there is no significant further decrease in the number of macrophages in both the LC and HC+SAL treated groups from 8 to 16 weeks of intervention. Our data are in line with a study reporting a very strong decrease in the number of macrophages from existing lesions already after a few days,[7] indicating that the decrease in macrophage number is a very early process in lesion regression. On the other hand, our data show that the plaque stability index as determined by the collagen-to-macrophage ratio increased during the first 8 weeks of intervention in both HC+SAL treated and LC diet fed mice, and further increased during the next 8 week period in both groups. This is in line with studies that reported a reduction in the macrophage content of lesions and an increase in the collagen-producing cell types fibroblasts [4] or smooth muscle cells (SMCs) [5,12] after periods of regression ranging from 6 weeks until 6 months, although these studies did not evaluate lesion regression in time. While the decrease in macrophage number occurs in an early stage of lesion regression, the increase in plaque stability index occurs both in the 0-8 and 8-16 week periods of intervention. These data thus indicate that lesion regression is a long-term process that is characterized by various stages. In addition, we show that the effects on macrophage content and on plaque stability index are more pronounced in HC+SAL treated mice as compared to the LC group, indicating that suppressing NF-κB activity has a beneficial effect beyond cholesterol lowering alone in modulating both macrophage content and lesion stability during regression of pre-existing atherosclerotic lesions.

We investigated the effect of suppressing NF-κB activity on atherosclerosis regression. While, to our knowledge, there are no other reported studies evaluating the role of NF-κB in atherosclerosis regression, several studies addressed the role of NF-κB in atherosclerosis development. However, this did not lead to unequivocal results. Studies investigating the effect of a macrophage-specific reduction in NF-κB activity on atherosclerosis development in hyperlipidemic LDLr deficient mice showed that

this leads to increased numbers of inflammatory cells in atherosclerotic lesions.[13,14] This was thought to be the consequence of reduced lipid uptake by macrophages, leading to an inflammatory response of the non-phagocytosed lipids and consequently to enhanced recruitment of leukocytes to the site of the lesion.[14] In contrast, our study did not show an increase in inflammatory cells upon inhibiting NF-κB activity. However, our regression study was carried out under conditions of low plasma cholesterol. Since there is little lipid deposition in the vessel wall under low cholesterol conditions, this may explain why there is no increase in lesional macrophages in our study upon suppressing NF-κB activity. On the other hand, our data are in line with a study evaluating a genetic model with whole body increased NF-κB activity showing increased atherosclerosis development and increased numbers of inflammatory cells in the lesions.[15] Furthermore, a study evaluating the effect of acetyl salicylic acid (aspirin) on the development of atherosclerosis in LDLr deficient mice showed that aspirin reduced NF-κB activity in aortic tissue, which was associated with reduced lesion size, a reduced number of macrophages and increased smooth muscle cells and collagen, also reflecting increased plaque stability.[16] However, since aspirin also reduces inflammation by reducing prostaglandin synthesis, the specific contribution of NF-κB inhibition in this study was not clear. The latter two studies show a beneficial effect of suppressing NF-κB activity even under high plasma cholesterol conditions. In those studies, whole body NF-κB activity was suppressed compared to a macrophage-specific reduction in the studies showing that suppressing NF-κB activity leads to more inflammatory cells in atherosclerotic lesions. Together, these data suggest that reducing NF-κB activity can have a beneficial effect on atherosclerosis development and atherosclerosis regression, depending on plasma cholesterol levels and/or the cell type in which NF-κB is suppressed.

Our data indicate that cholesterol lowering and suppressing NF-κB activity with SAL leads to a reduction in the macrophage content of lesions beyond the effect obtained by cholesterol lowering alone. This reduction could be due to a decreased influx of monocytes or faster turnover or increased emigration of macrophages. We found that the number of monocytes adhering to the endothelium was not different for LC or HC+SAL treated mice (data not shown), suggesting that the monocyte influx is not different in HC+SAL treated mice compared to LC diet fed mice. Regarding emigration of macrophages, suppression of NF-κB activity has been reported to coincide with decreased expression of C-C chemokine receptor 7 (CCR7),[17] a factor that is required for emigration of dendritic cell-like macrophages out of existing lesions.[7] As a consequence, suppressing NF-κB activity may lead to decreased rather than increased emigration of monocytes from lesions. It is thus not likely that increased emigration of monocytes lead to the decreased macrophage content in HC+SAL treated mice compared to LC diet fed mice. The reduction in macrophages could be related to increased macrophage turnover. We found that SAL treatment reduced NF-κB activity. NF-κB has been shown to regulate the expression of genes involved in apoptosis, and to stimulate cell survival.[18] In line with this, suppressing NF-κB activity may abolish cell survival signaling, leading

to induction of apoptosis. This was also reported in a study evaluating atherosclerosis development in mice lacking IKK2 specifically in macrophages. Deletion of IKK2 results in diminished NF-κB activation, and atherosclerotic lesions in mice lacking IKK2 expression in macrophages showed increased cell death.[13] Together, these data suggest that increased apoptosis as a consequence of suppressing NF-κB activity may be underlying the reduction in macrophage content of lesions we observed in HC+SAL treated mice compared to LC diet fed mice.

We found that HC+SAL treatment markedly down regulated hepatic NF-κB activity, and that this down regulation was more manifest in HC+SAL treated mice than in LC diet fed mice. Nevertheless, plasma levels of the hepatic inflammation marker serum amyloid A (SAA) were reduced to a lesser extent in the HC+SAL treated group than in the LC fed group compared to the HC group. In a recent study we showed that feeding of an HC, but not an LC diet induces a hepatic inflammatory reaction leading to increased plasma SAA levels. Three major pathways were identified that are involved in mediating HC-diet induced inflammation, namely pathways involving NF-κB, STAT3 and CCAAT-enhancer binding protein (C/EBP)α/β.[19] In the present study, we show that HC+SAL treatment specifically inhibits the NF-κB pathway in liver. Although NF-κB activity is suppressed in HC+SAL treated mice, the STAT3 pathway continues to be activated as a consequence of HC diet feeding. In contrast, switching mice from the HC to the LC diet reduces the activation of all cholesterol-induced inflammatory pathways. This could explain our observation that plasma SAA levels are lower in the LC group as compared to the HC+SAL group.

The clinical importance of gaining insight in processes that are involved in remodeling of pre-existing atherosclerosis to induce regression of atherosclerotic lesion has been increasingly recognized over the past years. Our data indicate that HC+SAL treatment has additive beneficial effects beyond cholesterol lowering alone in the regression of pre-existing lesions by further reducing the macrophage content of lesions and by further increasing lesion stability. These additive effects may at least partly be mediated by SAL's capacity to suppress NF-κB activity. Future studies are needed to determine if reducing cholesterol levels and NF-κB activity with SAL is also more beneficial than cholesterol lowering alone in the regression of pre-existing lesions in humans. In all, our data show that cholesterol-lowering and suppression of NF-κB activity by SAL promote lesion regression and increase plaque stability more efficiently than cholesterol lowering alone. Our data also illustrate that targeting multiple factors has additional value for the treatment of pre-existing atherosclerosis.

Acknowledgements:

This work was supported by the Centre for Medical Systems Biology [Grant number 115] within the framework of the Netherlands Genomics Initiative (NGI).

References

1. Steinberg D. Hypercholesterolemia and inflammation in atherogenesis: two sides of the same coin. *Mol Nutr Food Res* 2005;**49**:995-998.
2. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;**105**:1135-1143.
3. Lusis AJ. Atherosclerosis. *Nature* 2000;**407**:233-241.
4. Gijbels MJ, van der CM, 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.
5. Tsukamoto K, Tangirala R, Chun SH, Pure E, Rader DJ. Rapid regression of atherosclerosis induced by liver-directed gene transfer of ApoE in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 1999;**19**:2162-2170.
6. Verschuren L, de Vries-van der Weij, Zadelaar S, Kleemann R, Kooistra T. LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms. *J Lipid Res* 2009;**50**:301-311.
7. Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc Natl Acad Sci U S A* 2006;**103**:3781-3786.
8. Gottlieb I, Agarwal S, Gautam S, Desai M, Steen H, Warren WP, Xavier SS, Lima JA. Aortic plaque regression as determined by magnetic resonance imaging with high-dose and low-dose statin therapy. *J Cardiovasc Med (Hagerstown)* 2008;**9**:700-706.
9. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 1998;**396**:77-80.
10. Zadelaar S, Kleemann R, Verschuren L, de Vries-van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol* 2007;**27**:1706-1721.
11. Westerterp M, de HW, Berbee JF, Havekes LM, Rensen PC. Endogenous apoC-I increases hyperlipidemia in apoE-knockout mice by stimulating VLDL production and inhibiting LPL. *J Lipid Res* 2006;**47**:1203-1211.
12. Viles-Gonzalez JF, Fuster V, Corti R, Valdiviezo C, Hutter R, Corda S, Anand SX, Badimon JJ. Atherosclerosis regression and TP receptor inhibition: effect of S18886 on plaque size and composition--a magnetic resonance imaging study. *Eur Heart J* 2005;**26**:1557-1561.
13. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 2003;**112**:1176-1185.
14. Kanters E, Gijbels MJ, van dM, I, Vergouwe MN, Heeringa P, Kraal G, Hofker MH, de Winther MP. Hematopoietic NF-kappaB1 deficiency results in small atherosclerotic lesions with an inflammatory phenotype. *Blood* 2004;**103**:934-940.
15. Wolfrum S, Teupser D, Tan M, Chen KY, Breslow JL. The protective effect of A20 on atherosclerosis in apolipoprotein E-deficient mice is associated with reduced expression of NF-kappaB target genes. *Proc Natl Acad Sci U S A* 2007;**104**:18601-18606.
16. Cyrus T, Sung S, Zhao L, Funk CD, Tang S, Pratico D. Effect of low-dose aspirin on vascular inflammation, plaque stability, and atherogenesis in low-density lipoprotein receptor-

deficient mice. *Circulation* 2002;**106**:1282-1287.
17. Chen T, Guo J, Yang M, Han C, Zhang M, Chen W, Liu Q, Wang J, Cao X. Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. *Blood* 2004;**103**:413-421.
18. Bellas RE, FitzGerald MJ, Fausto N, Sonenshein GE. Inhibition of NF-kappa B activity induces apoptosis in murine hepatocytes. *Am J Pathol* 1997;**151**:891-896.
19. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der GJ, van OB, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol* 2007;**8**:R200.

Chapter 4

LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in ApoE*3-Leiden mice: time course and potential mechanisms

Lars Verschuren [1,2], Jitske de Vries-van der Weij [3], Susanne Zadelaar [1], Robert Kleemann [1], Teake Kooistra [1]

[1] TNO-Quality of Life, Biosciences-Gaubius Laboratory, Leiden, The Netherlands
[2] Dept. of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands
[3] Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

Journal of Lipid Research 2009; 50: 301-311

Abstract

Background: The aim of this study was to define the anti-atherosclerotic role of liver-X-receptors (LXRs) under both lesion progressive and lesion regressive conditions, to establish a temporal line of events, and to gain insights into the mechanisms underlying the anti-atherogenic potency of LXRs.

Methods and results: We used ApoE*3-Leiden mice to comprehensively and time-dependently dissect how T0901317, an LXR-agonist, inhibits initiation and progression of atherosclerotic lesions, and regresses existing lipid- and macrophage-rich lesions. T0901317 strongly suppresses lesion evolution and promotes lesion regression regarding lesion number, area, and severity. Quantitative plasma and vessel wall analyses corroborated by immunohistochemical evaluation of the aortic lesions revealed that under progressive (high-cholesterol diet) as well as regressive (cholesterol-free diet) conditions T0901317 (i) significantly increases plasma triglyceride and total cholesterol levels; (ii) does not affect the systemic inflammation marker, SAA; (iii) suppresses endothelial monocyte adhesion; and (iv) induces the expression of the cholesterol efflux-related genes ApoE, ABCA1 and ABCG1. Furthermore, under progressive conditions, T0901317 suppresses the vascular inflammatory status (NF-κB) and the vascular expression of adhesion molecules (E-selectin, ICAM-1, and CD44), lowers lesional macrophage accumulation, and blocks lesion evolution at the transition from lesional stage II to III. Under regressive conditions, T0901317 induces lesional macrophage disappearance and increases the expression of the chemokine receptor CCR7, a factor functionally required for regression.

Conclusions: The LXR-agonist T0901317 retards vascular lesion development and promotes lesion regression at several levels. The findings support that vascular LXR is a potential anti-atherosclerotic target.

Introduction

Coronary atherosclerosis represents the leading cause of morbidity and mortality of men and women throughout the Western world. Hypercholesterolemia is a well-established risk factor for the incidence of atherosclerosis and its pathologic complications[1]. However, despite the success of cholesterol-lowering statins in reducing cardiovascular causes of death, two thirds of the statin-treated patients still experience cardiovascular events. Consequently, focus has switched to risk factors other than hypercholesterolemia.

Atherosclerosis is now recognized as a multifactorial, multistep disease with numerous etiologies which have to act in concert to initiate and promote the atherosclerotic process. In addition to traditional risk factors such as hypertriglyceridemia, low high-density lipoprotein (HDL), and hypertension, inflammation is accepted as a major driving force of atherosclerotic lesion development. Inflammation has a key role in lesion initiation and evolution, and encompasses both cellular and molecular components[2].

Liver-X-receptor (LXR) belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors. Two LXR isoforms have been described so far, LXRa and LXRb. LXRb has a ubiquitous tissue distribution, whereas LXRa predominates in liver, adipose tissue, intestinal tissue, and macrophages; both isoforms respond to the same natural and synthetic ligands[3]. LXRs control genes involved in intestinal cholesterol absorption, hepatic bile acid synthesis, cholesterol efflux, vascular foam cell formation, and inflammation[4]. These properties make LXRs a potential target for therapeutic intervention in the atherosclerotic process. Studies performed over the last several years have established that LXRs are anti-atherosclerotic factors owing to their ability to regulate cholesterol and lipid homeostasis and to inhibit inflammation within the arterial wall[5-8]. However, these studies mainly focused on lesion growth but not lesion regression, did not establish a temporal line of events, and only partly addressed the mechanisms underlying the anti-atherogenic potency of LXRs.

Here we describe the impact of an LXR-agonist, T0901317, on the progression and regression of atherosclerosis in transgenic ApoE*3-Leiden (E3L) mice, a well-established mouse model for atherosclerosis.[9,10] E3L mice are highly responsive to cholesterol-containing diets, resulting in strongly elevated plasma cholesterol and triglyceride levels, with a prominent increase in VLDL- and LDL-sized lipoprotein particles. E3L mice do not develop atherosclerosis on a regular chow diet, but atherosclerosis in E3L mice can be initiated by feeding of a cholesterol-containing, Western type diet[10], whereas regression of pre-existing lesions can be induced using cholesterol-depleted diets[11]. E3L mice respond to hypolipidemic drugs with cholesterol-lowering and appear useful in predicting effects of pharmaceutical modifiers in humans[10]. Our study confirms the results previously published by other groups in different mouse models (ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice) regarding some of the effects of LXR-agonists on lesion development, but adds some important novel information with regard to the action of the LXR-agonist on the vasculature, in particular to vascular inflammation, adhesion molecule expression and under regressive conditions, potential mechanisms involved

in macrophage disappearance. Also, the analysis of the experiments at different time points allowed establishing a temporal line of events.

Methods

Animals

Female ApoE*3-Leiden transgenic (E3L) mice (TNO-Pharma, Gaubius Laboratory, Leiden, The Netherlands) were characterized for expression of human ApoE by enzyme-linked immunosorbent assay (ELISA). The animals were housed in wire-topped Macrolon cages with a layer of sawdust as bedding, and diets and water were given *ad libitum*.

T0901317-Atherosclerosis studies

Progression studies: To establish a time course of events, three separate progression studies were performed, with mice (age 10-12 weeks at start of experiment) sacrificed after t=2 weeks, t=10 weeks, or t=15 weeks of experimental treatment, respectively.
Study 1: (main study; sacrifice at 10 weeks). During a run-in period of 3 weeks, 24 female E3L mice received an atherogenic, 1% (w/w) cholesterol-containing Western-type diet (Hope Farms, Woerden, The Netherlands), further referred to as high-cholesterol (HC) diet. Then, mice were subdivided into 2 experimental groups (n=12) and matched for plasma cholesterol and triglycerides. In one group, HC was continued for another 10 weeks (HC group). The other, T0901317-treated group (HC+T0901317 group) received HC supplemented with 0.01% w/w T0901317 (Sigma Aldrich). Based on food intake in the HC+T0901317 group, the daily dose of T0901317 was 11 mg/kg bodyweight.
Study 2: as study 1, but sacrifice at 15 weeks; n=15 per group.
Study 3: (short study; sacrifice at 2 weeks). E3L mice were treated with HC-diet supplemented with or without 0.01%(w/w) T0901317 (n=8 per group; no run-in) and were sacrificed after 2 weeks of treatment.
Regression study: Twenty-six female E3L mice received HC for 18 weeks to develop atherosclerotic lesions. Then, 10 mice were sacrificed for atherosclerotic lesion analysis. The 16 remaining mice were subdivided into two groups of 8 mice each and switched for 8 weeks to a regressive, cholesterol-depleted diet (RD), supplemented with 0.01% (w/w) T0901317 (RD+T0901317 group) or not (RD group).

Analysis of plasma lipids, lipoproteins and plasma inflammation markers

Total plasma cholesterol and triglyceride levels were measured after 4 hours of fasting, using kits No. 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands), respectively. For lipoprotein profiles, pooled plasma was fractionated using an ÄKTA FPLC system (Pharmacia, Roosendaal, The Netherlands).[12] The plasma levels of E-selectin (R&D Systems) and SAA (Biosource) were determined by ELISA as reported.[13]

Atherosclerotic lesion analysis

At the end of a treatment period, mice were euthanized to collect hearts and aortas. Hearts were fixed and embedded in paraffin to prepare serial cross sections (5 μm-thick) throughout the entire aortic root area for (immuno)histological analysis.[12,14] Cross-sections were stained with hematoxylin-phloxine-saffron and atherosclerosis was analyzed blindly in 4 cross-sections of each specimen (at intervals of 40 μm). QWin-software (Leica)[12,14] was used for morphometric computer-assisted analysis of lesion number, lesion area and lesion severity according to the classification of the American Heart Association[15] as reported.[12,14] Monocyte adhesion and macrophage area were determined essentially as described previously.[14,16,17] For immunostaining of ICAM, LXRα/β, p50-NF-κB, p65-NF-κB, CD44 and CCR7 antibodies GTX76543 (Genetex), sc-1000, sc-114, sc-109, sc-18849 and sc-9701 (Santa Cruz Biotechnology) were used, respectively.[18]

Nucleic acid extraction and Real-time PCR

Total RNA was extracted from individual aortas (n=5 per group) using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads according to the manufacturer's instructions. The integrity of each RNA sample obtained was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). Then, cDNA was prepared using kit #A3500 (Promega, Leiden, The Netherlands) for real-time polymerase chain reaction (RT-PCR) analysis. Mastermix (Eurogentec, Seraing, Belgium), the ABI-7700 system (Perkin-Elmer Biosystems, Nieuwekerk a/d IJssel, the Netherlands), and established primer/probe sets were used according to the manufacturer's instructions. Cyclophilin A (PE Biosystems) was used as a reference.

Statistical methods

Significance of difference was calculated by analysis of variance (ANOVA) test followed by a least significant difference post hoc analysis using SPSS 11.5 for Windows (SPSS, Chicago, USA). The level of statistical significance was set at $P<0.05$.

Results

T0901317 enhances plasma lipid levels in E3L mice

Food intake of mice fed HC+T0901317 for ten weeks was slightly lower than that in animals on HC (2.2±0.2 grams per day (g/day) vs. 2.5±0.2 g/day; $P<0.05$; Table 1). At the end of the treatment period, there was no significant difference in bodyweight between the two groups (HC+T0901317, 19.6±0.5 g; HC, 20.8±0.4 g; Table 1). Plasma cholesterol levels in HC+T0901317 (23.4±4.1 mM) were significantly increased compared with HC (18.7±3.6 mM, $P<0.05$; Table 1). This rise in plasma total cholesterol levels in HC+T0901317 can be mainly ascribed to a particular subfraction (fractions 10-17; Figure 1A), co-eluting with particles in the IDL/LDL range. T0901317 strongly

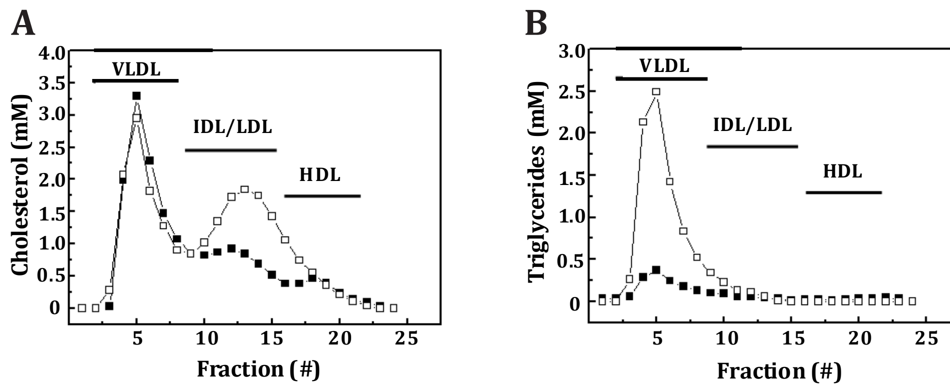


Figure 1: Lipoprotein profiles of control and T0901317-treated, cholesterol-fed E3L mice. (A), Cholesterol content in fractionated plasma samples. (B), Triglyceride content in fractionated plasma samples. Solid squares indicate HC, open squares indicate HC+T0901317.

(6.8 times) increased fasting plasma triglyceride levels (HC+T0901317, 12.3±4.4 mM; HC, 1.8±0.4 mM; P<0.05; Table 1), which rise is entirely confined to particles in the VLDL-range (Figure 1B). Quantitative RT-PCR analysis of liver samples indicates that the strong increase in triglyceride levels in the T0901317-treated group may at least partly be explained by the 2.2-fold (P<0.01) elevated expression of the lipogenic gene, sterol regulatory binding protein (SREBP)-1c.

Table 1. Metabolic parameters of control and T0901317-treated, cholesterol-fed E3L mice.

	HC	HC+ T0901317
Average food intake (g/day)	2.5 ± 0.2	2.2 ± 0.2*
Average body weight (g)	20.8 ± 0.4	19.6 ± 0.5
Average total cholesterol level (mM)	18.7 ± 3.6	23.4 ± 4.1*
Average triglyceride level (mM)	1.8 ± 0.4	12.3 ± 4.4*

Metabolic parameters of control and T0901317-treated, cholesterol-fed E3L mice. Data are expressed as means ± SD; n=12 for each group; *P<0.05 vs. HC

T0901317 reduces atherosclerotic lesion number, size and severity
After 10 weeks of experimental treatment, *early* atherosclerotic lesion formation was analyzed in cross-sections of the aortic valve area. The average lesion number per mouse was 18.3±2.3 in HC and reduced by 64% to 6.5±1.3 in HC+T0901317 (P< 0.01; Figure 2A). Measurement of the total cross-sectional lesion area showed a similar picture (Figure 2B): HC displayed a cross-sectional lesion area of 37027±7450 μm^2 and HC+T0901317 reduced the lesion area by 85% to 5484±2019 μm^2 (P<0.05).

To assess whether these differences merely reflect a difference in time of onset

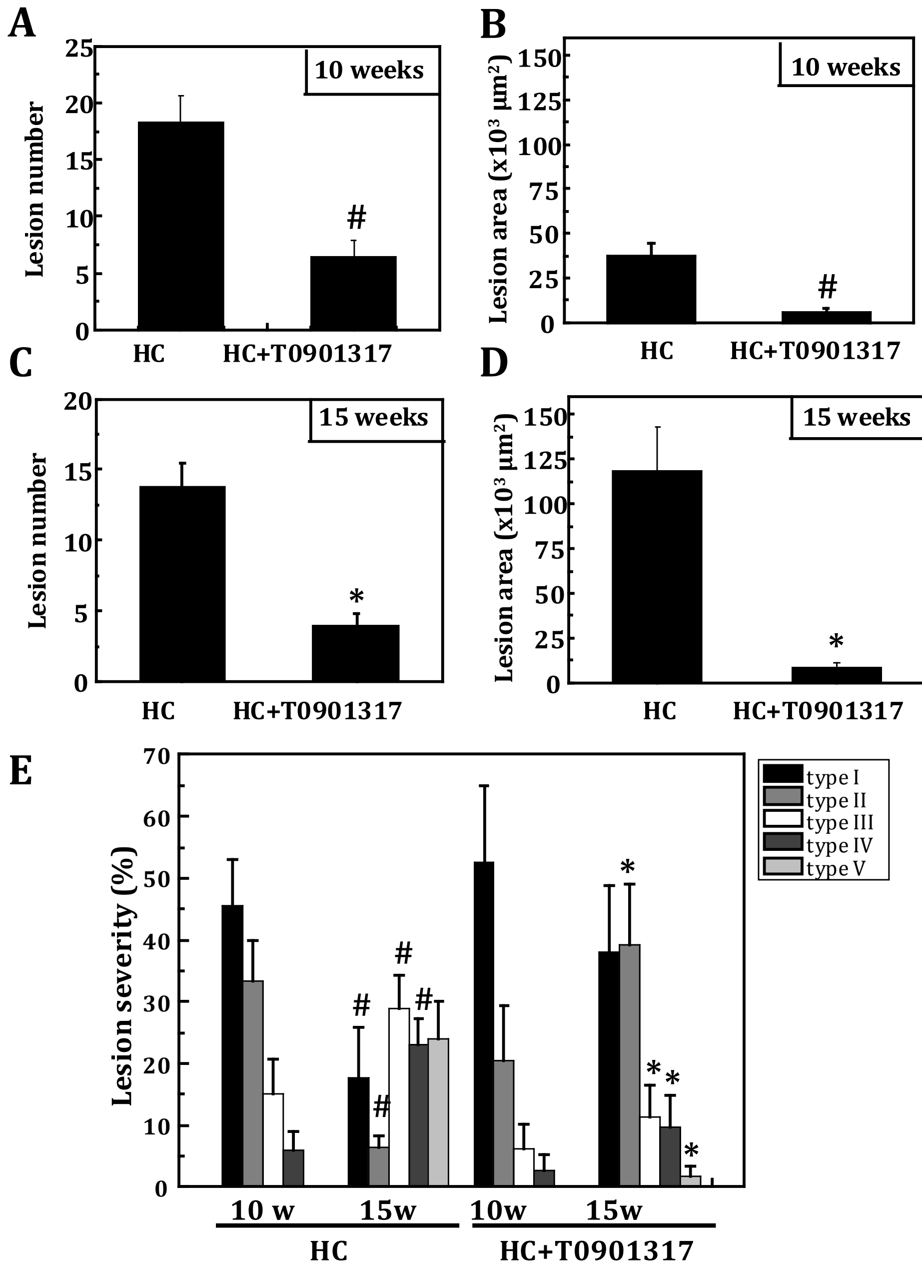


Figure 2: Effect of T0901317 on atherosclerosis development in aortic valve area. Shown are effects of T0901317 on total lesion number after 10 weeks (A) and 15 weeks (C) of treatment and effects of T0901317 on total cross-sectional lesion area after 10 weeks and 15 weeks (D) of treatment. The effect of T0901317 on lesion severity is presented as percentage of cross-sections analyzed (E). Data represent mean values ± SEM (n≥10 mice per group) #P<0.05 vs. HC-10 weeks, *P<0.05 vs. HC-15 weeks.

of the disease process or are the result of diminished lesion initiation and progression, lesion number and size were also analyzed (in a separate experiment) after a 15-weeks-treatment. The difference in lesion number of the two groups resembled that of the 10-week experiment (Figure 2C), while the lesion area was substantially increased in HC (3.2 fold), but only marginally (1.5 fold) in HC+T0901317 after 15 weeks (Figure 2D), indicating that T0901317 is interfering with both lesion initiation and lesion development rather than retarding the onset of the disease process.

Table 2. Plasma inflammation markers of control and T0901317-treated, cholesterol-fed E3L mice.

	t=0	HC	HC+ T0901317
Serum Amyloid A (µg/ml)	10.1 ± 4.6	21.7 ± 15.3#	24.5 ± 12.1#
E-Selectin (ng/ml)	84.4 ± 9.7	97.2 ± 13.7#	84.4 ± 12.2*

Plasma inflammation markers of control and T0901317-treated, cholesterol-fed E3L mice. Shown are average plasma levels of Serum Amyloid A and E-Selectin at the start (t=0) and the end (t=10 weeks) treatment period (means ± SD n=12 for each group; #P<0.05 vs. t=0 and *P<0.05 vs. HC).

This conclusion was further strengthened by evaluating lesion severity. Cross-sections of the aortic root were morphologically analyzed and lesions were graded according to the classification of the American Heart Association.[15] Grading after 10 weeks demonstrated that lesions from both HC and HC+T0901317 mainly contained mild type I/II lesions (~80% of all classified lesions) and hardly type IV/V lesions (Figure 2E). After 15 weeks, a clear shift in lesion severity was observed in HC but not in HC+T0901317. In HC, 24%±6% of all cross-sections contained type I/II lesions and 76%±5% contained type III/IV/V lesions (Figure 2E). In contrast, in HC+T0901317 treated mice 77%±10% of all cross-sections contained type I/II lesions and 23%±4% contained type III/IV/V lesions, thereby remarkably resembling the findings obtained after the 10-weeks-treatment. Importantly, T0901317 blocked lesion progression at the transition from lesion severity stage II to III, which is characterized by the absence of smooth muscle cell (SMC) proliferation and migration to the cap, a hallmark of type III lesions. Thus, T0901317 strongly suppresses atherogenesis, both at the initiation and the progression phase of disease. The question therefore arises by what mechanism T0901317 interferes with the atherosclerotic process.

The vessel wall as target for T0901317

To evaluate whether the anti-atherosclerotic effects of T0901317 are paralleled by anti-inflammatory effects, we analyzed systemic inflammatory markers originating from liver (SAA) or vasculature (E-selectin). HC markedly and significantly elevated plasma levels of both SAA (2.1-fold) and E-selectin (1.2-fold) (Table 2). T0901317 significantly suppressed HC-induced E-selectin but not SAA plasma levels, pointing to a direct anti-inflammatory effect of T0901317 on the vascular endothelium. To seek further evidence for a direct effect of T0901317 on the vessel wall we evaluated vascular expression of

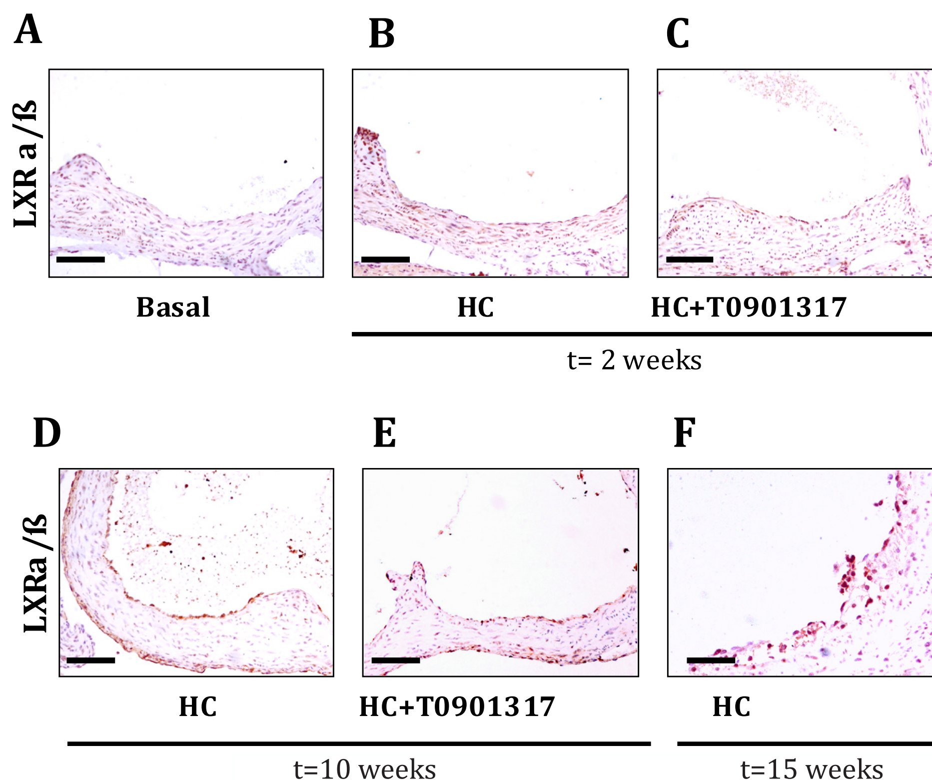


Figure 3: Vascular LXRα/β expression is induced in cholesterol-fed E3L mice. Representative photomicrographs of immunohistochemical staining of LXRα/β-immunoreactivity (IR) in cross-sections of the aortic root: in basal chow-fed E3L mice (A); after 2 weeks of HC (B) or HC+T0901317 (C); after 10 weeks of HC (D) or HC+T0901317 (E); after 15 weeks of HC (F). Bar= 50 µm.

LXRα/β inmmunohistochemically using an antibody recognizing both LXR isoforms (see Methods section). In chow-fed E3L mice no LXRα/β expression was detectable in the vasculature (Figure 3A), but 2 weeks after starting the HC diet, expression of LXRα/β was demonstrable in both the HC and HC+ T0901317 group (Figure 3B-C) and remained so for the rest of the experimental treatment period (Figure 3D-E). In both experimental groups expression of LXRα/β was mainly confined to the endothelium and to a lesser extent to SMC; no notable difference in staining intensity was seen between the two groups (Figure 3B-E). Upon lesion progression, accumulating monocyte-derived macrophages in the lesions also stained positive for LXRα/β (Figure 3F). QRT-PCR analysis revealed that both LXRα and LXRβ were about two-fold up-regulated by dietary cholesterol.

Together, these data demonstrate, for the first time, that dietary cholesterol induces LXR protein expression in the vasculature (endothelium, SMC, monocyte-derived macrophages), thus enabling T0901317 to specifically suppress HC-induced expression of endothelial E-selectin and thereby to lower plasma levels of E-selectin.

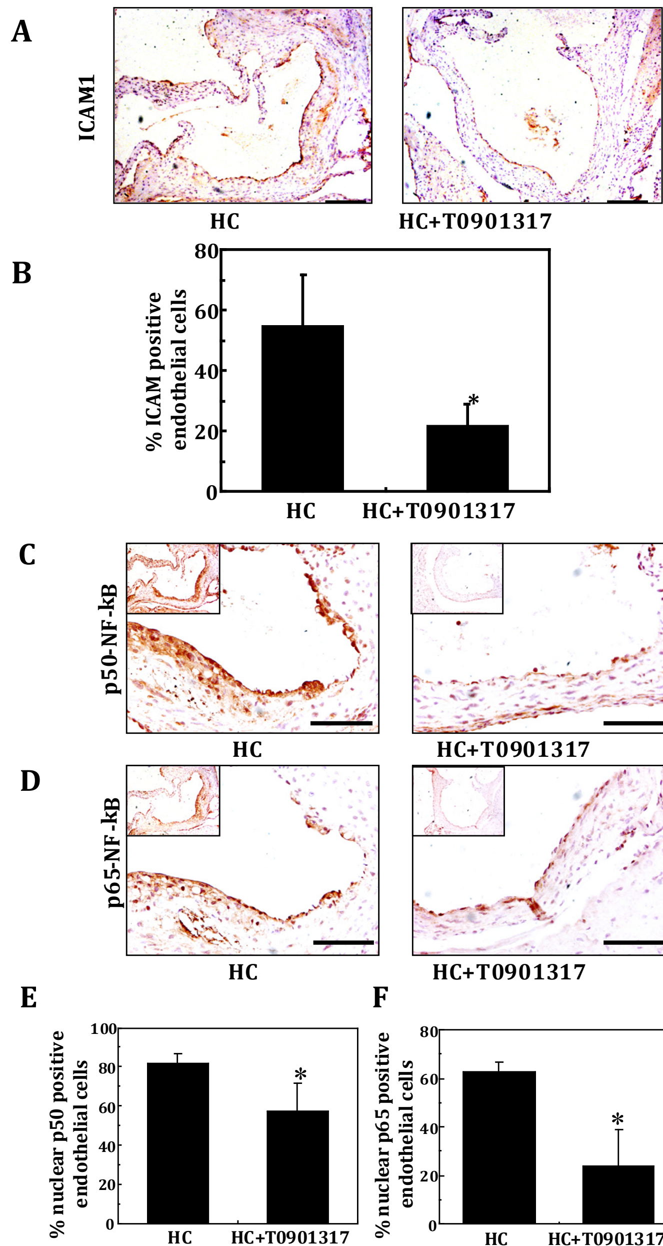


Figure 4: Effect of T0901317 on vascular ICAM-1 and NF-κB expression. Representative photomicrographs of immunohistochemical staining of ICAM-1-IR (A) after 10 weeks of treatment with HC or HC+T0901317. Analyzed percentage of ICAM-1-IR positive EC in control vs treatment group (B). Representative photomicrographs of immunohistochemical staining of p50-NF-κB-IR (C), and p65-NF-κB-IR (D) after 10 weeks of treatment with HC or HC+T0901317. (*$P<0.05$ vs. HC). Bar= 100 μm. Analyzed percentage of nuclear p50-NF-κB-IR positive EC (E), and nuclear p65-NF-κB-IR positive EC (F) after 10 weeks of treatment with HC or HC+T0901317.

Anti-atherogenic action of T0901317 in the vasculature
The anti-inflammatory effect of T0901317 in the vasculature is not restricted to E-selectin. Immunohistochemical staining of ICAM-1 underlines that T0901317 suppresses endothelial adhesion molecule expression more generally. Whereas in HC 55%±17% of the aortic endothelial cells (EC) expressed ICAM-1, in HC+T0901317 only 22%±7% (P<0.05) of the cells did so (Figures 4A and 4B). Furthermore, the total number of EC per cross-section with positive p65-NF-κB- or p50-NF-κB-staining (in cytosol and/or nucleus) was strongly reduced in HC+T0901317 (Figure 4C-D). Also, the number of EC showing nucleus-associated (i.e. active) p65-NF-κB or p50-NF-κB immunoreactivity was strongly and significantly (P<0.05) reduced in HC+T0901317 compared with HC (Figure 4E-F). These results are further evidence that T0901317 not only lessens the number of NF-κB-positive EC, but also the number of active NF-κB-containing EC, thus providing a rationale for the reduced expression of cellular adhesion molecules and the overall suppressive effect on lesion initiation with T0901317. The

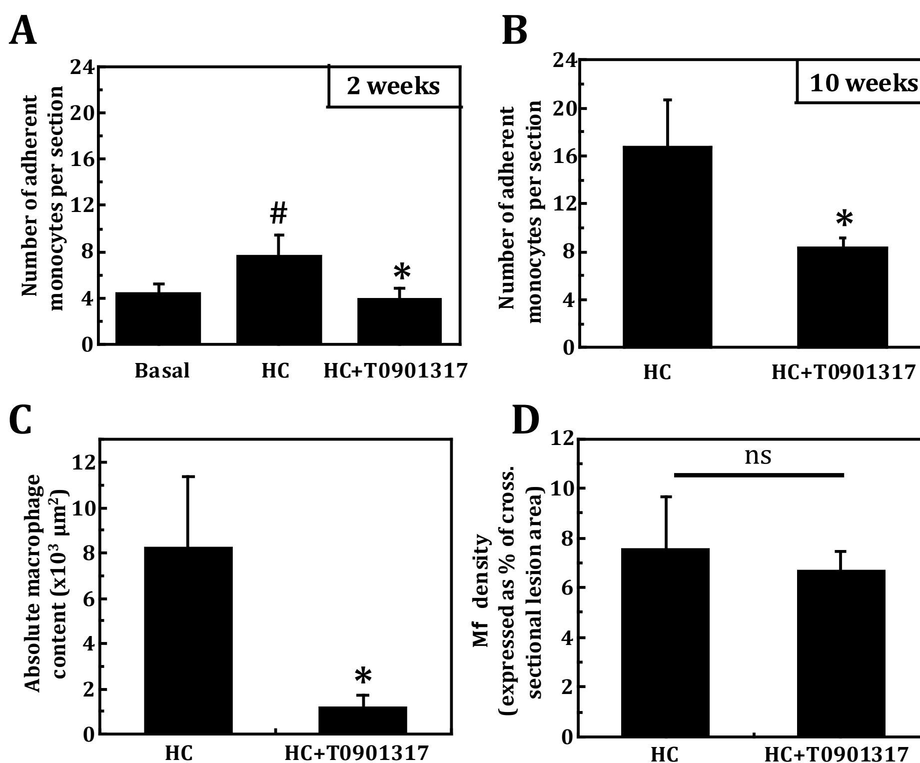


Figure 5. Effect of T0901317 on aspects of atherogenesis in the aortic valve area. Shown are effects of T0901317 on number of monocytes adhering to endothelium after 2 weeks (A) and 10 weeks of HC (B), on absolute macrophage containing lesion area after 10 weeks (C), and on macrophage density (i.e. macrophage area expressed as percentage of cross-sectional lesion area) after 10 weeks of HC. Data are presented as mean ± SD, #P<0.05 vs. chow-fed E3L mice (basal) and *P<0.05 vs. HC.

T0901317-reduced expression of adhesion molecules is paralleled by diminished recruitment of blood monocytes. As early as 2 weeks after starting HC-treatment, the number of adherent monocytes was significantly increased in HC (7.7±1.7 compared with 4.5±0.7 in chow fed E3L mice (Figure 5A)), but not so in HC+T0901317 (4.0±0.9). After 10 weeks, monocyte adhesion was further increased in HC (16.7±4.0; Figure 5B); monocyte adherence was also elevated in HC+T0901317 (8.3 ± 2.1 vs. 4.0±0.9 at t=2 weeks; P<0.05), but remained significantly lower than in HC. Since monocyte-adhesion is a prerequisite, but not sufficient, for formation of early lesions, we subsequently determined lesional macrophage content and macrophage density. Figure 5C shows that the absolute macrophage-containing lesion area at 10 weeks was substantially (by 85%; P<0.05) reduced in HC+T0901317 compared with HC (1227±507 μm^2 vs. 8238±3168 μm^2). On the other hand, the macrophage density (the macrophage area expressed as percentage of the cross-sectional lesion area) is very similar in the two groups (Figure 5D).

Markedly, in HC+T0901317 lesions are less severe, with lesion progression seemingly blocked at the transition from lesion severity stage II to III (Fig. 2E), a phenotype that is also seen in CD44-deficient mice.[19] CD44 is a cellular adhesion molecule that promotes adhesion and recruitment of monocytes/macrophages and regulates the migration and proliferation of SMC.[19,20] CD44 expression after 10 weeks of HC-treatment is mainly confined to monocytes and macrophage/foam cells in the intima, and expression is further increased and extended to SMC during subsequent atherogenesis progression after 15 weeks (Figure 6A-B). T0901317 strongly and significantly reduced CD44 expression in the vasculature (Figures 6A-C), both at the mRNA (RT-PCR analysis) and protein (immuno-histochemical analysis) level. In contrast to CD44, T0901317 strongly increased the expression of genes reportedly involved in reverse cholesterol efflux (ApoE, ABCA1 and ABCG1; Table 3). Collectively, the data show that in HC+T0901317 lesion number, area, and severity are strongly suppressed with a parallel decrease in monocyte adhesion and SMC migration and proliferation. These effects can at least partly be explained by diminished expression of the adhesion molecules E-selectin, ICAM-1, and CD44, and by an up-regulation of factors promoting cholesterol efflux, ApoE, ABCA1 and ABCG1.

Table 3. Vascular gene expression of control and T0901317-treated, cholesterol-fed E3L mice.

	HC	HC+ T0901317
apoe	100% ± 11%	211% ± 22.3% *
abca1	100% ± 15%	821% ± 211% *
abcg1	100% ± 13%	491% ± 109% *

Vascular gene expression of control and T0901317-treated, cholesterol-fed E3L mice. Shown are the average aortic gene expression levels of *apoe, abca1,* and *abcg1* at the end of the 10-weeks treatment period as percentage values relative to corresponding values at the start (t=0) of the treatment (means ± SD; n=12 for each group; *P<0.05 vs. HC).

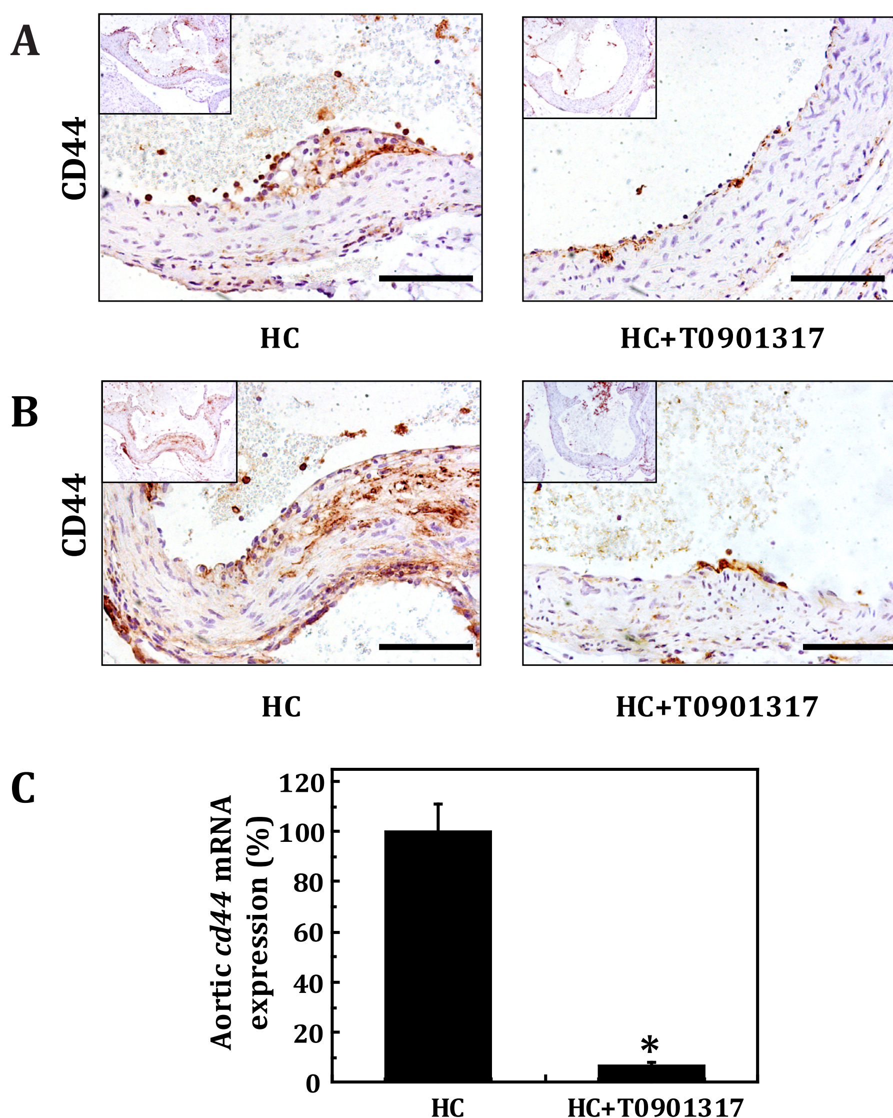


Figure 6. Effect of T0901317 on vascular CD44 expression. Representative photomicrographs of immunohistochemical staining of CD44-IR in cross-sections of the aortic root after treatment with HC or HC+T0901317 for 10 weeks (A) and 15 weeks (B). Bar= 100 µm. Shown is also aortic *cd44* mRNA expression after 10 weeks of HC or HC+T0901317 (C). *P<0.05 vs. HC.

Effect of T0901317 on pre-existing lesions

Considering the anti-inflammatory and anti-atherosclerotic activities of T0901317, the question arises whether T0901317 can also promote lesion regression. To address this question E3L mice were fed HC-diet for 18 weeks to induce moderately severe (type III-IV) lesions. One group of animals was then sacrificed (reference group) and

two others received a cholesterol-free regression diet (RD) for 8 weeks, supplemented (RD+T0901317) or not (RD) with T0901317. The switch from HC to RD resulted in a fall in total plasma cholesterol from 21.2±5.6 mM to 6.4±2.3 mM (P<0.05, Figure 7A) in the RD group, but no significant change in plasma cholesterol levels was seen in RD+T0901317 (20.7±2.4 mM). Lipoprotein distribution profile analysis (Figure 7B) shows that the decrease in plasma cholesterol in RD is mainly in the VLDL range and to a lesser extent in the IDL/LDL range, but not in the HDL fractions, whereas the profile in RD+T0901317 very much resembles that seen in HC+T0901317, with an increase of cholesterol in fractions 10-17, corresponding to particles in the IDL/LDL range. Whereas lesion area in RD (85924±31016 μm^2) was not significantly decreased as compared to the reference group (97203±16432 μm^2), it was markedly reduced (by 76%) in RD+T0901317 (20699±7995 μm^2; P<0.05; Figure 7C).

In both RD and RD+T0901317, a strong decrease in the plaque content of foam cells was observed: from 18040±3205 μm^2 in the reference group to 2876±315 μm^2 in RD (P<0.05) and 363±351 μm^2 in RD+T0901317 (P<0.05; Figure 7D). Similarly, a significant drop (P<0.05; Figure 7E) in monocyte adherence was seen in the two groups: from 23±5 monocytes per section (reference) to 16±4 (RD) and 10±2 (RD+T0901317) monocytes per section.

A number of molecular mechanisms underlying regression in mice have been proposed, including macrophage apoptosis,[21] CCR7-mediated migration of foam cells to lymph nodes[22,23] and reverse cholesterol transport.[24] RT-PCR gene expression analysis (Table 4; values normalized for the number of macrophages and reference mice set at 100%) revealed strongly increased expression of the apoptotic genes BCL-2, Caspase-3, BAX, and FAS in aortas from RD mice, and even further enhanced in RD+T0901317 mice. The presence of condensed nuclei in the lesions (Figure 7F) illustrates the occurrence of ongoing apoptosis in RD+T0901317 mice.

Recently, Trogan et al.[23] reported that the chemokine receptor CCR7 is expressed in foam cells and functionally required for regression. QRT-PCR analysis shows that CCR7 gene expression is significantly increased in RD (225%±47%; P<0.05) and even more so in
RD+T0901317 (861%±132%; P<0.01), indicating that CCR7 could also be a key factor in atherosclerosis regression in the present study. This is underlined by demonstrating enhanced CCR7-stained leukocytes accumulated in the adventitial space (Figure 7G).

Finally, we sought evidence supportive for increased reverse cholesterol efflux by measuring gene expression of cholesterol efflux pathway-related factors. QRT-PCR analysis of ApoE (87%±10%), ABCA1 (24%±3%; P<0.05) and ABCG1 (62%±6%; P<0.05) showed decreased gene expression in RD as compared with the reference group, while T0901317 strongly induced these genes under regression conditions (ApoE: 306%±20%; ABCA1: 149%±24% and ABCG1: 314%±29%; P<0.05, Table 4), even if the macrophage content in RD+T0901317 was stronger decreased than that in RD.

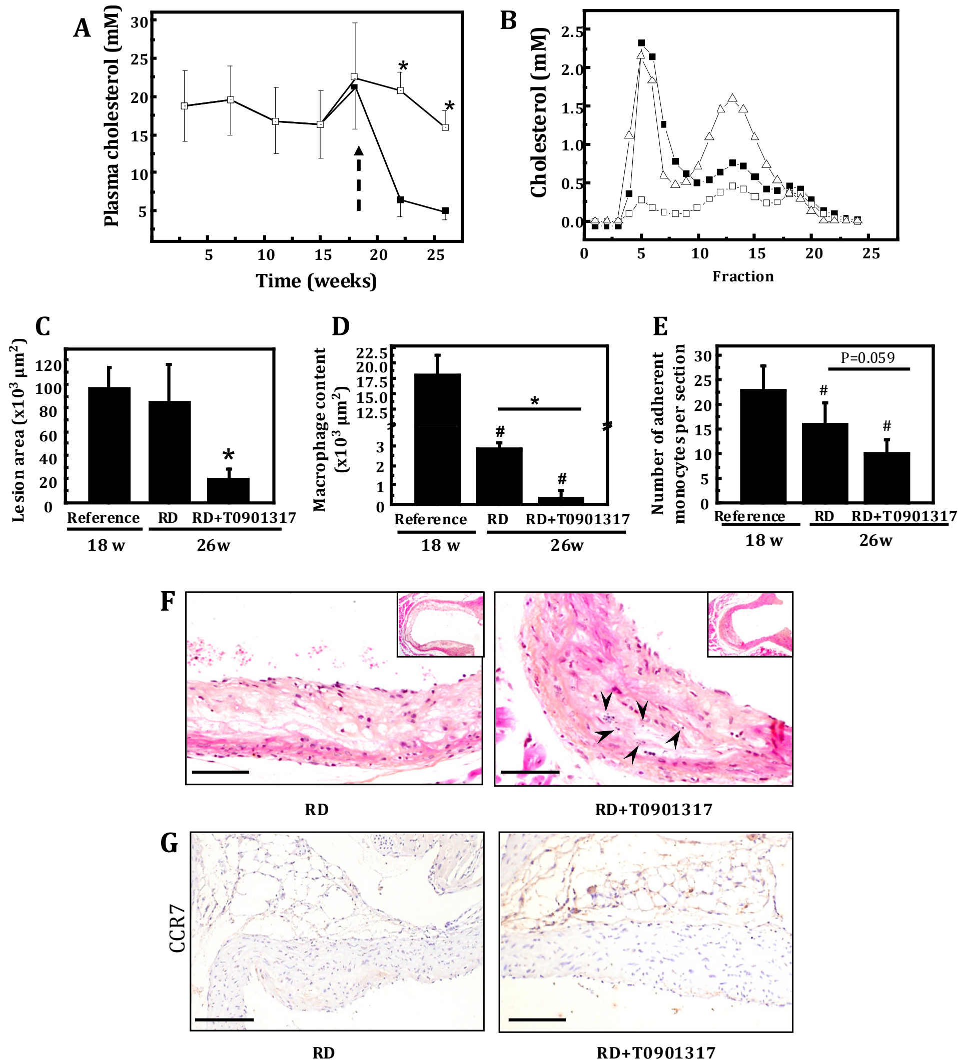


Figure 7: Effect of T0901317 on aspects of atherosclerotic lesion regression. Shown are (A) plasma cholesterol concentrations during regression period (arrow indicates start of RD feeding), with solid squares indicating RD treatment group and open squares indicating RD+T0901317 group. Data are represented as mean ± SD, *P<0.05 vs. RD; (B) Lipoprotein distribution profiles of the two experimental groups (n=8 for each group) after 8 weeks of RD (open squares) or RD+T0901317 (solid squares). For comparison, the lipoprotein profile of pooled plasma of the reference group (n=10; t= 18 weeks) is also shown (triangles); (C) total cross-sectional lesion area of reference, RD, and RD+T0901317 groups, with data representing mean values ± SEM (n=8 per group); (D) absolute macrophage containing lesion area, with data representing mean values ± SEM (n=8 mice per group); (E) number of monocytes adhering to endothelium, with data representing mean values ± SEM (n=8 mice per group); (F) Representative photomicrographs illustrating condensed nuclei (arrowheads), (G) Representative photomicrographs of immunohistochemical staining of adventitial CCR7-IR.

Table 4: Vascular gene expression of control and T0901317-treated E3L mice on a cholesterol-free, regressive diet (RD).

	t=18 weeks	Regression t=26 weeks	
	Reference	RD	RD+T0901317
fas	100% ± 4%	291% ± 11%*	2726% ± 148%*,#
bax	100% ± 6%	876% ± 87%*	3072% ± 374%*,#
bcl-2	100% ± 6%	897% ± 83%*	6162% ± 376%*,#
caspase-3	100% ± 5%	434% ± 34%*	2173% ± 386%*,#
ccr7	100% ± 16%	225% ± 47%*	861% ± 132%*,#
apoe	100% ± 11%	87% ± 10%	306% ± 20%*,#
abca1	100% ± 8%	24% ± 3%*	149% ± 24%*,#
abcg1	100% ± 7%	62% ± 6%*	314% ± 29%*,#

Vascular gene expression of control and T0901317-treated E3L mice on a cholesterol-free, regressive diet (RD). Shown are average aortic gene expression levels of cholesterol-efflux-related (*apoe, abca1,* and abcg1) and apoptosis/regression-related genes (*fas, bax, bcl-2, caspase-3,* and *ccr7*) *at* the end of the regression period (t= 26 weeks) relative to reference group which was set at 100%. Values are normalized for aortic monocyte/macrophage content (means ± SD; *P<0.05 vs. Reference and #P<0.05 vs. RD).

Discussion

We evaluated the effects of an LXR agonist, T0901317 on specific aspects of atherosclerotic lesion progression and regression in E3L mice, a model for atherosclerosis with predictive value for the human situation. In E3L, vascular LXRα and LXRβ expression was induced in aortic EC, SMC and macrophages under atherogenic dietary conditions. T0901317 strongly suppresses atherosclerotic lesion evolution and promotes lesion regression with respect to lesion number, area, and severity, despite elevated plasma total cholesterol and triglyceride levels. T0901317 exerts its actions at several levels. Under lesion-*progressive* conditions T0901317 suppresses dietary cholesterol-induced vascular expression of the inflammatory transcription factor, NF-κB, adhesion molecules (E-selectin, ICAM-1, and CD44), monocyte adhesion, and lesional macrophage content. T0901317 also induces the expression of the cholesterol efflux-related genes ApoE, ABCA1 and ABCG1, and blocks lesion evolution at the transition from stage II to III.

The ability of T0901317 to promote regression of preexisting atherosclerotic lesions under regressive conditions is linked mechanistically to i) increased cholesterol efflux from macrophages in the aortic lesions (through induction of expression of ApoE, ABCA1 and ABCG1); ii) reduction of macrophage content of the lesions by apoptosis; and iii) increased expression of the chemokine receptor CCR7, a factor functionally required for regression.

The T0901317-induced increase in plasma cholesterol and triglycerides in E3L

has also been described previously[25] and is similar to findings in ApoE$^{-/-}$ mice[26]. The T0901317-induced rise in cholesterol in E3L is confined to particles co-eluting with the IDL/LDL fraction and very much resembles findings described for fenofibrate-treated E3L mice[14]. The induced fraction contains mainly the apolipoprotein ApoE, to a minor extent ApoB, and no ApoA1. In contrast, the T0901317-induced increase in cholesterol in ApoE$^{-/-}$ was in HDL and accompanied by a marked increase in ApoA1, but not ApoB levels[26]. Interestingly, the apolipoprotein composition and size of the T0901317-induced particle in E3L is comparable to that induced by T0901317 in C57/Bl6 mice which was designated "enlarged HDL"[27]. The particle also shows the characteristics of HDL1, which has been suggested to be involved in ApoA1-independent cholesterol efflux, possibly sustained by ApoE[28,29].

Different effects of LXR agonists on cholesterol have been described in Ldlr$^{-/-}$ mice. In one report, T0901317 did not affect plasma total cholesterol levels in Ldlr$^{-/-}$ mice fed an atherogenic diet[8], and in other studies a decrease in plasma cholesterol levels was observed with LXR agonists[5,6].

A common finding in studies using T0901317 or other LXR ligands is the rise in triglyceride levels, which can be ascribed to the induction of the transcription factor SREBP-1 and its target genes FAS and SCD, two key enzymes involved in lipogenesis[3,4]. Noteworthy, T0901317 also is a high-affinity ligand for the xenobiotic receptor, pregnane–X-receptor (PXR). Because PXR and the LXRs are co-expressed in liver and PXR plays an important role in lipid metabolism, some of the hepatic effects of T0901317 may have resulted from simultaneous stimulation of LXR and PXR activation[30]. Importantly, vascular effects of T0901317 reported here most likely reflect LXR activation only since CD36, a PXR target [31] was not induced by T0901317 in E3L mice (data not shown).

Our finding that T0901317 strongly suppresses atherosclerotic lesion evolution in E3L mice is in line with previous gain-of-function and loss-of-function studies[7] and results obtained in ApoE$^{-/-}$ and Ldlr$^{-/-}$ mice with T0901317 and the LXR agonist GW3965[5,8]. Relevantly, the present study reveals several levels of anti-atherosclerotic action of T0901317. Firstly, a strong reduction of aortic inflammation as reflected by diminished vascular endothelial NF-κB activity, adhesion molecule expression (E-selectin, ICAM-1, and CD44), monocyte adhesion, and lesional macrophage content, all in the absence of systemic inflammation (cf. SAA). Secondly, T0901317 strongly increases gene expression of factors important for cholesterol efflux from vascular macrophages (ApoE, ABCA1, and ABCG1). ABCA1 mediates cholesterol efflux from macrophages to lipid-free ApoA1[32] whereas ABCG1 is a mediator of macrophage cholesterol efflux to mature HDL *in vitro*[33]. Up-regulation of ABCA1 and ABCG1 in the vasculature has previously been suggested to contribute to the anti-atherogenic capacity of T0901317[5,8]. Another LXR agonist, GW3965 was shown to increase aortic expression of ABCA1 and ABCG1 in ApoE$^{-/-}$ mice[5].

Thirdly, T0901317 blocked lesion progression from lesion severity stage II to III, characterized by the absence of SMC in the cap[15]. Proliferation and migration of SMC from the media towards the intima, a hallmark of type III lesions, is a key event in

lesion progression and regulated by CD44[34,35]. The detected down-regulation of CD44 by T0901317 in our study may thus provide an explanation for the observed blockage of lesion progression.

Atherosclerosis regression is an important clinical goal, but mouse studies on regression are relatively few. We observed that under dietary cholesterol-free ("regressive") conditions, there was a rapid loss of plaque foam cells from existing plaques, and this process was further enhanced by T0901317. In line with a previous study in E3L mice macrophage disappearance preceded reduction in plaque size under regressive conditions[11]. Remarkably, the effect of T0901317 on plaque regression occurred while plasma cholesterol level and lipoprotein profile were very similar to those found with T0901317 under progressive (HC) conditions. Also, monocyte adherence in the presence of T0901317 was not different between regressive and progressive conditions.

Three processes were identified that could be relevant for explaining the T0901317-stimulated disappearance of macrophages. First, the disappearance of macrophages could be the result of apoptosis. Indeed, we found evidence for increased number of apoptotic bodies with a parallel up-regulation of pro-apoptotic genes (Caspase-3, BAX, and FAS) in the aorta. Secondly, T0901317 enhances gene expression of reverse cholesterol transport markers, ApoE, ABCA1 and ABCG1. This is of relevance because it is thought that the elevated plasma membrane cholesterol content in foam cells inhibits cell migration[36]. Since plaque progression may result in part from long-term retention of foam cells in the plaque, promoting emigration of foam cells from atherosclerotic lesions through lowering cellular cholesterol content might lead to plaque regression. Thirdly, findings from a study by Trogan *et al*.[23] raised the possibility that, during regression, foam cells acquire dendritic cell (DC) characteristics that permit them to migrate to draining lymph nodes. This migration depends on the chemokine receptor CCR7, which might become up-regulated in foam cells in plaques undergoing regression. When CCR7 function was abrogated *in vivo* by treatment with antibodies to CCR7 ligands CCL19 and CCL21, lesion size and foam cell content were substantially preserved. In line with this, we found that CCR7 expression in the vasculature is located at the adventitial space and is increased 2-fold when regression is induced by RD and 8-fold by RD+T0901317, despite the decreases in macrophage content. Concomitantly, increases in hepatic CCL19 and CCL21 expression were observed (data not shown).

In summary, our findings show a comprehensive analysis of the beneficial effects of T0901317 on lesion evolution and regression in E3L mice and provide plausible mechanisms for its mode of action, including stimulation of cholesterol efflux-related genes and suppression of the inflammatory state. The data also revealed CCR7 induction in foam cells, a chemokine receptor reportedly functionally required for regression. LXR agonists could thus contribute in abrogating (pre-existing) atherosclerosis.

Acknowledgements

This study was supported by the Dutch Heart Foundation (NHS; grant 2002B102 to L.V.), the Center for Medical Systems Biology CMSB (grant 115 to J.d.V.v.d.W), and the Dutch Organization for Scientific Research (NWO; grant VENI 016. 036.061 to R.K.).

References

1. Braunwald E. Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. *N. Engl. J. Med.* 1997; 337: 1360-1369.
2. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002; 105: 1135-1143.
3. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B. Role of LXRs in control of lipogenesis. *Genes Dev.* 2000; 14: 2831-2838.
4. Zelcer N and Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J. Clin. Invest*. 2006; 116: 607-614.
5. Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc. Natl. Acad. Sci. U. S. A*. 2002; 99: 7604-7609.
6. Levin N, Bischoff ED, Daige CL, Thomas D, Vu CT, Heyman RA, Tangirala RK, Schulman IG. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25: 135-142.
7. Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc. Natl. Acad. Sci. U. S. A*. 2002; 99: 11896-11901.
8. Terasaka N, Hiroshima A, Koieyama T, Ubukata N, Morikawa Y, Nakai D, Inaba T. T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS Lett.* 2003; 536: 6-11.
9. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der GJ, van OB, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol.* 2007; 8: R200.
10. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1706-1721.
11. Gijbels MJ, van der CM, 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. Verschuren L, Kleemann R, Offerman EH, Szalai AJ, Emeis SJ, Princen HM, Kooistra T. Effect of low dose atorvastatin versus diet-induced cholesterol lowering on atherosclerotic lesion progression and inflammation in apolipoprotein E*3-Leiden transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25: 161-167.

13. Rein D, Schijlen E, Kooistra T, Herbers K, Verschuren L, Hall R, Sonnewald U, Bovy A, Kleemann R. Transgenic flavonoid tomato intake reduces C-reactive protein in human C-reactive protein transgenic mice more than wild-type tomato. *J. Nutr.* 2006; 136: 2331-2337.
14. Kooistra T, Verschuren L, de Vries-Van der Weij, Koenig W, Toet K, Princen HM, Kleemann R. Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple antiatherogenic effects besides lowering plasma cholesterol. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2322-2330.
15. Stary HC. Natural history and histological classification of atherosclerotic lesions: an update. *Arterioscler. Thromb. Vasc. Biol.* 2000; 20: 1177-1178.
16. Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat. Med.* 2007; 13: 587-596.
17. Kleemann R, Princen HM, Emeis JJ, Jukema JW, Fontijn RD, Horrevoets AJ, Kooistra T, Havekes LM. Rosuvastatin reduces atherosclerosis development beyond and independent of its plasma cholesterol-lowering effect in APOE*3-Leiden transgenic mice: evidence for antiinflammatory effects of rosuvastatin. *Circulation*. 2003; 108: 1368-1374.
18. Verschuren L, Lindeman JH, van Bockel JH, bdul-Hussien H, Kooistra T, Kleemann R. Up-regulation and coexpression of MIF and matrix metalloproteinases in human abdominal aortic aneurysms. *Antioxid. Redox. Signal.* 2005; 7: 1195-1202.
19. Zhao L, Hall JA, Levenkova N, Lee E, Middleton MK, Zukas AM, Rader DJ, Rux JJ, Pure E. CD44 regulates vascular gene expression in a proatherogenic environment. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 886-892.
20. Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Pure E. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J. Clin. Invest*. 2001; 108: 1031-1040.
21. Wang BY, Ho HK, Lin PS, Schwarzacher SP, Pollman MJ, Gibbons GH, Tsao PS, Cooke JP. Regression of atherosclerosis: role of nitric oxide and apoptosis. *Circulation*. 1999; 99: 1236-1241.
22. Llodra J, Angeli V, Liu J, Trogan E, Fisher EA, Randolph GJ. Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. *Proc. Natl. Acad. Sci. U. S. A*. 2004; 101: 11779-11784.
23. Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc. Natl. Acad. Sci. U. S. A*. 2006; 103: 3781-3786.
24. Cuchel M and Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation*. 2006; 113: 2548-2555.
25. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J. Biol. Chem.* 2002; 277: 34182-34190.
26. Dai X, Ou X, Hao X, Cao D, Tang Y, Hu Y, Li X, Tang C. Effect of T0901317 on hepatic proinflammatory gene expression in apoE-/- mice fed a high-fat/high-cholesterol diet.

Inflammation. 2007; 30: 105-117.

27. Jiang XC, Beyer TP, Li Z, Liu J, Quan W, Schmidt RJ, Zhang Y, Bensch WR, Eacho PI, Cao G. Enlargement of high density lipoprotein in mice via liver X receptor activation requires apolipoprotein E and is abolished by cholesteryl ester transfer protein expression. *J. Biol. Chem.* 2003; 278: 49072-49078.
28. Gruen ML, Plummer MR, Zhang W, Posey KA, Linton MF, Fazio S, Hasty AH. Persistence of high density lipoprotein particles in obese mice lacking apolipoprotein A-I. *J. Lipid Res.* 2005; 46: 2007-2014.
29. Vaisman BL, Lambert G, Amar M, Joyce C, Ito T, Shamburek RD, Cain WJ, Fruchart-Najib J, Neufeld ED, Remaley AT, Brewer HB, Jr., Santamarina-Fojo S. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J. Clin. Invest*. 2001; 108: 303-309.
30. Mitro N, Vargas L, Romeo R, Koder A, Saez E. T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS Lett.* 2007; 581: 1721-1726.
31. Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, Ren S, Evans RM, Xie W. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J. Biol. Chem.* 2006; 281: 15013-15020.
32. Wang N, Silver DL, Costet P, Tall AR. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J. Biol. Chem.* 2000; 275: 33053-33058.
33. Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. U. S. A*. 2004; 101: 9774-9779.
34. Jain M, He Q, Lee WS, Kashiki S, Foster LC, Tsai JC, Lee ME, Haber E. Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. *J. Clin. Invest*. 1996; 98: 877.
35. Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA, Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J. Exp. Med.* 1994; 180: 807-816.
36. Nagao T, Qin C, Grosheva I, Maxfield FR, Pierini LM. Elevated cholesterol levels in the plasma membranes of macrophages inhibit migration by disrupting RhoA regulation. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1596-1602.

Chapter 5

Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more pro-inflammatory lesions than atorvastatin

Jitske de Vries-van der Weij [1,4,*], Willeke de Haan [2,*],
José W.A. van der Hoorn [3,4], Thomas Gautier [5], Caroline C. van der Hoogt [2],
Marit Westerterp [2], Johannes A. Romijn [2], J. Wouter Jukema [3],
Louis M. Havekes [2,3,4], Hans M.G. Princen [4], Patrick C.N. Rensen [2]

* Both authors contributed equally

[1] Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
[2] Dept. of General Internal Medicine, Endocrinology, and Metabolic Disease,
Leiden University Medical Center, Leiden, The Netherlands
[3] Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands
[4] The Netherlands Organization for Applied Scientific Research (TNO)-Biosciences,
Gaubius Laboratory, Leiden, The Netherlands
[5] Center for Liver, Digestive, and Metabolic Diseases, Dept. of Pediatrics,
University Medical Center Groningen, Groningen, The Netherlands

Circulation 2008; 117: 2515-2522

Abstract

Background: Although CETP inhibition is regarded as a promising strategy to reduce atherosclerosis by increasing HDL-cholesterol, the CETP inhibitor torcetrapib given on top of atorvastatin had no effect on atherosclerosis and even increased cardiovascular death in the recent ILLUMINATE trial. Therefore, we evaluated the anti-atherogenic potential and adverse effects of torcetrapib in humanized *APOE*3-Leiden.CETP* (*E3L.CETP*) mice.

Methods and Results: *E3L.CETP* mice were fed a cholesterol-rich without drugs or with torcetrapib (12 mg/kg/day), atorvastatin (2.8 mg/kg/day) or both for 14 weeks. Torcetrapib decreased CETP activity both in the absence and presence of atorvastatin (-74% and -73% respectively, $P<0.001$). Torcetrapib decreased plasma cholesterol (-20%, $P<0.01$), albeit to a lesser extent than atorvastatin (-42%, $P<0.001$) or the combination of torcetrapib and atorvastatin (-40%, $P<0.001$). Torcetrapib increased HDL-cholesterol in the absence (+30%) and in the presence (+34%) of atorvastatin. Torcetrapib and atorvastatin alone both reduced atherosclerotic lesion size (-43% and -46%, $P<0.05$), but combination therapy did not reduce atherosclerosis as compared to atorvastatin alone. Remarkably, as compared to atorvastatin, torcetrapib induced enhanced monocyte recruitment and expression of monocyte chemoattractant protein-1 and resulted in lesions of a more inflammatory phenotype, as reflected by an increased macrophage content and reduced collagen content.

Conclusions: CETP inhibition by torcetrapib *per se* reduces atherosclerotic lesion size but does not enhance the anti-atherogenic potential of atorvastatin. However, as compared to atorvastatin, torcetrapib introduces lesions of a less stable phenotype.

Introduction

The cholesteryl ester transfer protein (CETP) is an important regulator of the HDL-C level. CETP is secreted predominantly by the liver and mainly associates with HDL in plasma, where it transports cholesteryl esters (CE) from HDL to (V)LDL in exchange for triglycerides,[1,2] and thus lowers HDL-C. HDL is atheroprotective as it mediates reverse cholesterol transport (i.e. transport of cholesterol from the vessel wall to the liver) and it has anti-inflammatory, anti-thrombotic and anti-oxidative properties.[3,4] Therefore, CETP inhibition is regarded as a promising strategy to increase HDL-C levels and to reduce atherosclerosis.[1] However, the effect of CETP activity on atherosclerosis in humans has not been unequivocally determined. Mutations in the CETP gene that reduce CETP mass and activity (e.g. D442G and Int14 G(+1)>A) lead to elevated HDL-C levels,[5,6] but the effects of these mutations on atherosclerosis are still in dispute.[7-10]
Torcetrapib, which forms an inactive complex between CETP and HDL[1] has been the first CETP inhibitor tested in large human trials, in which it was shown to increase HDL-C levels by approx. 60%.[11-13] The resulting HDL particles were able to mediate cellular cholesterol efflux more efficiently.[14] However, the large scale ILLUMINATE trial was stopped prematurely because of an excess of deaths in patients receiving torcetrapib with atorvastatin as compared to those receiving atorvastatin alone, mainly related to cardiovascular events.[15] In addition, the RADIANCE and ILLUSTRATE trials revealed no therapeutic benefit of combining torcetrapib with atorvastatin with respect to atherosclerosis progression as assessed by coronary intima-media thickness (IMT) and intravascular ultrasonography (IVUS) measurements.[11-13]
The effect of torcetrapib alone on atherosclerosis, however, has not yet been evaluated in humans, and the mechanism underlying the increased death rate associated with torcetrapib treatment has not been elucidated as yet. Therefore, we now examined the effect of torcetrapib with or without atorvastatin on atherosclerosis development in humanized *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice.[16] *E3L* mice show a human-like response to lipid-lowering therapies.[17] Cross-breeding with *CETP* transgenic mice, which express human CETP under control of its natural flanking regions, resulted in *E3L.CETP* mice that also respond to HDL-modulating intervention.[18,19]

Methods

Animals
Human CETP transgenic mice which express CETP under control of its natural flanking regions (strain 5203)[20] were obtained from Jackson laboratories (Bar Harbor, MC) and crossbred with *E3L* mice[21] to obtain *E3L.CETP* mice.[16] All mice used in this study were heterozygous *E3L.CETP* transgenic females on a C57Bl/6 background. Mice were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water unless indicated otherwise. Mice were fed regular chow (Ssniff, Soest, Germany) or a diet with 15% (w/w) cacao butter (diet T, Hope Farms, Woerden, the Netherlands)

supplemented with 0.1% or 0.25% (w/w) cholesterol (Sigma) with or without torcetrapib (2R,4S)-4-[[[3,5bis(trifluoromethyl) phenyl]methyl]-(methoxycarbonyl) amino]-2-ethyl-3,4-dihydro-6-trifluoromethyl)-3-phenyl-1(2H)-quinolinecarboxylic acid, ethyl ester ($C_{26}H_{25}N_2O_4F_9$), (kindly provided by Roche, Basel, Switzerland) and/or atorvastatin ([R-(R*,R*)]-2-(4-fluorophenyl)-beta,delta-dihydroxy-5-(1-methylethyl)-3-phenyl-4[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid ($C_{33}H_{24}FN_2O_5$) (Lipitor). Unless indicated otherwise, blood was drawn after 4 h fasting in EDTA-containing cups by tail bleeding and plasma was isolated. All animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Single Torcetrapib Treatment
To verify that torcetrapib inhibits CETP activity in *E3L.CETP* mice *in vivo*, mice on a chow diet were given a single intragastric gavage of torcetrapib (0, 1, 3 and 10 mg/kg) in approx. 200 µL of ethanol: solutol: saline 10:10:80 (v:v:v). Blood was drawn before gavage and at 1, 2, 4, 6, 8 and 24 h after gavage. During the first 8 h after the gavage mice were fasted. Plasma was assayed for total CETP activity as described below. Alternatively, mice were fed a diet containing 15% cacao butter with 0.1% or 0.25% cholesterol, and the effect of 10 mg/kg torcetrapib was determined on plasma CETP activity at 2 h after gavage.

Total Plasma CETP Activity, Endogenous CETP Activity and CETP Mass
Total plasma CETP activity was measured as the transfer of [^{3}H]cholesteryl oleate (CO) from LDL to HDL.[16] Briefly, 5 µL (diluted) mouse plasma was incubated with human [^{3}H]CO-labeled LDL and HDL in sodium phosphate buffer containing 5,5'-dithio-bis(2-nitrobenzoic acid) to inhibit lecithin-cholesterol acyltransferase (LCAT) activity. After overnight incubation, LDL was precipitated. The supernatant containing [^{3}H]CO-HDL was counted for ^{3}H activity. CETP activity was calculated as nmol CE transfer/ mL plasma/ h. Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole (NBD)-labeled cholesteryl esters (RB-CETP, Roar Biomedical, NY, USA), as described.[22] CETP mass was determined using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi, Tokyo, Japan).

Long-term Torcetrapib Treatment
To determine the effect of torcetrapib without and with atorvastatin on atherosclerosis development and plasma cholesterol, *E3L.CETP* mice were fed a diet containing 0.25% cholesterol to increase plasma cholesterol levels to ~16 mM. After 4 weeks, mice were randomized into four groups according to their plasma cholesterol levels. Mice were fed a control diet, a diet with atorvastatin (0.0023% ~ 2.8 mg/kg/day), torcetrapib (0.01% ~ 12 mg/kg/day) or both. Blood was drawn one week before randomization and at week 6, 9 and 14 of drug treatment, and was assayed for lipids, CETP mass and activity. After 14 weeks, mice were euthanized and atherosclerosis development was assessed

as described below.

Plasma Lipids and Lipoprotein Profiles
Plasma was assayed for cholesterol and phospholipids (PL) using commercially available enzymatic kits according to the manufacturer's protocols (236691, Roche Molecular Biochemicals, Indianapolis IN, USA, and phospholipids B Wako Chemicals, Neuss, Germany, respectively). To determine the lipid distribution over plasma lipoproteins, lipoproteins were separated using fast protein liquid chromatography (FPLC). Plasma was pooled per group and 50 μL of each pool was injected onto a Superose 6 HR 10/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 μL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 μL were collected and assayed for cholesterol and PL as described above.

Atherosclerosis Quantification
After 14 weeks of drug intervention, mice were sacrificed by CO_2 inhalation. Blood was drawn via cardiac puncture and hearts were isolated. Hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin and were cross-sectioned (5 μm) throughout the aortic root area. Per mouse 4 sections with 50 μm intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxin-saffron (HPS) for histological analysis. Lesions were categorized for severity according to the American Heart system adapted for mice.[23,24] Various types of lesions were discerned: type 0 (no lesions), type 1-3 (early fatty streak-like lesions containing foam cells) and type 4-5 (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization and/or necrosis). Lesion area was determined using Leica Qwin image analysis software (EIS, Asbury NJ). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY) was used to quantify the macrophage area and the number of monocytes adhering to the endothelium. Sirius Red was used to quantify the collagen area, and the antibody M0851 (1:800, DAKO) against smooth muscle cell actin to quantify the smooth muscle cell area. Monocyte chemoattractant protein-1 (MCP-1) was detected using goat anti-mouse MCP-1 (M18, 1:300; Santa Cruz Biotechnology, Santa Cruz, Calif).

Statistical Analysis
Data are presented as means ± SD unless indicated otherwise. Statistical differences were assessed using the Mann Whitney U test. For lesion typing, differences were assessed by the Chi Square test. SPSS 14.0 was used for statistical analysis. Values of $P<0.05$ was regarded as statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Torcetrapib Inhibits CETP Activity in E3L.CETP mice

To verify that *E3L.CETP* mice appropriately respond to CETP inhibition, *E3L.CETP* mice on a chow diet received an oral garage of torcetrapib (1, 3 and 10 mg/kg) or vehicle. As expected, torcetrapib time- and dose-dependently reduced plasma CETP activity, reaching a minimum at 2 h after gavage (-59±8%, -83±4%, and -96±4%; *P*<0.01). At 3 and 10 mg/kg, significant reductions were still observed after 8 h (-45±25% and -45±17% respectively; *P*<0.01) (Fig. 1A). Because cholesterol-feeding of *E3L.CETP* mice increases plasma CETP mass and activity,[16] we next measured the inhibitory capacity of torcetrapib on plasma CETP activity in mice fed a diet without or with 0.1% (w/w) or 0.25% (w/w) cholesterol, which increased plasma CETP activities (3.4-fold and 4.3- fold, respectively). Despite the increase in plasma CETP activity, an oral gavage of torcetrapib (10 mg/kg) still profoundly decreased CETP activity in the presence of 0.1% (-64±11%; *P*<0.05) and 0.25% (-59±13%; *P*<0.05) cholesterol in the diet (Fig. 1B).

Torcetrapib Reduces Plasma Cholesterol Levels to a Lesser Extent than Atorvastatin

To determine the effect of torcetrapib on plasma lipid levels in the absence or presence of atorvastatin, *E3L.CETP* mice were fed a diet containing 0.25% (w/ w) cholesterol without or with torcetrapib and/or atorvastatin. Addition of torcetrapib, atorvastatin or both to the diet did not affect food intake or body weights of *E3L.CETP* mice (not shown). The cholesterol-rich diet resulted in a plasma cholesterol level of 16.1±3.5 mM in the control group. Torcetrapib decreased plasma cholesterol (-20%; *P*<0.01)

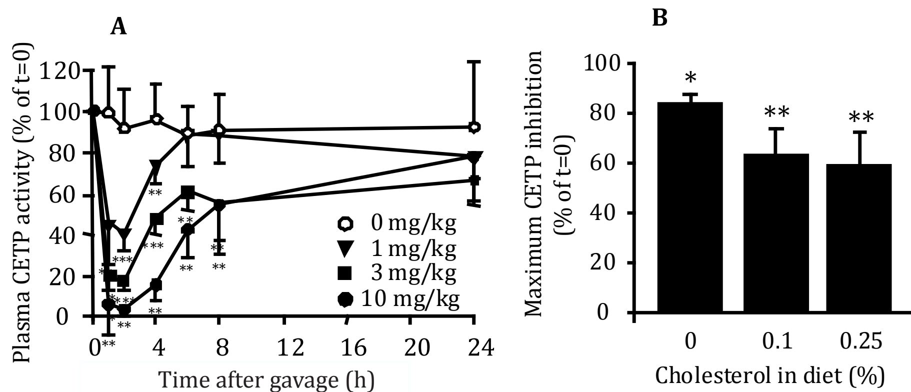


Figure 1. A single dose of torcetrapib inhibits CETP *in vivo*. *E3L.CETP* mice fed a chow diet received the indicated amounts of torcetrapib via intragastric gavage. Blood was drawn at the indicated time points and plasma was assayed for CETP activity (A). *E3L.CETP* mice, fed a chow diet or a diet containing 0.1% and 0.25% cholesterol, received torcetrapib (10 mg/kg) by intragastric gavage and total CETP activity was measured 2 h after gavage (B). Values are means ± SD (n=4-6); *P<0.05, **P<0.01, ***P<0.001 as compared to the control group.

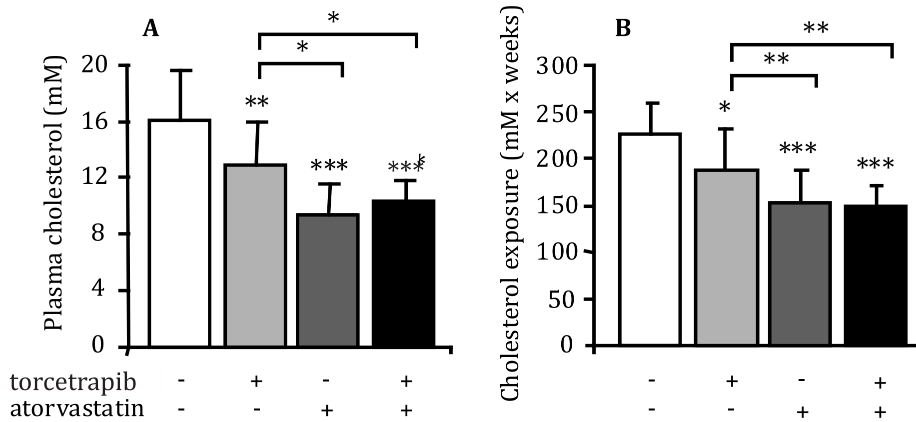


Figure 2. Torcetrapib reduces plasma cholesterol to a lesser extent than atorvastatin. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 9 weeks of drug intervention, blood was drawn and plasma was assayed for cholesterol (A). Blood was drawn at additional time points (0, 6, 9, and 14 weeks) and TC was measured. Total cholesterol exposure during the study was calculated (B). Values are means ± SD (n=14-15); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

to a lesser extent as compared to atorvastatin (-42%; *P*<0.001). The combination of torcetrapib and atorvastatin did not decrease plasma cholesterol further as compared to atorvastatin alone (-40% vs -42%) (Fig. 2A). Since torcetrapib and atorvastatin consistently lowered plasma cholesterol throughout the study, they similarly decreased total cholesterol exposure (Fig. 2B). Thus, torcetrapib alone reduced total cholesterol exposure to a lower extent as compared to atorvastatin and combination therapy (Fig. 2B).

To determine the distribution of lipids over lipoproteins, lipoproteins were fractionated by FPLC and cholesterol and PL were measured in the individual fractions (Fig. 3). Torcetrapib reduced (V)LDL-C (-26%) (Fig. 3A) to a lesser extent than atorvastatin (-42%) (Fig. 3A and 3B), and torcetrapib did not enhance the (V)LDL-C reducing effect of atorvastatin (Fig. 3B). In addition, torcetrapib increased plasma HDL-C levels by +30% in the absence of atorvastatin (Fig. 3A) and by +34% in the presence of atorvastatin, as judged from the cholesterol content of the FPLC fractions 17-22 (Fig. 3B). This torcetrapib-induced increase in HDL-C was paralleled by an increase in PL in the HDL fractions (Fig. 3C and 3D). Despite these increased HDL-C levels, apoAI levels were not altered by torcetrapib treatment (not shown).

Torcetrapib Reduces CETP Activity and Increases CETP Mass Whereas Atorvastatin Decreases Both CETP Activity and Mass

Torcetrapib decreased CETP activity efficiently both in the absence (-73%; *P*<0.001) and presence of atorvastatin (-74%; *P*<0.001) (Fig. 4A). Atorvastatin alone also decreased CETP activity, but to a lesser extent (-32%; *P*<0.001). Despite the decreased

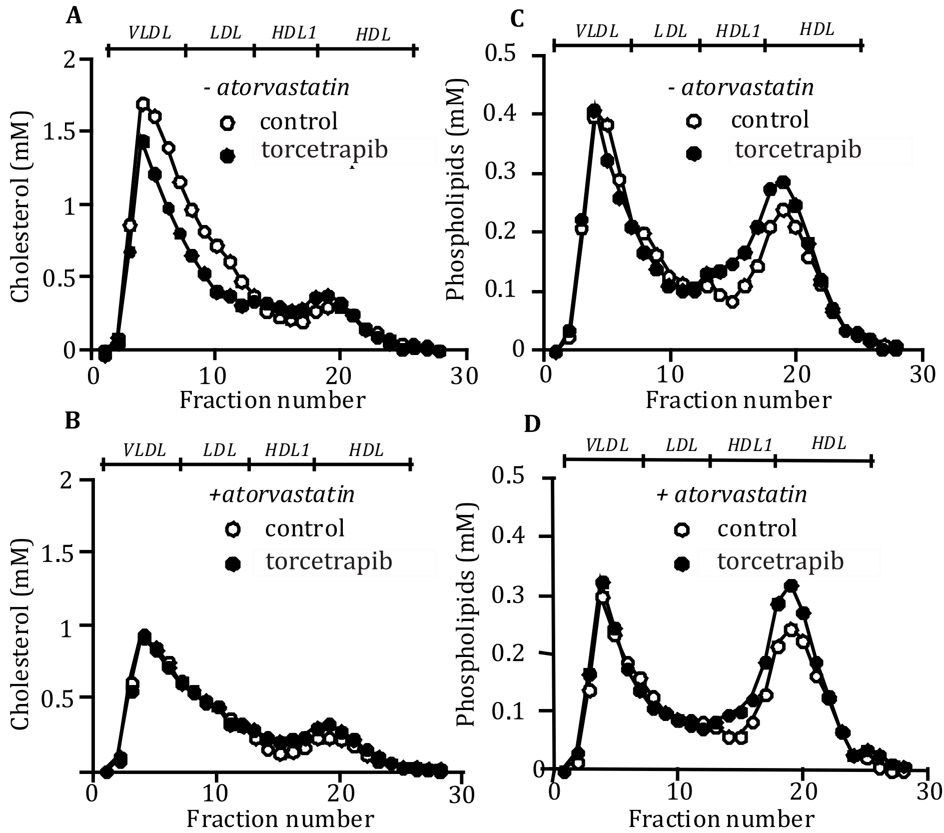


Figure 3. Torcetrapib reduces plasma VLDL and increases HDL levels. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 14 weeks of drug intervention, blood was drawn and plasma was pooled per treatment group (n=14-15). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for total cholesterol (A, B) and phospholipid (C, D).

CETP activity, torcetrapib treatment increased CETP mass (+33%; $P<0.001$). On the contrary, atorvastatin decreased CETP mass (-24%; $P<0.001$), whereas the combination therapy did not significantly affect CETP mass as compared to untreated mice (Fig. 4B). These data are in line with previous observations that torcetrapib increases CETP mass in humans despite the decrease in CETP activity[25] and that atorvastatin decreases CETP levels[26,27] by decreasing CETP expression.[18]

Torcetrapib Reduces Atherosclerotic Lesion Severity and Lesion Area but Does Not Enhance the Anti-Atherogenic Effect of Atorvastatin

To determine the effect of torcetrapib on atherosclerosis development in the absence or in the presence of atorvastatin, the 4 groups of mice were euthanized after 14 weeks and atherosclerosis severity and lesion size were measured in the aortic root. Representative

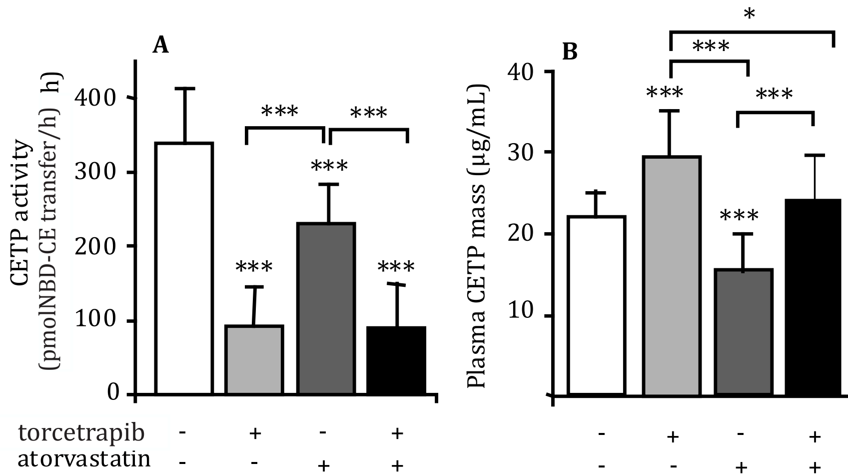
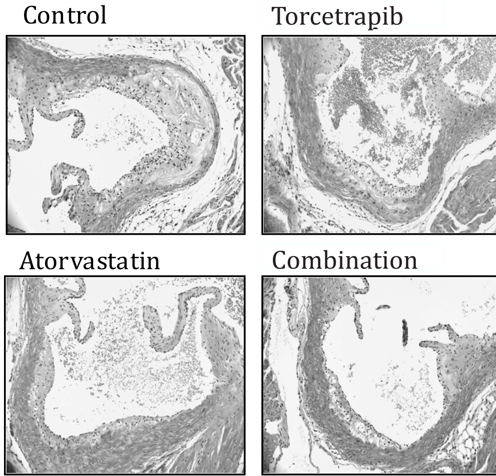


Figure 4. **Torcetrapib reduces plasma CETP activity and increases CETP mass.** Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 9 weeks of drug intervention, blood was drawn and plasma was assayed endogenous CETP activity (A) and CETP mass (B). Values are means ± SD (n=14-15); *P<0.05, ***P<0.001 vs the control group.

pictures of each group are shown in Fig. 5A. As compared to the control group, mice treated with torcetrapib, atorvastatin or both had more lesion-free sections and fewer severe lesions of type 4 to 5. Thus, torcetrapib, atorvastatin and the combination of both reduced lesion severity similarly (Fig. 5B). Accordingly,
torcetrapib and atorvastatin alone induced a similar reduction in lesion area (-43%
torcetrapib and atorvastatin alone induced a similar reduction in lesion area (-43% and -46% respectively; *P*<0.05). Combination treatment also reduced atherosclerosis as compared to the control group (-60%; *P*<0.001), but did not significantly enhance the atherosclerosis-reducing potency of atorvastatin alone (Fig. 5C).

Torcetrapib Induces Monocyte Recruitment and Results in a More Pro-Inflammatory Lesion Phenotype as Compared to Atorvastatin

We next evaluated the effect of torcetrapib, atorvastatin and the combination of both on monocyte recruitment and lesion composition with respect to the macrophage area, smooth muscle cell area and collagen area. Torcetrapib alone and in combination with atorvastatin increased the adherence of monocytes to the vessel wall as compared to the control and atorvastatin- treated group (Fig 6A). Although torcetrapib did not significantly raise MCP-1 as compared to the control group, torcetrapib significantly increased MCP-1 as compared to atorvastatin (+99%; P<0.05) (Fig. 6B). The increase in adhering monocytes as induced by torcetrapib was accompanied by an increased area of macrophages in the intima (Fig. 6C). Although torcetrapib did not appear to affect the smooth muscle cell content (Fig. 6D), torcetrapib alone and in combination with atorvastatin tended to decrease the area of collagen (*P*=0.14 and *P*=0.13, resp.)



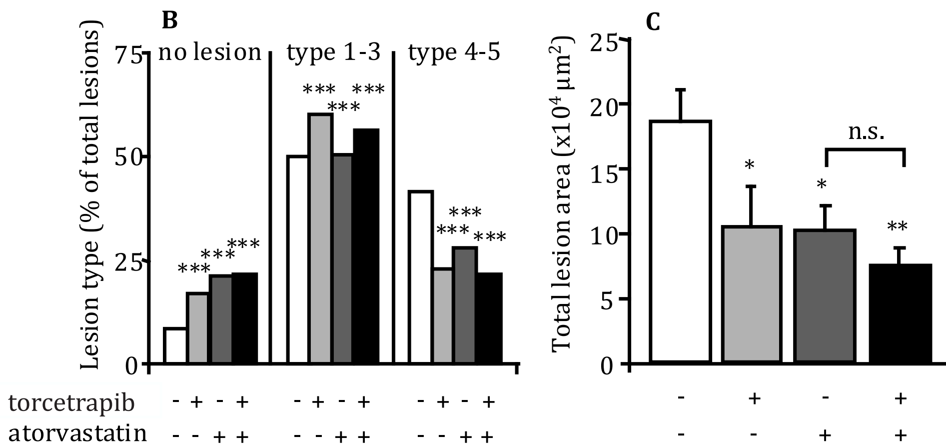


Figure 5. Torcetrapib reduces atherosclerosis development but does not enhance the atherosclerosis reducing effect of atorvastatin. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 14 weeks of drug intervention, hearts were isolated, fixed, dehydrated and embedded in paraffin and representative HPS stained pictures of each group are shown (A). Four sections per mouse with 50 μm intervals were typed and categorized according to lesion severity (B), and total lesion area was calculated (C). Values are means ± SEM (n=14-15) *P<0.05, **P<0.01, ***P<0.001 as compared to the control group.

(Fig. 6E). Thus, whereas atorvastatin reduces lesion size and *P*=0.13, resp.) (Fig. 6E). Thus, whereas atorvastatin reduces lesion size without affecting lesion composition as compared to untreated mice, torcetrapib reduces lesion size accompanied by a more pro-inflammatory torcetrapib reduces lesion size accompanied by a more pro-inflammatory lesion phenotype, reflected by an increased macrophage-to-collagen ratio, as compared to control-treated mice (+75%) and atorvastatin-treated mice (+67%).

Discussion

Torcetrapib has been shown to markedly raise HDL-C and was, therefore, expected to reduce atherosclerosis in humans. Despite this, the recent RADIANCE, ILLUSTRATE AND ILLUMINATE trials have concluded that torcetrapib was ineffective in reducing

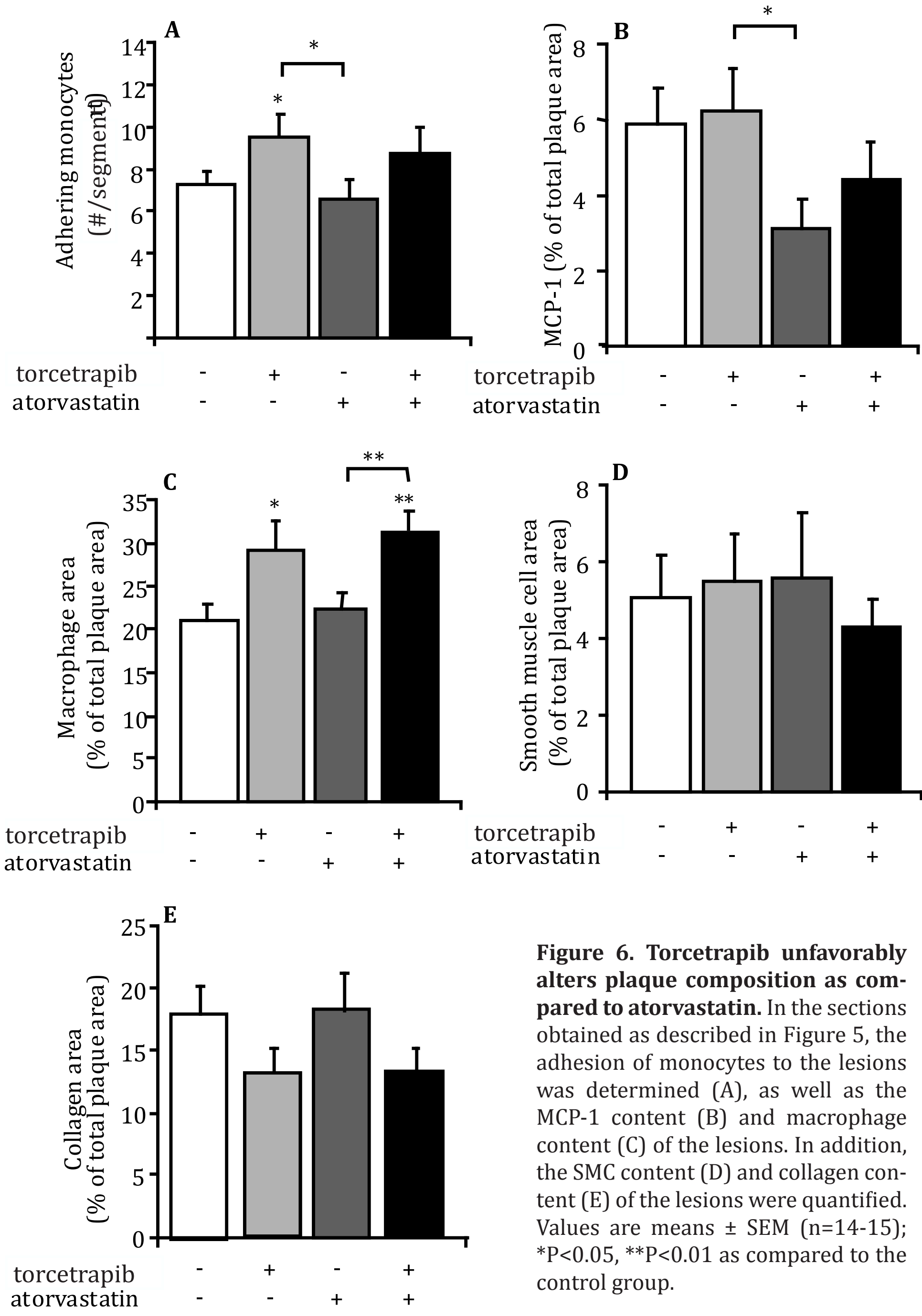


Figure 6. Torcetrapib unfavorably alters plaque composition as compared to atorvastatin. In the sections obtained as described in Figure 5, the adhesion of monocytes to the lesions was determined (A), as well as the MCP-1 content (B) and macrophage content (C) of the lesions. In addition, the SMC content (D) and collagen content (E) of the lesions were quantified. Values are means ± SEM (n=14-15); *P<0.05, **P<0.01 as compared to the control group.

atherosclerosis[11-13] and increased clinical event rate.[15] However, it should be realized that the effectiveness of torcetrapib has only been assessed in dyslipidemic patients who also received atorvastatin. Therefore, in the present study we examined the effect of torcetrapib *per se* on atherosclerosis development. In our study we show that torcetrapib alone reduces the progression of atherosclerosis, but does not enhance the anti-atherosclerotic potency of atorvastatin and that torcetrapib results in a more pro-inflammatory lesion phenotype as compared to atorvastatin.

Torcetrapib reduced total cholesterol exposure to a lesser extent (-17%) as compared to atorvastatin (-41%), whereas torcetrapib and atorvastatin equally reduced atherosclerotic lesion size (both ~-45%). Previous diet-induced atherosclerosis studies in mice have consistently demonstrated that atherosclerotic lesion area could generally be reliably predicted from cholesterol exposure (H.M.G. Princen PhD and P.C.N. Rensen PhD, unpublished data, 2007). Therefore, torcetrapib decreased atherosclerosis development more drastically than could be expected based merely on the observed reduction in cholesterol exposure. Since torcetrapib treatment results in increased HDL levels, it is likely that HDL is involved in the atheroprotective effect of torcetrapib. In line with this hypothesis, we have observed previously that *E3L.CETP* mice show a 7-fold increased atherosclerotic lesion area as compared to *E3L* only mice, which was much more than could be expected based on a modest increase in total plasma cholesterol *per se*. In fact, we showed that plasma from *E3L.CETP* mice was less effective in mediating SR-BI-dependent cholesterol efflux than plasma from *E3L* mice, as accompanied by a large reduction in HDL-1.[16] In the present study, we did not detect an effect of torcetrapib on either SR-BI or ABCA1-mediated cholesterol efflux (not shown), possibly related to the relatively mild effect of torcetrapib on the HDL level as compared to total CETP deficiency. We therefore speculate that effects of torcetrapib on other properties of HDL, including its anti-inflammatory, anti-oxidative and/or anti-thrombotic properties may have resulted in the more prominent reduction in atherosclerotic lesion size than could be expected merely on the basis of a reduction in total cholesterol.

The fact that torcetrapib alone reduced atherosclerosis development is in line with a previous study showing that torcetrapib treatment alone reduces atherosclerosis in rabbits.[28] However, we also show that torcetrapib did not significantly enhance the anti-atherogenic potential of atorvastatin. We have evaluated the effects of torcetrapib and atorvastatin in E3L.CETP mice with a relatively high plasma cholesterol level of approx. 16 mM, to avoid the possibility that the combined cholesterol-lowering actions of atorvastatin and torcetrapib would result in a plasma cholesterol level below that required for atherosclerosis development in E3L.CETP mice (*i.e.* 6-8 mM). Despite this limitation, torcetrapib *per se* (*i.e.* without concomitant administration of atorvastatin) may thus have an anti-atherosclerotic effect in humans as well.

From the recent clinical trials, it has become clear that torcetrapib has several adverse effects. The ILLUMINATE trial showed that torcetrapib elevated blood pressure, increased cardiovascular events and increased death rate, mainly related to cardiovascular causes.[15] However, the mechanisms underlying these unexpected adverse effects have

not completely been elucidated yet. In the present study, we did not detect a significant effect of torcetrapib on blood pressure, probably because of small experimental groups (data not shown). However, compared with atorvastatin, torcetrapib enhanced monocyte adherence to the vessel wall, enhanced vascular MCP-1 expression, and increased the macrophage area within the lesions. Torcetrapib thus appears to enhance the recruitment of monocytes to the endothelium and transmigration of the monocytes into the intima resulting in an enhanced macrophage content of the plaque, compared with similarly sized lesions resulting from atorvastatin treatment. The observation that torcetrapib tended to reduce the collagen content of the plaque independent of the smooth muscle cell content can be explained by induction of collagen breakdown by macrophages, (e.g., via secretion of metalloproteinases). Although plaque rupture is a rare phenomenon in mice, such inflammatory lesions with a high macrophage to collagen ratio are more unstable and may well have caused an increased incidence of plaque rupture in humans, thereby explaining increased cardiovascular death. It would be interesting to evaluate in future studies whether these effects of torcetrapib are compound-specific or related to its effect on lipoprotein metabolism, by comparison with other CETP inhibitors that are currently under development (e.g. JTT-705 and anacetrapib).

Interestingly, recent data from the ILLUMINATE trial indicate that torcetrapib increased plasma aldosterone levels via an as yet unknown mechanism.[15] In addition to increasing blood pressure,[29] aldosterone increases atherosclerosis development in mice.[30-32] This is related to its pro-inflammatory properties including increased MCP-1 expression, increased monocyte infiltration into the coronary artery, increased lipid loading of macrophages, and increased expression of matrix metalloproteinases.[29,30] Preliminary data on aldosterone levels in pooled plasma of the various mouse groups indicated that the average aldosterone level is higher in the torcetrapib-treated group (+15%) and combination-treated group (+48%) than in the atorvastatin-treated group. This suggests that the torcetrapib-induced increase in aldosterone levels may causally increase the inflammatory plaque phenotype in mice.

In conclusion, torcetrapib inhibits the progression of atherosclerosis, but does not enhance the anti-atherosclerotic potency of atorvastatin. In addition, as compared to atorvastatin, torcetrapib causes a more pro-inflammatory and unstable lesion phenotype.

Acknowledgments

We thank Dr. C. Maugeais (Hoffman La Roche, Basel, Switzerland) for scientific discussion and E.H. Offerman, C.M. van den Hoogen and A. van Nieuwkoop (TNO-Biosciences, Leiden, The Netherlands) for their excellent technical assistance. This work was performed in the framework of the Leiden Center for Cardiovascular Research LUMC-TNO, and supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.), the Centre for Medical Systems Biology (CMSB project 115) in

the framework of the Netherlands Genomics Initiative (NGI) and the Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.). J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (NHS grant 2001D032).

References

1. Barter PJ and Kastelein JJ. Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease. *J. Am. Coll. Cardiol.* 2006; 47: 492-499.
2. Ha YC, Calvert GD, Barter PJ. In vivo metabolism of esterified cholesterol and apoproteins in rabbit plasma low density lipoproteins. *Atherosclerosis*. 1979; 34: 451-455.
3. Barter P, Gotto AM, LaRosa JC, Maroni J, Szarek M, Grundy SM, Kastelein JJ, Bittner V, Fruchart JC. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *N. Engl. J. Med.* 2007; 357: 1301-1310.
4. Chapman MJ. Therapeutic elevation of HDL-cholesterol to prevent atherosclerosis and coronary heart disease. *Pharmacol. Ther.* 2006; 111: 893-908.
5. Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, . Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J. Clin. Invest*. 1994; 94: 1872-1882.
6. Takahashi K, Jiang XC, Sakai N, Yamashita S, Hirano K, Bujo H, Yamazaki H, Kusunoki J, Miura T, Kussie P, . A missense mutation in the cholesteryl ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. *J. Clin. Invest*. 1993; 92: 2060-2064.
7. Boekholdt SM, Kuivenhoven JA, Wareham NJ, Peters RJ, Jukema JW, Luben R, Bingham SA, Day NE, Kastelein JJ, Khaw KT. Plasma levels of cholesteryl ester transfer protein and the risk of future coronary artery disease in apparently healthy men and women: the prospective EPIC (European Prospective Investigation into Cancer and nutrition)-Norfolk population study. *Circulation*. 2004; 110: 1418-1423.
8. Kuivenhoven JA, Jukema JW, Zwinderman AH, de KP, McPherson R, Bruschke AV, Lie KI, Kastelein JJ. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. *N. Engl. J. Med.* 1998; 338: 86-93.
9. Moriyama Y, Okamura T, Inazu A, Doi M, Iso H, Mouri Y, Ishikawa Y, Suzuki H, Iida M, Koizumi J, Mabuchi H, Komachi Y. A low prevalence of coronary heart disease among subjects with increased high-density lipoprotein cholesterol levels, including those with plasma cholesteryl ester transfer protein deficiency. *Prev. Med.* 1998; 27: 659-667.
10. Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR. Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. *J. Clin. Invest*. 1996; 97: 2917-2923.
11. Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial. *Lancet*. 2007; 370: 153-160.
12. Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. *N. Engl. J. Med.* 2007; 356: 1620-1630.

13. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. *N. Engl. J. Med.* 2007; 356: 1304-1316.
14. Yvan-Charvet L, Matsuura F, Wang N, Bamberger MJ, Nguyen T, Rinninger F, Jiang XC, Shear CL, Tall AR. Inhibition of cholesteryl ester transfer protein by torcetrapib modestly increases macrophage cholesterol efflux to HDL. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1132-1138.
15. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *N. Engl. J. Med.* 2007; 357: 2109-2122.
16. Westerterp M, van der Hoogt CC, de HW, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2552-2559.
17. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij, van der HJ, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1706-1721.
18. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM, Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008; 197: 57-63.
19. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM, Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *J. Lipid Res.* 2007; 48: 1763-1771.
20. Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. *J. Clin. Invest.* 1992; 90: 1290-1295.
21. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de B, I, van VB, van der BH, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. *J. Biol. Chem.* 1993; 268: 10540-10545.
22. Gautier T, Tietge UJ, Boverhof R, Perton FG, Le GN, Masson D, Rensen PC, Havekes LM, Lagrost L, Kuipers F. Hepatic lipid accumulation in apolipoprotein C-I-deficient mice is potentiated by cholesteryl ester transfer protein. *J. Lipid Res.* 2007; 48: 30-40.
23. Gijbels MJ, van der CM, 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. 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.
25. Clark RW, Sutfin TA, Ruggeri RB, Willauer AT, Sugarman ED, Magnus-Aryitey G, Cosgrove PG, Sand TM, Wester RT, Williams JA, Perlman ME, Bamberger MJ. Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. *Arterioscler. Thromb. Vasc. Biol.* 2004; 24: 490-497.
26. Guerin M, Lassel TS, Le GW, Farnier M, Chapman MJ. Action of atorvastatin in combined hyperlipidemia : preferential reduction of cholesteryl ester transfer from HDL to VLDL1

particles. *Arterioscler. Thromb. Vasc. Biol.* 2000; 20: 189-197.
27. van Venrooij FV, Stolk RP, Banga JD, Sijmonsma TP, van TA, Erkelens DW, linga-Thie GM. Common cholesteryl ester transfer protein gene polymorphisms and the effect of atorvastatin therapy in type 2 diabetes. *Diabetes Care*. 2003; 26: 1216-1223.
28. Morehouse LA, Sugarman ED, Bourassa PA, Sand TM, Zimetti F, Gao F, Rothblat GH, Milici AJ. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *J. Lipid Res.* 2007; 48: 1263-1272.
29. Hirono Y, Yoshimoto T, Suzuki N, Sugiyama T, Sakurada M, Takai S, Kobayashi N, Shichiri M, Hirata Y. Angiotensin II receptor type 1-mediated vascular oxidative stress and proinflammatory gene expression in aldosterone-induced hypertension: the possible role of local renin-angiotensin system. *Endocrinology*. 2007; 148: 1688-1696.
30. Hashikabe Y, Suzuki K, Jojima T, Uchida K, Hattori Y. Aldosterone impairs vascular endothelial cell function. *J. Cardiovasc. Pharmacol.* 2006; 47: 609-613.
31. Keidar S, Kaplan M, Pavlotzky E, Coleman R, Hayek T, Hamoud S, Aviram M. Aldosterone administration to mice stimulates macrophage NADPH oxidase and increases atherosclerosis development: a possible role for angiotensin-converting enzyme and the receptors for angiotensin II and aldosterone. *Circulation*. 2004; 109: 2213-2220.
32. Suzuki J, Iwai M, Mogi M, Oshita A, Yoshii T, Higaki J, Horiuchi M. Eplerenone with valsartan effectively reduces atherosclerotic lesion by attenuation of oxidative stress and inflammation. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 917-921.

Chapter 6

Human CETP aggravates atherosclerosis by increasing VLDL-cholesterol rather than by decreasing HDL-cholesterol in ApoE*3-Leiden mice

Jitske de Vries-van der Weij [1,2], Susanne Zadelaar [1], Karin Toet [1], Louis M. Havekes [1,3], Teake Kooistra [1], Patrick C.N. Rensen [4]

[1] The Netherlands Organization for Applied Scientific Research (TNO) - BioSciences, Gaubius Laboratory, Leiden, The Netherlands
[2] Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands.
[3] Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands.
[4] Dept. of General Internal Medicine, Endocrinology, and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands.

Atherosclerosis 2009; 206: 153-158

Abstract

Objective: CETP adversely affects the plasma lipoprotein profile by increasing VLDL-cholesterol and decreasing HDL-cholesterol. The relative contribution of either of these changes to atherosclerosis development is not known. We investigated to what extent the increase in VLDL-cholesterol can explain the atherogenic action of human CETP expression in APOE*3-Leiden (E3L) mice, a model for human-like lipoprotein metabolism.
Methods and Results: E3L and E3L.CETP mice were fed a low cholesterol (LC) diet, resulting in a 4-fold increased VLDL-cholesterol level as well as a 9-fold increased atherosclerotic lesion area in the aortic root in E3L.CETP mice compared to E3L-LC mice. E3L mice fed a high cholesterol (HC) diet to match for the increased VLDL-cholesterol levels in E3L.CETP mice, displayed a similar atherosclerotic lesion area as observed in E3L.CETP mice. Hence, the CETP-induced raise in atherosclerosis can largely be explained by increased VLDL-cholesterol. Despite similar atherosclerosis development, E3L.CETP mice had lower HDL-cholesterol as compared to E3L-HC mice (-49%) indicating that the HDL-cholesterol lowering effect of CETP is unlikely to contribute to atherosclerosis development in this experimental setting. Remarkably, atherosclerotic lesions in CETP-expressing mice were enriched in collagen, suggesting a role of CETP or the diet in modifying lesion collagen content.
Conclusions: In this experimental setting, the pro-atherogenic effect of CETP is largely explained by increased VLDL-cholesterol.

Introduction

Atherosclerosis is a disease affecting the large arteries and is one of the leading causes of death in the Western world. An important risk factor for the development of atherosclerosis is dyslipidemia, as characterized by high levels of (very) low density lipoprotein-cholesterol ((V)LDL-C) and low levels of high density lipoprotein-cholesterol (HDL-C). The cholesteryl ester transfer protein (CETP) plays a role in the regulation of both (V)LDL-C and HDL-C levels, as CETP transfers cholesteryl esters from HDL to (V)LDL in exchange for triglycerides (TG) [1]. In this way, CETP lowers HDL-C and at the same time increases (V)LDL-C, thereby unfavorably modifying two risk factors for atherosclerosis development [2].

Indeed, in established hyperlipidemic mouse models for atherosclerosis, CETP expression aggravated atherosclerosis development [3,4]. However, contradicting effects of CETP on atherosclerosis development have been reported in humans. In studies where a reduced CETP mass led to increased HDL-C a beneficial effect on CHD was found only with concomitantly reduced (V)LDL-C [2,5-7], while in studies where (V)LDL-C was not affected an increased CHD prevalence was observed [8-10]. These studies suggest that (V)LDL-C levels are of great importance in determining the effect of CETP on CHD. On the other hand, a recent meta-analysis suggests that mutations in CETP that are associated with reduced CETP activity and increased HDL-C coincided with a reduced CHD risk [11]. Together, these studies demonstrate the complex role of CETP in atherosclerosis.

This complexity is further illustrated by the RADIANCE 1 [12] and 2 [13], ILLUSTRATE [14] and ILLUMINATE [15] trials, which evaluated the CETP inhibitor torcetrapib in combination with the LDL-C lowering drug atorvastatin. Combination therapy led to a strong increase in HDL-C (up to +63%) that was accompanied by a mild decrease in LDL-C (up to -20%) compared to atorvastatin only, but no beneficial effect on atherosclerosis progression was seen [12-14]. The ILLUMINATE trial was even stopped prematurely because of an excess of deaths in the torcetrapib/atorvastatin-treated group, attributed largely to cardiovascular death [15]. The mechanism behind the increased death rate is still unclear. Unexpected effects could have occurred due to adverse effects of CETP inhibition or to off-target drug effects of torcetrapib treatment, such as an increased blood pressure, increased plasma aldosterone levels [16] and potentially increased plaque instability [17].

Despite the large number of studies that have been performed to get insight into the effect of CETP on atherosclerosis, the precise role of CETP in atherosclerosis development is thus still unclear. Firstly, as CETP affects (V)LDL-C as well as HDL-C levels, it is difficult to distinguish between the relative contributions of either of these factors to lesion development. Secondly, it is not known whether CETP produced in atherosclerotic lesions by macrophages [18,19] and smooth muscle cells [20] has a local effect on atherosclerosis development.

In the current study we aimed at determining whether the CETP-induced increase in atherosclerosis as evident from experimental studies [3,4] can be explained

by an increase in VLDL-C or if other effects of CETP, such as a decrease in HDL-C or a local effect of CETP in atherosclerotic lesions, also affect atherosclerosis development. To this end, we used APOE*3-Leiden (E3L) mice and E3L.CETP mice, models for human-like lipoprotein metabolism in which plasma cholesterol levels can be titrated to desired levels by adjusting the dietary cholesterol content. By comparing atherosclerosis development between E3L mice and E3L.CETP mice that were matched for VLDL-C, we show that increased VLDL-C can largely explain the proatherogenic effect of CETP in E3L mice.

Methods

Animals

E3L mice were crossbred with mice expressing human CETP under control of the natural flanking regions, resulting in E3L.CETP mice [3]. Female E3L mice and E3L.CETP mice of 12-14 weeks old (n=14-15 per group) were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water unless indicated otherwise. Mice were fed a diet containing 15% (w/w) cacao butter (diet T, Hope Farms, Woerden, the Netherlands) supplemented with 0.1% or 0.4% (w/w) cholesterol (Sigma) for 15 weeks. Blood was drawn after 4 h of fasting at t=0, 4, 8, 13 and 15 weeks after the start of experimental diet feeding. After 15 weeks of experimental diet feeding, the mice were sacrificed by CO_2 inhalation and hearts were isolated for atherosclerosis analysis. All animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Analysis of plasma cholesterol, cholesterol distribution over lipoproteins and inflammation markers

Plasma total cholesterol and TG were measured (kit no. 1489437 and kit no. 1488872, Roche Diagnostics, Mannheim, Germany respectively). For lipoprotein profiles, plasma was fractionated using an ÄKTA fast protein liquid chromatography (FPLC) system (Pharmacia, Roosendaal, The Netherlands) as described [21]. Fractions of 50 μL were collected and assayed for cholesterol (using kit no. 1489437, Roche Diagnostics, Mannheim, Germany).

Plasma levels of fibrinogen (in house assay) [22], serum amyloid A (SAA) (Tridelta, Ireland, according to manufacturer's instructions) and E-selectin (R&D systems) [23] were determined by ELISA.

Atherosclerosis Quantification

After isolation, hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin, and cross-sectioned throughout the aortic root area. For each mouse, 4 sections with 50 μm intervals were used for atherosclerosis measurements. Sections were stained with hematoxylin-phloxin-saffron (HPS) for histological analysis. Lesions were categorized for severity according to the American Heart system adapted

for mice [24]. Various types of lesions were discerned: no lesions, mild lesions (type 1-3) and severe lesions (type 4-5). Lesion area was determined using Cell D imaging software (Olympus Soft Imaging Solutions). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific) was used to quantify the macrophage area and the number of monocytes adhering to the endothelium and Sirius Red was used to quantify the collagen area.

Statistical Analysis
Significance of difference was calculated by one-way analysis of variance (ANOVA) test followed by a least significant difference post hoc analysis. For lesion typing, differences were assessed by the Chi Square test. SPSS 14.0 for Windows (SPSS, Chicago, USA) was used for statistical analysis.

Results

Plasma total cholesterol and cholesterol distribution
To compare atherosclerotic lesion development between female E3L mice and E3L. CETP mice, both groups of mice were fed a Western-type diet containing 0.1% (w/w) cholesterol (LC-diet). A third group consisting of E3L mice was fed a Western-type diet supplemented with 0.4% (w/w) cholesterol (HC-diet) in order to match for the elevated plasma VLDL-C levels in the E3L.CETP group. Lipoprotein profiling confirmed that VLDL-C levels were comparable for E3L.CETP and E3L-HC mice and were about 4-fold higher than in the E3L-LC group (Figure 1A). The HDL fractions of E3L-LC and

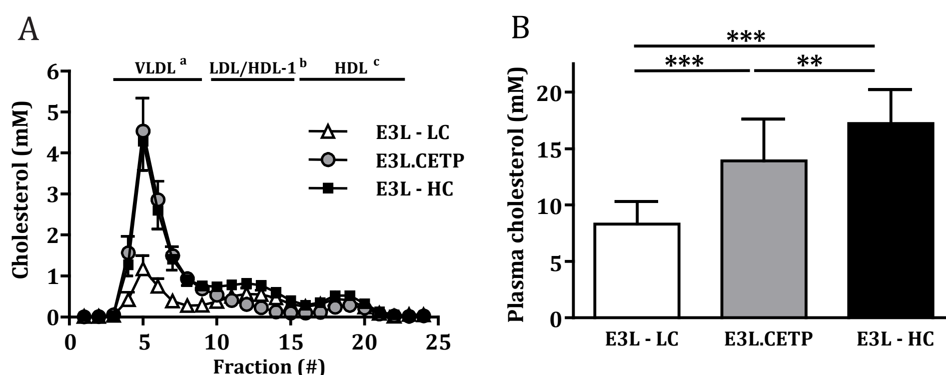


Figure 1. Plasma cholesterol and triglyceride distributions and total cholesterol. Two groups of mice were fed a diet containing 0.1% cholesterol (E3L-LC and E3L.CETP), whereas a third group of mice was fed a diet containing 0.4% cholesterol (E3L-HC) for 15 weeks. After 15 weeks, blood was drawn, and the distribution of cholesterol over the individual lipoproteins was determined after separation by FPLC (A), and total plasma cholesterol (B) was measured. Values are means ± SD (n=14-15). a: fractions 4-9 of the E3L.CETP group and the E3L-HC group are significantly different from the E3L-LC group (P<0.001). b: fractions 10-15 of the E3L.CETP group and the E3L-HC group are significantly different from each other and from the E3L-LC group (P<0.01). c: fractions 16-20 of the E3L-HC group are significantly different from both the E3L-LC and E3L.CETP groups (P<0.01), **P<0.01, ***P<0.001.

E3L-HC mice had an increased cholesterol content compared to E3L.CETP mice. These concomitant effects resulted in plasma total cholesterol levels that were increased in the E3L-HC (+107%, P<0.001) and E3L.CETP groups (+67%, P<0.001) compared to the E3L-LC group (Figure 1B). The data in Figure 1, obtained at 15 weeks, are representative for the whole experimental period (t=4, 8, 13 and 15). Plasma TG levels did not differ between the E3L-LC (1.6±0.5 mM) and E3L-HC (1.6±0.6 mM) groups, but were somewhat increased in E3L.CETP mice (2.7±1.2 mM; P<0.001).

Systemic inflammation markers do not differ between the experimental groups

Because inflammation is increasingly recognized as an important risk factor for atherosclerosis development, we evaluated whether or not the three experimental groups differed in circulating levels of the systemic inflammation markers SAA, fibrinogen, and sE-selectin. As shown in Table 1, plasma SAA levels were slightly increased in E3L-HC mice compared to E3L-LC mice (+37%, P<0.05), but were not different from E3L.CETP mice. Fibrinogen levels increased in time in all groups (+16-23%, P<0.01) and were increased in E3L.CETP mice compared to E3L-LC mice (+13%, P<0.05), but not compared to E3L-HC mice. As compared to t=0 values, sE-selectin levels were slightly increased in E3L-LC mice (+20%, P<0.01) and in E3L-HC mice (+32%, P<0.001), but not in E3L.CETP mice. These data show that systemic inflammation markers are not or only slightly increased upon experimental treatment. There is no indication for a consistent difference in systemic inflammation between the three groups.

Table 1. Plasma levels of systemic inflammation markers

	Plasma SAA (μg/ml)		Plasma fibrinogen (mg/ml)		Plasma sE-selectin (ng/ml)	
Group	t=0	t=13	t=0	t=13.	t=0	t=13
E3L-LC	6.6 (0.8)	6.3 (1.9)	1.8 (0.3)	2.1 (0.3) **	91 (11)	109 (11) **
E3L.CETP	6.2 (1.3)	7.7 (3.2)	1.9 (0.2)	2.4 (0.3) **,#	93 (10)	96 (10) ##
E3L-HC	7.1 (1.0)	8.7 (2.6) #	1.8 (0.2)	2.2 (0.4) **	90 (9)	119(14) **,#,$$$

Two groups of mice were fed a diet containing 0.1% cholesterol (E3L-LC and E3L.CETP), whereas a third group of mice was fed a diet containing 0.4% cholesterol (E3L-HC). After 0 and 13 weeks of experimental diet feeding, blood was drawn and plasma levels of serum amyloid A (SAA), fibrinogen and soluble E-selectin (sE-selectin) were determined. Values are means (SD). **Significantly different compared to t=0 from same group, P<0.01. #Significantly different from E3L-LC at same time point, P<0.05. ##Significantly different from E3L-LC at same time point, P<0.01. $$$Significantly different from E3L.CETP at same time point, P<0.001.

Atherosclerosis development is strongly increased in E3L.CETP and E3L-HC mice compared to E3L-LC mice, but is not different between VLDL-C matched groups

After 15 weeks of experimental diet feeding, all mice were sacrificed to analyze atherosclerosis development in the aortic root, with particular attention to comparing

lesion formation in the VLDL-C matched E3L.CETP and E3L-HC groups. Representative pictures of each group are shown in Figure 2A. Lesion area was 7-fold increased in E3L-HC mice (P<0.001) and 9-fold increased in E3L.CETP mice (P<0.001) compared to E3L-LC mice. There was no significant difference in lesion area between VLDL-C-matched E3L.CETP mice and E3L-HC mice (Figure 2B). A similar picture was seen for lesion severity analysis: E3L-LC mice mostly developed mild or no lesions, whereas the VLDL-C-matched E3L.CETP mice and E3L-HC mice showed less lesion-free segments and developed considerably more severe lesions (Figure 2C: P<0.001 and P<0.01, respectively). Again, no difference in lesion severity was noted between the VLDL-C-matched E3L.CETP and E3L-HC groups.

Collagen content is strongly increased in E3L.CETP mice compared to E3L-LC and E3L-HC mice

One of the initial processes in lesion formation is the adhesion of monocytes to the endothelium. To assess whether there is a difference in monocyte adhesion between

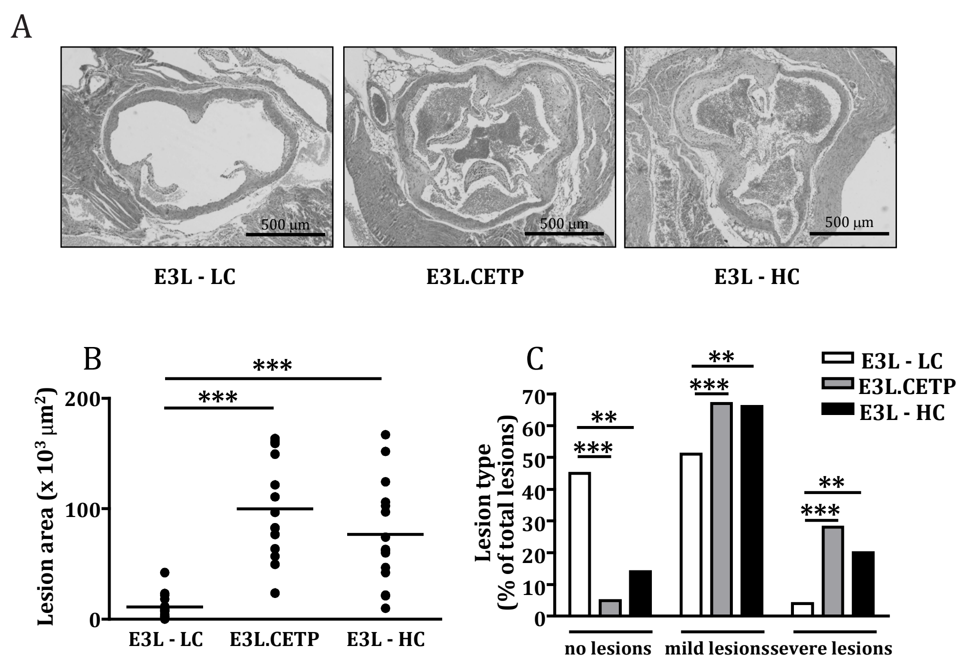


Figure 2. Analysis of atherosclerosis development. Two groups of mice were fed a diet containing 0.1% cholesterol (E3L-LC and E3L.CETP), whereas a third group of mice was fed a diet containing 0.4% cholesterol (E3L-HC). After 15 weeks of experimental diet feeding, hearts were isolated to analyze atherosclerosis development. Representative HPS stained pictures of each group are shown (A). Four sections per mouse with 50 μm intervals were analyzed. Total lesion area was determined (B) and lesions were typed and categorized according to lesion severity (C). Horizontal bars indicate mean lesion area. Each point corresponds to one mouse; **P<0.01, ***P<0.001.

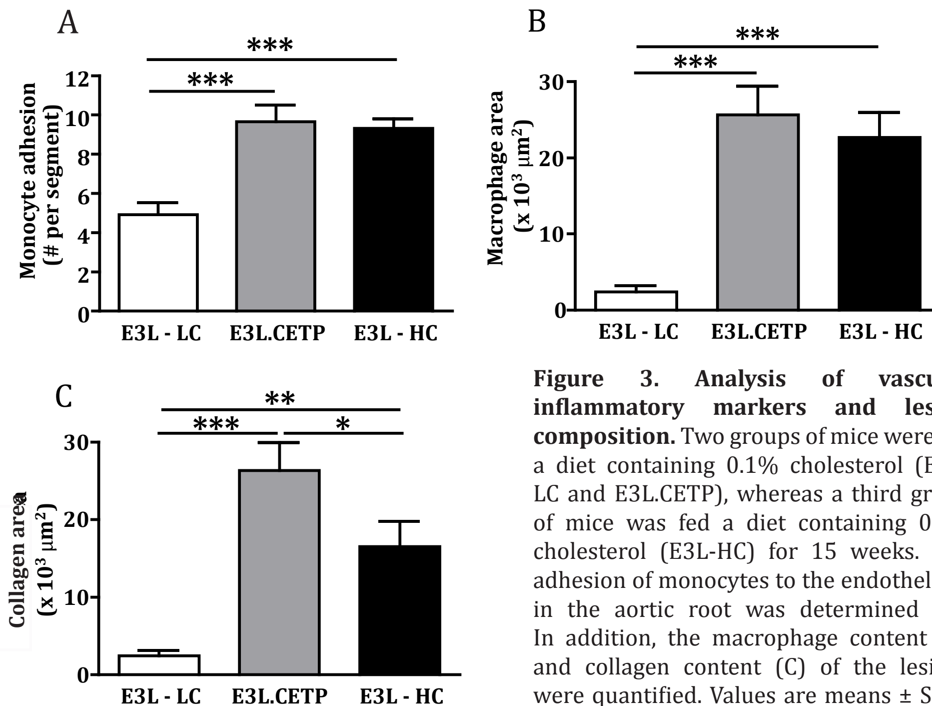


Figure 3. Analysis of vascular inflammatory markers and lesion composition. Two groups of mice were fed a diet containing 0.1% cholesterol (E3L-LC and E3L.CETP), whereas a third group of mice was fed a diet containing 0.4% cholesterol (E3L-HC) for 15 weeks. The adhesion of monocytes to the endothelium in the aortic root was determined (A). In addition, the macrophage content (B) and collagen content (C) of the lesions were quantified. Values are means ± SEM; *P<0.05, **P<0.01, ***P<0.001.

the three treatment groups, the amount of monocytes adhering to the endothelium was determined. As shown in Figure 3A, monocyte adhesion was increased to the same extent in the E3L.CETP (+92%, P<0.001) and E3L-HC (+85%, P<0.001) groups compared to E3L-LC mice. Similarly, macrophage area was not significantly different between E3L. CETP mice and E3L-HC mice, and was increased 11-fold and 9-fold compared to E3L-LC mice respectively (Figure 3B). Notably, collagen content of the lesions was increased in E3L.CETP mice compared to both the E3L-LC group (10-fold, P<0.001) and VLDL-C-matched E3L-HC mice (1.6-fold, P<0.05) (Figure 3C).

Discussion

In a previous study we showed that CETP expression in E3L mice led to an increase in VLDL-C and a decrease in HDL-C, accompanied with a strong increase in atherosclerosis development [3]. In the current study we provide evidence that this increase in atherosclerotic lesion formation can be explained by the rise in VLDL-C levels in E3L. CETP mice, rather than by reduction of HDL-C levels, or than by changing the systemic inflammatory status. Furthermore, the collagen content of atherosclerotic lesions in E3L.CETP mice is strongly increased compared to lesions in VLDL-C matched E3L-HC mice, indicating that CETP has a previously unknown role in increasing collagen levels in atherosclerotic lesions.

First, our present data show that, under the current experimental conditions, the CETP-induced increase in VLDL-C levels largely explains the observed increase in atherosclerosis development in E3L mice. In fact, atherosclerotic lesion area appeared to be well predicted by changes in VLDL-C (R^2 = 0.694; P<0.001), but not by changes in LDL/HDL-1-C (R^2 = 0.000; P=0.909) or HDL-C (R^2 = 0.045; P=0.259) (Figure 4). In contrast, our recent study in E3L.CETP mice investigating the effect of torcetrapib and/or atorvastatin on plasma lipid levels and atherosclerosis showed that atorvastatin more potently decreased VLDL-C levels than torcetrapib, while both compounds similarly reduced atherosclerotic lesion area [17]. These previous data thus suggest that besides VLDL-C, other factors may also influence atherosclerosis development, including HDL. Our present finding that the CETP-induced rise in VLDL-C is the most important determinant for atherosclerotic lesion development is in line with studies in rabbits. Administration of the CETP inhibitor JTT-705 to rabbits similarly caused a decrease in (V)LDL-C in addition to an increase in HDL-C and decreased atherosclerosis

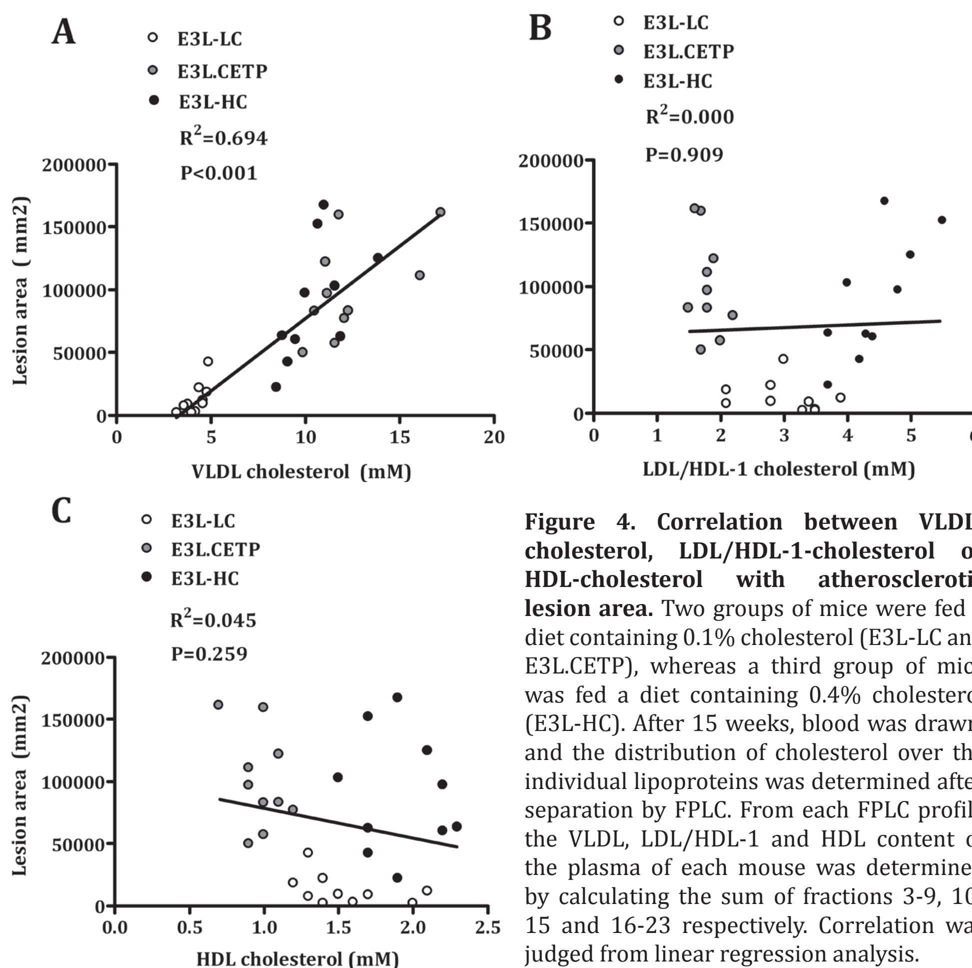


Figure 4. Correlation between VLDL-cholesterol, LDL/HDL-1-cholesterol or HDL-cholesterol with atherosclerotic lesion area. Two groups of mice were fed a diet containing 0.1% cholesterol (E3L-LC and E3L.CETP), whereas a third group of mice was fed a diet containing 0.4% cholesterol (E3L-HC). After 15 weeks, blood was drawn, and the distribution of cholesterol over the individual lipoproteins was determined after separation by FPLC. From each FPLC profile the VLDL, LDL/HDL-1 and HDL content of the plasma of each mouse was determined by calculating the sum of fractions 3-9, 10-15 and 16-23 respectively. Correlation was judged from linear regression analysis.

development [25]. Interestingly, treatment of rabbits with JTT-705 in a subsequent study increased HDL-C without affecting (V)LDL-C, and did not affect atherosclerosis development [26].

Second, our data show that CETP expression in E3L mice increases the collagen composition of atherosclerotic lesions. These data solidify findings of a recent study in which we investigated the effect of torcetrapib on atherosclerosis development in E3L.CETP mice, and found that partial CETP inhibition tended to decrease the collagen content of atherosclerotic lesions [17]. From that study it was not clear if the effect on collagen content was a compound-specific effect of torcetrapib or a general effect of CETP inhibition. Our current data suggest that the torcetrapib-induced reduction in collagen content of the lesions was a direct result of CETP inhibition, albeit that the mechanism is still unknown. A decrease in the collagen content of atherosclerotic lesions by CETP inhibition, thereby destabilizing these lesions, could theoretically explain the increased cardiovascular death rate in the torcetrapib/atorvastatin-treated group compared to the atorvastatin-treated group in the ILLUMINATE trial [15], but more research is needed to confirm this theory.

It should be noted that LDL is the most abundant atherogenic lipoprotein in humans, whereas larger cholesterol-enriched VLDL remnants accumulate in E3L and E3L.CETP mice, which may thus limit the extrapolative value of our data to the human situation. Despite the different nature of the accumulating atherogenic lipoprotein, the apparently contradicting observations in human studies investigating the effect of mutations in CETP, leading to a decreased CETP mass and increased HDL-C levels, on CHD could be explained by different effects on atherogenic lipoprotein levels (i.e. LDL). When LDL-C did not change, there was an increase in CHD prevalence [8-10], while a decrease in LDL-C levels coincided with a reduction in the incidence of CHD [2,5-7]. Although some human studies indicate a protective role of CETP-related changes in HDL-C and not LDL-C on CHD [11,27], in the RADIANCE 1 and 2 trials, carotid intima-media thickness progression correlated with changes in LDL-C and not HDL-C [28], which is in line with the data from our study.

As the aim of this study was to evaluate the role of the CETP-induced increase in VLDL-C on atherosclerosis development, this unavoidably led to differences in levels, size and composition of HDL between the experimental groups. Although the differences in HDL do not seem to have a large impact on atherosclerosis development, we should point out that this study was not designed to investigate the specific role of CETP-induced changes in HDL on atherosclerosis development. Therefore, future studies should demonstrate if CETP-induced differences in HDL affect HDL functionality and thereby also influence atherosclerosis development.

In this study we show that, under the current experimental conditions, the proatherogenic role of CETP is mainly the consequence of its VLDL-C increasing effect, suggesting that CETP inhibition could lead to a reduction in atherosclerosis development mainly by lowering (V)LDL-C levels. As a consequence, CETP inhibition may not be a good alternative or additive for current LDL-C lowering therapies, e.g.

with statins. CETP inhibition may even interfere with the beneficial effect of CETP on stabilization of atherosclerotic lesions by increasing collagen [17]. Thus, favorable effects of CETP inhibition on plasma (V)LDL-C and HDL-C levels, may not outweigh possible detrimental effects on plaque stability. Therefore, the strategy of CETP inhibition as a novel strategy to reduce atherosclerosis development in the general dyslipidemic population should be pursued with care.

In conclusion, our data indicate that the increase in VLDL-C is the most important factor contributing to the proatherogenic effect of CETP in E3L mice. Furthermore, our data argue that CETP may positively influence atherosclerotic lesion stability by enhancing the collagen content of atherosclerotic lesions.

Acknowledgements

This work was performed within the framework of the Leiden Center for Cardiovascular Research LUMC-TNO and supported by the Leiden University Medical Center (Gisela Thier Fellowship to Dr Rensen), the Netherlands Heart Foundation (NHS grant 2003B136 to Dr Rensen), the Centre for Medical Systems Biology (CMSB project 115 to Dr Havekes) within the framework of the Netherlands Genomics Initiative (NGI), the Netherlands Organization for Scientific Research (NWO grant 908-02-097 to Dr Havekes and NWO VIDI grant 917.36.351 to Dr Rensen) and The European Nutrigenomics Organization (NuGO, CT-2004-505944 to Dr Kooistra).

References

1. Ha YC, Calvert GD, Barter PJ. In vivo metabolism of esterified cholesterol and apoproteins in rabbit plasma low density lipoproteins. *Atherosclerosis*. 1979; 34: 451-455.
2. Moriyama Y, Okamura T, Inazu A, Doi M, Iso H, Mouri Y, Ishikawa Y, Suzuki H, Iida M, Koizumi J, Mabuchi H, Komachi Y. A low prevalence of coronary heart disease among subjects with increased high-density lipoprotein cholesterol levels, including those with plasma cholesteryl ester transfer protein deficiency. *Prev. Med.* 1998; 27: 659-667.
3. Westerterp M, van der Hoogt CC, de HW, Offerman EH, Dallinga-Thie GM, Jukema JW, Havekes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2552-2559.
4. Plump AS, Masucci-Magoulas L, Bruce C, Bisgaier CL, Breslow JL, Tall AR. Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arterioscler. Thromb. Vasc. Biol.* 1999; 19: 1105-1110.
5. Boekholdt SM, Kuivenhoven JA, Wareham NJ, Peters RJ, Jukema JW, Luben R, Bingham SA, Day NE, Kastelein JJ, Khaw KT. Plasma levels of cholesteryl ester transfer protein and the risk of future coronary artery disease in apparently healthy men and women: the prospective EPIC (European Prospective Investigation into Cancer and nutrition)-Norfolk population study. *Circulation*. 2004; 110: 1418-1423.
6. de Grooth GJ, Smilde TJ, Van WS, Klerkx AH, Zwinderman AH, Fruchart JC, Kastelein JJ, Stalenhoef AF, Kuivenhoven JA. The relationship between cholesteryl ester transfer protein levels and risk factor profile in patients with familial hypercholesterolemia.

Atherosclerosis. 2004; 173: 261-267.
7. Kuivenhoven JA, Jukema JW, Zwinderman AH, de KP, McPherson R, Bruschke AV, Lie KI, Kastelein JJ. The role of a common variant of the cholesteryl ester transfer protein gene in the progression of coronary atherosclerosis. The Regression Growth Evaluation Statin Study Group. *N. Engl. J. Med.* 1998; 338: 86-93.
8. Agerholm-Larsen B, Nordestgaard BG, Steffensen R, Jensen G, Tybjaerg-Hansen A. Elevated HDL cholesterol is a risk factor for ischemic heart disease in white women when caused by a common mutation in the cholesteryl ester transfer protein gene. *Circulation*. 2000; 101: 1907-1912.
9. Borggreve SE, Hillege HL, Wolffenbuttel BH, de Jong PE, Zuurman MW, van der SG, van TA, Dullaart RP. An increased coronary risk is paradoxically associated with common cholesteryl ester transfer protein gene variations that relate to higher high-density lipoprotein cholesterol: a population-based study. *J. Clin. Endocrinol. Metab*. 2006; 91: 3382-3388.
10. Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR. Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. *J. Clin. Invest*. 1996; 97: 2917-2923.
11. Thompson A, Di AE, Sarwar N, Erqou S, Saleheen D, Dullaart RP, Keavney B, Ye Z, Danesh J. Association of cholesteryl ester transfer protein genotypes with CETP mass and activity, lipid levels, and coronary risk. *JAMA*. 2008; 299: 2777-2788.
12. Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. *N. Engl. J. Med.* 2007; 356: 1620-1630.
13. Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial. *Lancet*. 2007; 370: 153-160.
14. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. *N. Engl. J. Med.* 2007; 356: 1304-1316.
15. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *N. Engl. J. Med.* 2007; 357: 2109-2122.
16. Forrest MJ, Bloomfield D, Briscoe RJ, Brown PN, Cumiskey AM, Ehrhart J, Hershey JC, Keller WJ, Ma X, McPherson HE, Messina E, Peterson LB, Sharif-Rodriguez W, Siegl PK, Sinclair PJ, Sparrow CP, Stevenson AS, Sun SY, Tsai C, Vargas H, Walker M, III, West SH, White V, Woltmann RF. Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone. *Br. J. Pharmacol.* 2008; 154: 1465-1473.
17. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM, Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation*. 2008; 117: 2515-2522.
18. Faust RA, Tollefson JH, Chait A, Albers JJ. Regulation of LTP-I secretion from human monocyte-derived macrophages by differentiation and cholesterol accumulation in vitro.

Biochim. Biophys. Acta. 1990; 1042: 404-409.

19. Gotoda T, Kinoshita M, Shimano H, Harada K, Shimada M, Ohsuga J, Teramoto T, Yazaki Y, Yamada N. Cholesteryl ester transfer protein deficiency caused by a nonsense mutation detected in the patient's macrophage mRNA. *Biochem. Biophys. Res. Commun.* 1993; 194: 519-524.
20. Ishikawa Y, Ito K, Akasaka Y, Ishii T, Masuda T, Zhang L, Akishima Y, Kiguchi H, Nakajima K, Hata Y. The distribution and production of cholesteryl ester transfer protein in the human aortic wall. *Atherosclerosis*. 2001; 156: 29-37.
21. Westerterp M, de Haan W, Berbee JF, Havekes LM, Rensen PC. Endogenous apoC-I increases hyperlipidemia in apoE-knockout mice by stimulating VLDL production and inhibiting LPL. *J. Lipid Res.* 2006; 47: 1203-1211.
22. Koopman J, Maas A, Rezaee F, Havekes J, Verheijen J, Gijbels M, Haverkate F. Fibrinogen and atherosclerosis: A study in transgenic mice. *Fibrinol. Proteol.* 1997; 11: 19-21.
23. Rein D, Schijlen E, Kooistra T, Herbers K, Verschuren L, Hall R, Sonnewald U, Bovy A, Kleemann R. Transgenic flavonoid tomato intake reduces C-reactive protein in human C-reactive protein transgenic mice more than wild-type tomato 2. *J. Nutr.* 2006; 136: 2331-2337.
24. Zadelaar AS, Boesten LS, Jukema JW, van Vlijmen BJ, Kooistra T, Emeis JJ, Lundholm E, Camejo G, Havekes LM. Dual PPARalpha/gamma agonist tesaglitazar reduces atherosclerosis in insulin-resistant and hypercholesterolemic ApoE*3Leiden mice. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2560-2566.
25. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. 2000; 406: 203-207.
26. Huang Z, Inazu A, Nohara A, Higashikata T, Mabuchi H. Cholesteryl ester transfer protein inhibitor (JTT-705) and the development of atherosclerosis in rabbits with severe hypercholesterolaemia. *Clin. Sci. (Lond)*. 2002; 103: 587-594.
27. Nicholls SJ, Tuzcu EM, Brennan DM, Tardif JC, Nissen SE. Cholesteryl ester transfer protein inhibition, high-density lipoprotein raising, and progression of coronary atherosclerosis: insights from ILLUSTRATE (Investigation of Lipid Level Management Using Coronary Ultrasound to Assess Reduction of Atherosclerosis by CETP Inhibition and HDL Elevation). *Circulation*. 2008; 118: 2506-2514.
28. Vergeer M, Bots ML, van Leuven SI, Basart DC, Sijbrands EJ, Evans GW, Grobbee DE, Visseren FL, Stalenhoef AF, Stroes ES, Kastelein JJ. Cholesteryl ester transfer protein inhibitor torcetrapib and off-target toxicity: a pooled analysis of the rating atherosclerotic disease change by imaging with a new CETP inhibitor (RADIANCE) trials. *Circulation*. 2008; 118: 2515-2522.

Chapter 7

Bexarotene induces dyslipidemia by increased VLDL production and cholesteryl ester transfer protein (CETP)-mediated reduction of HDL

Jitske de Vries-van der Weij [1,2], Willeke de Haan [3], Lihui Hu [3], Maarten Kuif [3], H. Ling D.W. Oei [3], José W.A. van der Hoorn [2,4], Louis M. Havekes [2,3,4], Hans M.G. Princen [2], Johannes A. Romijn [3], Johannes W.A. Smit [3], Patrick C.N. Rensen [3]

[1] Dept. of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
[2] The Netherlands Organization for Applied Scientific Research - BioSciences, Gaubius Laboratory, Leiden, The Netherlands.
[3] Dept. of General Internal Medicine, Endocrinology, and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands
[4] Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands

Endocrinology 2009; 150: 2368-2375

Abstract

A common dose-limiting side effect of treatment with the RXR agonist bexarotene is dyslipidemia. We evaluated the effects of bexarotene on plasma lipid metabolism in patients with metastatic differentiated thyroid carcinoma (DTC), and investigated the underlying mechanism(s) in APOE*3-Leiden mice without (E3L) and with human CETP (E3L.CETP). To this end, ten patients with metastatic DTC were treated with bexarotene (300 mg/day) for 6 weeks. Bexarotene increased plasma TG (+150%), primarily associated with VLDL, and raised plasma total cholesterol (TC) (+50%). However, while bexarotene increased VLDL-C and LDL-C (+63%), it decreased HDL-C (-30%) and tended to decrease apoAI (-18%) concomitant with an increase in endogenous CETP activity (+44%). To evaluate the cause of the bexarotene-induced hypertriglyceridemia and the role of CETP in the bexarotene-induced shift in cholesterol distribution, E3L and E3L.CETP mice were treated with bexarotene through dietary supplementation (0.03% w/w). Bexarotene increased VLDL-associated TG in both E3L (+47%) and E3L.CETP (+29%) mice, by increasing VLDL-TG production (+68%). Bexarotene did not affect the TC levels or distribution in E3L mice, but increased VLDL-C (+11%) and decreased HDL-C (-56%) as well as apoAI (-31%) in E3L.CETP mice, concomitant with increased endogenous CETP activity (+41%). This increased CETP activity by bexarotene-treatment is likely due to the increase in VLDL-TG, a CETP substrate that drives CETP activity. In conclusion, bexarotene causes combined dyslipidemia as reflected by increased TG, VLDL-C and LDL-C and decreased HDL-C, which is the result of an increased VLDL-TG production that causes an increase of the endogenous CETP activity.

Introduction

Bexarotene (Targretin[0], Ligand Pharmaceuticals Inc., LGD1069) is used as a chemotherapeutic agent for the treatment of cutaneous T-cell lymphoma [1], and is evaluated as a strategy to treat patients with metastasis of differentiated thyroid carcinoma (DTC) [2], breast cancer [3], non-small-cell lung cancer [4] and psoriasis [5]. Bexarotene is a selective retinoid X receptor (RXR) agonist, which class of agonists is also called rexinoids. When activated, RXR can form homodimers or heterodimers with other nuclear hormone receptors, including the liver X receptor (LXR) [6]. These RXR homo- or heterodimers can bind to specific responsive elements in target genes to alter their transcription.

Hypertriglyceridemia, an important risk factor for the development of cardiovascular disease [7], is one of the most frequently occurring side-effects in patients treated with bexarotene [8]. Some studies have been performed to unravel the underlying mechanism, but this did not lead to unequivocal results. A study in APOE2 knock-in mice showed that bexarotene increased the expression of hepatic genes involved in TG synthesis, which may imply that bexarotene increases VLDL production [9]. However, bexarotene also increases the expression of angiopoietin-like protein 3 (Angptl3) *in vivo* [9] and of apoCIII *in vitro* [10], factors that are both inhibitors of the TG-hydrolyzing activity of lipoprotein lipase (LPL) and could thus affect TG clearance. The relevance of either of these findings for the effect of bexarotene on triglyceride metabolism has not been evaluated *in vivo*. Thus, as yet it is not clear whether the bexarotene induced hypertriglyceridemia is caused by increased TG production, decreased TG clearance or both.

Hypercholesterolemia, besides hypertriglyceridemia, is another reported side effect of bexarotene [8]. Notably, the distribution of cholesterol over the various lipoproteins, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL), has not yet been studied in humans. Furthermore, mouse studies investigating the effect of RXR agonists on cholesterol distribution showed inconsistent data. In db/db mice, an RXR agonist was shown to increase HDL-C [11], whereas in APOE2 knock-in mice, bexarotene had no effect on HDL-C levels [9]. However, in contrast to humans, mice do not naturally express the cholesteryl ester transfer protein (CETP). CETP plays an important role in the metabolism of both TG and cholesterol, as it transfers TG from VLDL and LDL to HDL in exchange for cholesteryl esters (CE) [12]. In this way, CETP increases the cholesterol content of VLDL and LDL, while decreasing HDL-C levels. The importance of CETP in HDL-C metabolism has been illustrated by studies showing that the HDL-C-raising effect of the drugs atorvastatin [13], fenofibrate [14], niacin [15] and torcetrapib [16] is CETP-dependent. The contradicting data on the effect of bexarotene on cholesterol distribution obtained in previous studies may thus relate to the absence of CETP.

The aim of our study was to investigate the effect of bexarotene on lipoprotein levels in humans treated with bexarotene in more detail, and to study the underlying mechanism as well as the potential involvement of CETP therein in mice. We found that bexarotene increases VLDL-TG, increases VLDL-C and LDL-C and decreases

HDL-C levels in humans. The mechanisms underlying these changes were studied in APOE*3-Leiden mice without (E3L) and with expression of human CETP under control of its own promoter and regulatory flanking regions (E3L.CETP), as E3L and E3L.CETP mice are well-established models for human-like lipoprotein metabolism and have been shown to respond in a human-like manner to lipid-modifying drugs [13-17]. Using these mice we showed that bexarotene increases TG by increasing VLDL-TG production, and decreases HDL-C by increasing endogenous CETP activity.

Methods

Patients

The effects of bexarotene on lipid metabolism were studied in 10 patients with metastases of DTC who received 300 mg/day bexarotene (Targretin capsules, Ligand Pharmaceuticals, Crawley, UK) for 6 weeks. Exclusion criteria were pregnancy, contraindications for the application of recombinant human TSH (rhTSH), and contraindications for the use of bexarotene such as hematological malignancies, leukopenia or coagulopathy, a history of pancreatic disease and severe hypertriglyceridemia (fasting TG levels >4.5 mmol/L). Two patients had a history of well controlled hypertension, for which they used beta receptor blocking agents, angiotensin converting enzyme blocking agents and diuretics. Two other patients had well controlled type 2 diabetes mellitus, for which they used oral antidiabetic medication (metformin and glimepiride). Their fasting glucose levels were 5 and 5.7 mmol/L. One patient with type 2 diabetes mellitus also used simvastatin, an HMG-CoA reductase inhibitor. Baseline total cholesterol and triglycerides were 4.38 mmol/L and 1.87 mmol/L respectively. Another patient had a history of a mild myocardial infarction, with normal cardiac function. This patient also used simvastatin for secondary prevention. Baseline total cholesterol and triglycerides were 5.08 and 1.23 mmol/L. One patient had a history of obstructive pulmonary disease for which she used brochospasmolytic drugs. The institutional review board approved the study, and all patients gave written informed consent. This study was designed based on previously published studies [2,18,19]. Measurements were performed prior to, and after this intervention. Each patient served as his/her own control.

Animals

Male E3L mice and E3L.CETP mice (n=7-8 per group) were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water unless indicated otherwise. Mice were fed a standard chow diet (Sniff, Soest, Germany) or the same diet supplemented with 0.03% (w/w) bexarotene (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl) ethenyl] benzoic acid, $C_{24}H_{28}O_2$) (Targretin, Ligand Pharmaceuticals, Crawley, UK) for 3 weeks. Experiments were performed after 4 h of fasting, unless stated otherwise. All animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Analysis of plasma lipids and lipoproteins

In the human study, blood samples were collected after an overnight fast. In all mouse experiments, blood was collected from the tail vein after 4 hours of fasting. Plasma TG and total cholesterol (TC) were measured using kit no. 1488872 and kit no. 1489437 (Roche Diagnostics, Mannheim, Germany), respectively. HDL-C levels were measured after heparin/manganese chloride-induced precipitation of apoB-containing lipoproteins as described [15]. Lipoprotein profiles were measured after fractionation of plasma using an ÄKTA fast protein liquid (FPLC) system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described [20]. Fractions of 50 μL were collected and assayed for TC and TG as described above.

Analysis of apoAI levels in human and mouse plasma

Plasma levels of human apoAI were determined by ELISA. Shortly, goat anti-human apoAI antibody (Academy Biomedical Company, Inc., Houston, USA; 11A-G2b) was coated overnight onto Costar medium binding plates (Costar, Inc., New York, NY) at 4°C and subsequently incubated with diluted human plasma (dilution: 1:1,000,000) for 2 h at 37°C. Next, horse radish peroxidase (HRP)-conjugated goat anti-human apoAI (Academy Biomedical Company, Inc., Houston, USA; 11H-G1b) was added and incubated for 2 h at 37°C. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Human apoAI (Academy Biomedical Company, Inc., Houston, USA; 11P-101) was used as a standard. Plasma mouse apoAI levels were determined by ELISA as described [15].

Analysis of plasma CETP activity and mass and hepatic mRNA expression

Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (RB-CETP, Roar Biomedical, New York, NY) as described [21]. This method determines CETP activity by measuring the transfer of cholesteryl esters from donor liposomes to endogenous lipoproteins by endogenous CETP. CETP mass was measured with the Daiichi CETP ELISA kit according to the manufacturer's instructions (Daiichi, Tokyo, Japan). For analysis of CETP mRNA expression, RNA was isolated from livers as described [22] using RNA-Bee (Campro Scientific, Veenendaal, The Netherlands). Integrity of RNA obtained was confirmed by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (both from Agilent Technologies, Amstelveen, The Netherlands). Preparation of cDNA was performed using the Revert Aid First strand cDNA synthesis kit (Fermentas Life Sciences, St. Leon-Rot, Germany) and RNA expression was determined by RT-PCR on a MyiQ Single-Color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Primers for *CETP* [21] and *Apob* [23] have been described previously. Primers for *Scd1* (forward: 5'-GCGATACACTCTGGTGCTCA-3'; reverse: 5'-CCCAGGGAAACCAGGATATT-3') and *Fas* (forward: 5'- ATTGCATCAAGCAAGTGC AG-3'; reverse: 5'- GAGCCGTCAAACAGG AAGAG-3') were designed using Primer 3 Software and primer efficiency was 90-100%.

Analysis of in vivo clearance of VLDL-like TG-rich particles
VLDL-like TG-rich emulsion particles (80 nm) labeled with glycerol tri[^{3}H]oleate (triolein, TO) and [^{14}C]cholesteryl oleate (CO) were prepared and characterized as described previously [24]. After 3 weeks of experimental diet feeding, mice were anesthetized by intraperitoneal injection with 6.25 mg/kg vetranquil (Alfasan, Woerden, The Netherlands), 6.25 mg/kg dormicum (Midazolam, Delta Select GmbH, Dreieich, Germany) and 0.31 mg/kg fentanyl (Janssen-Cilag B.V., Tilburg, The Netherlands). Emulsion particles were injected intravenously (*i.v.*) at a dose of 1 mg TG per mouse. Blood samples were taken via tail bleeding at 2, 5, 10, 20 and 30 minutes after injection and ^{3}H and ^{14}C activities in plasma were counted. Plasma volumes (mL) were calculated as 0.04706 x bodyweight (g) as determined from ^{125}I-BSA clearance studies as described previously [25]. After 30 minutes, liver, heart, spleen, hind limb muscle and white adipose tissue (WAT) (*i.e.* gonadal, perirenal and intestinal) were isolated. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, UK) and then ^{3}H and ^{14}C activity were counted. Values were corrected for plasma radioactivity present in the respective tissues [24].

Analysis of hepatic VLDL production
To study hepatic VLDL production, mice were fed the experimental diets for 3 weeks. Mice were anesthetized as described above and received an *i.v.* injection of Tran ^{35}S label (150 mCi/mouse, MP Biomedicals, Eindhoven, The Netherlands) to label newly produced apoB, followed after 30 minutes by an *i.v.* injection of Triton WR-1339 to inhibit VLDL clearance (0.5 mg/g bodyweight, 10% solution in PBS). Blood samples were drawn at t=0, 15, 30, 60 and 90 minutes after injection. Plasma TG levels were measured as described above. After 120 minutes, mice were exsanguinated via the retro-orbital plexus. VLDL was quantitatively isolated from plasma after density gradient ultra centrifugation at d<1.006 g/mL. VLDL-apoB was counted for incorporated ^{35}S and VLDL-TG was measured as described above.

Statistical Analysis
Data are presented as means ± SD unless indicated otherwise. For patient data, statistical differences between pre- and post-treatment samples were assessed with the paired t-test. Statistical differences between control-treated and bexarotene-treated mice were assessed with the Mann-Whitney *U* test using SPSS 14.0 (SPSS Inc, Chicago, Ill). Values of P<0.05 were regarded as statistically significant.

Results

Bexarotene increases VLDL and LDL and decreases HDL in humans
To evaluate the effect of bexarotene treatment on plasma lipid levels, blood was drawn after an overnight fast from 10 patients with metastatic DTC before and after 6 weeks of treatment with 300 mg bexarotene per day. Baseline TSH levels were

0.24±0.68 mU/L. After bexarotene treatment, mean TSH levels were 0.13±0.33 mU/L (P=0.648). Bexarotene treatment resulted in a strong increase in plasma TG (+150%, P<0.001) (Figure 1A) and TC (+50%, P<0.001) (Figure 1B). In addition, bexarotene decreased HDL-C (-30%, P<0.05) (Figure 1C) and tended to decrease its most abundant apolipoprotein apoAI (-18%, P=0.09) (Figure 1D).

Lipoprotein profiling showed that the bexarotene-induced increase in TG was mainly present in VLDL (+173%, P<0.05) and to a lesser extent in LDL (+76%, P<0.05) (Figure 1E). The bexarotene-induced increase in TC was explained by an increase in

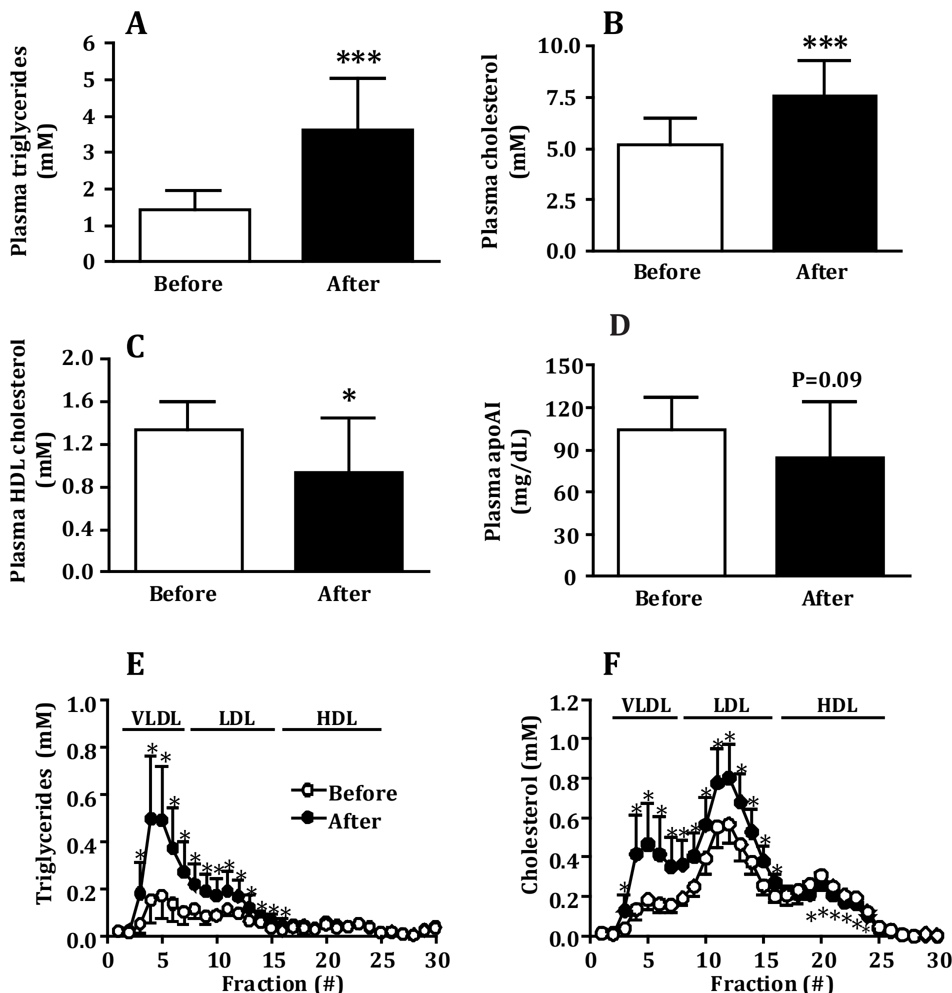


Figure 1. Bexarotene increases VLDL-TG, LDL-TG, VLDL-C and LDL-C but decreases HDL-C in humans. Blood was drawn from patients before and after 6 weeks of bexarotene treatment, and plasma was assayed for TG (A) and TC (B). Furthermore, HDL-C (C) and apoAI (D) levels were determined. Values are means ± SD (n=10, *P<0.05, ***P<0.001; paired t-test). Lipoproteins from individual plasma samples were fractionated using FPLC and assayed for TG (E) and TC (F).

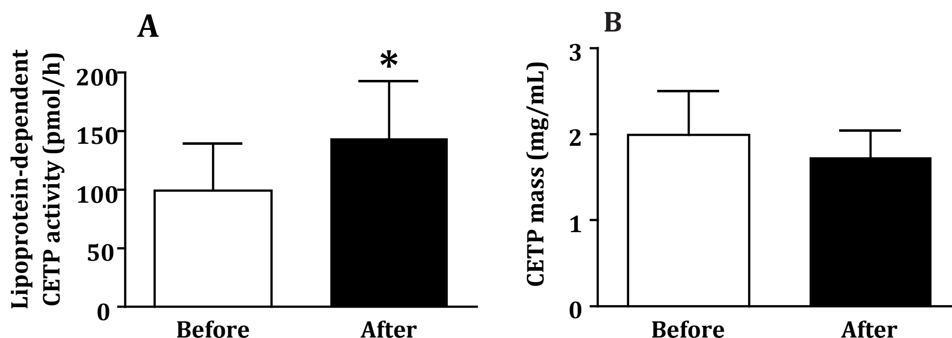


Figure 2. Bexarotene decreases endogenous plasma CETP activity without affecting CETP mass in humans. Blood was drawn from patients before and after 6 weeks of bexarotene treatment. In plasma, lipoprotein-dependent CETP activity (A) and CETP mass (B) were determined. Values are means ± SD (n=10, *P<0.05; paired t-test).

VLDL-C (+145%, P<0.05) and LDL-C (+41%, P<0.05), whereas HDL-C was decreased (-20%, P<0.05) (Figure 1F).

To investigate whether CETP could be involved in the shift in cholesterol distribution upon bexarotene treatment, plasma CETP activity and mass were measured. Bexarotene increased CETP activity (+44%, P<0.05) (Figure 2A) without changing CETP mass (Figure 2B). The effects of bexarotene on lipid profiles appeared not to be influenced by medications as mentioned in the Methods section.

Bexarotene increases VLDL-TG in both E3L and E3L.CETP mice and decreases HDL-C only in E3L.CETP mice

To investigate the mechanism behind the bexarotene-induced changes in plasma lipid metabolism, E3L and E3L.CETP mice were fed a regular chow diet with or without 0.03% (w/w) bexarotene for 3 weeks. Similarly as in humans, bexarotene increased plasma TG both in E3L mice (+47%, P<0.05) (Figure 3A) and in E3L.CETP mice (+29%, P<0.01) (Figure 3B) compared to control-treated mice. Although bexarotene did not affect plasma TC in E3L mice (Figure 3C) and E3L.CETP mice (Figure 3D), bexarotene strongly decreased HDL-C in E3L.CETP mice (-56%, P<0.01) (Figure 3F), but not in E3L mice (Figure 3E). Similarly, bexarotene decreased apoAI levels in E3L.CETP mice (-31%, P<0.05) (Figure 3H) but not in E3L mice (Figure 3G).

Lipoprotein profiling showed that the rise in TG upon bexarotene treatment was confined to VLDL in both E3L mice (Figure 4A) and E3L.CETP mice (Figure 4B). Whereas bexarotene did not affect the distribution of cholesterol over lipoproteins in E3L mice (Figure 4C), bexarotene shifted cholesterol from HDL to VLDL in E3L.CETP mice (Figure 4D). These combined data show that CETP expression does not influence the hypertriglyceridemic effect of bexarotene, but is crucial for the bexarotene-induced shift in cholesterol from HDL to VLDL.

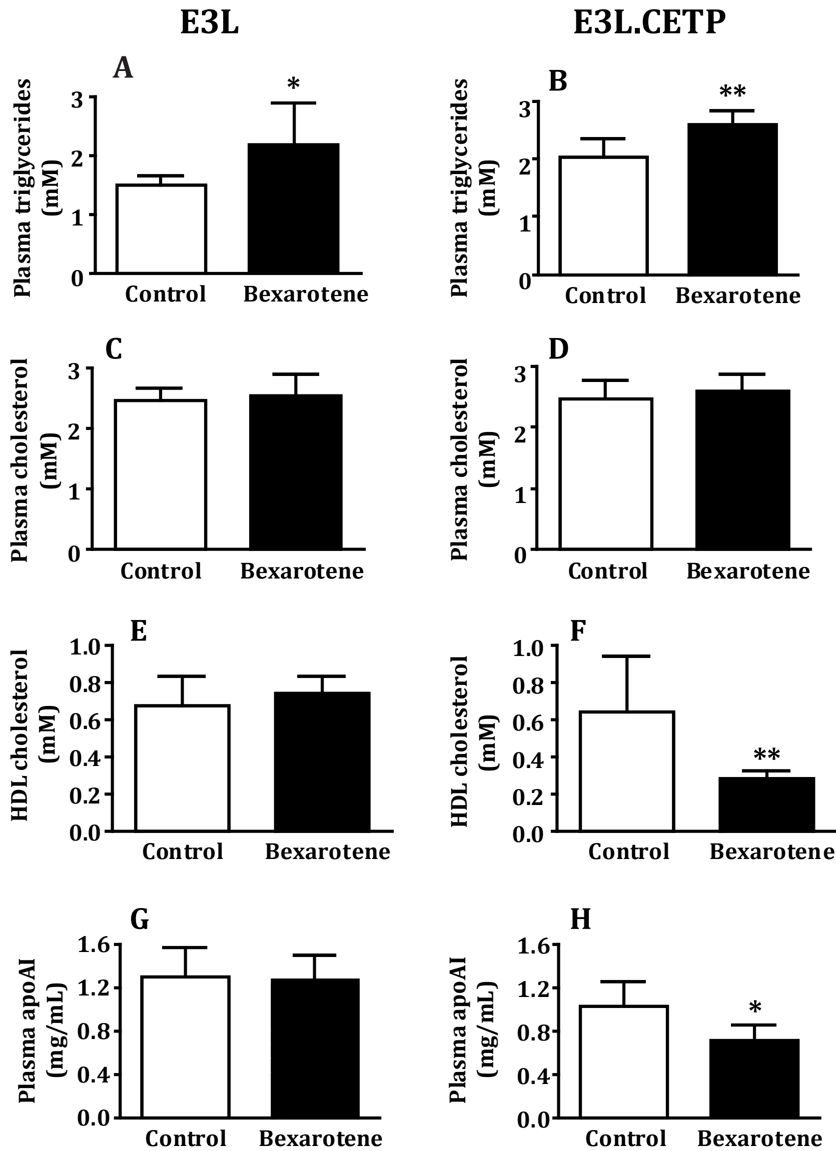


Figure 3. Bexarotene increases plasma TG in E3L and E3L.CETP mice, and decreases HDL-C and apoAI levels only in E3L.CETP mice. E3L (A, C, E, G) and E3L.CETP (B, D, F, H) mice were fed a diet without or with 0.03% bexarotene. After 3 weeks blood was drawn and plasma was assayed for TG (A, B) and cholesterol (C, D). Furthermore, plasma HDL-C (E, F) and apoAI (G, H) were determined. Values are means ± SD (n=7-8, $^*P<0.05$; $^{**}P<0.01$ compared to the control group, Mann-Whitney *U* test).

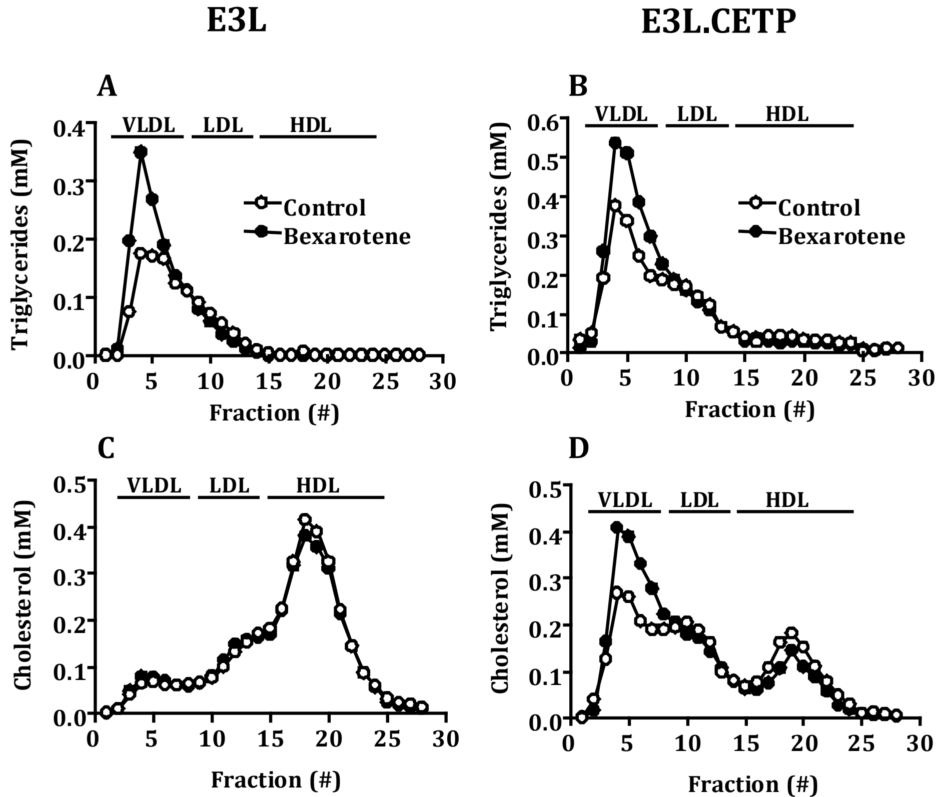


Figure 4. Bexarotene increases VLDL-TG in E3L and E3L.CETP mice, and decreases HDL-C only in E3L.CETP mice. E3L (A, C) and E3L.CETP (B, D) mice were fed a diet with or without 0.03% bexarotene. After 3 weeks blood was drawn and plasma was pooled per group. Pooled plasma samples were fractionated using FPLC and the individual fractions were assayed for TG (A) and TC (B).

Bexarotene does not affect VLDL-TG clearance in E3L.CETP mice

Since bexarotene similarly affected both TG levels and cholesterol distribution in E3L. CETP mice as in humans, further elucidation of the effects of bexarotene on plasma lipid metabolism was performed using E3L.CETP mice. First, we analyzed the cause of the bexarotene-induced hypertriglyceridemia. To investigate whether bexarotene decreased TG clearance, the effect of bexarotene on the clearance of [^{3}H]TO and [^{14}C]CO double-labeled TG-rich VLDL-like emulsion particles was investigated. Bexarotene treatment did not affect the plasma half-life of [^{3}H]TO as compared to control treatment (t½ = 6.0±0.8 min vs. 6.8±1.4 min) (Figure 5A), nor the uptake of [^{3}H]TO-derived fatty acid by the various organs (Figure 5B). [^{14}C]CO disappeared at a slower rate from plasma than [^{3}H]TO, but bexarotene treatment did not affect its plasma half-life (t½ = 11.5±1.8 min vs. 12.4±2.6 min) (Figure 5C). Likewise, the uptake of [^{14}C]CO was not different for any organ but for the liver (Figure 5D), which is explained by a slightly increased liver

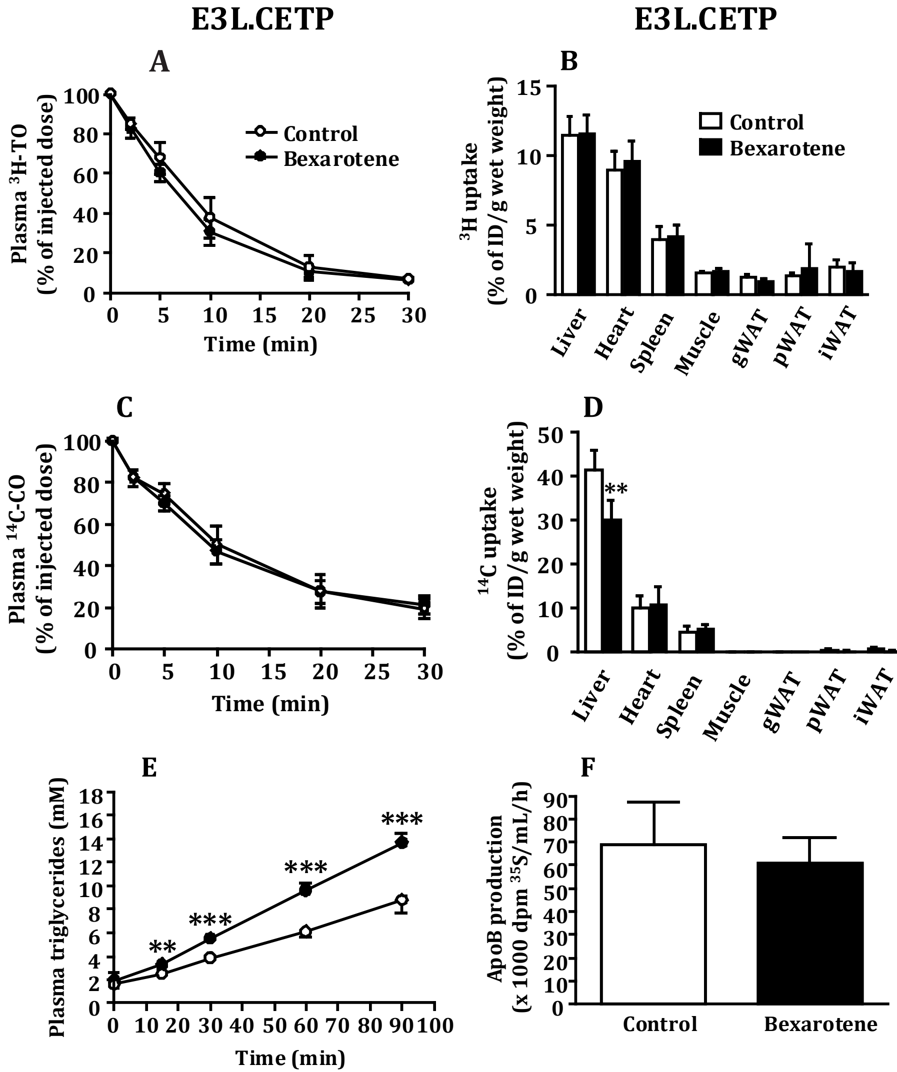


Figure 5. Bexarotene does not affect the clearance of VLDL-like TG-rich particles and increases VLDL-TG production without affecting VLDL-apoB production in E3L.CETP mice. E3L.CETP mice were fed a diet with or without 0.03% bexarotene for 3 weeks. For VLDL-like particle clearance studies, mice received an intravenous bolus of [^{3}H]TO and [^{14}C]CO double-labeled VLDL-like emulsion particles (1 mg TG per mouse). At 2, 5, 10, 20 and 30 minutes after injection, blood samples were taken and ^{3}H (A) and ^{14}C (C) activities in plasma were counted and calculated as a percentage of the injected dose (ID). At 30 minutes after injection, mice were euthanized, organs were isolated and uptake of ^{3}H (B) and ^{14}C (D) activity by the organs was measured. For VLDL production studies, mice received intravenous injections of Tran^{35}S to label protein and Triton WR1339 to block lipolysis. At 0, 15, 30, 60 and 90 minutes after injections, blood samples were taken and plasma was assayed for TG (E). TG production rates were determined by linear regression analysis. At 120 min, mice were exsanguinated and VLDL was isolated and assayed for ^{35}S-apoB (F). Values are means ± SD (n=7-8, **P<0.01; ***P<0.001 compared to control group).

weight observed in bexarotene-treated mice.

Bexarotene increases VLDL-TG production in E3L.CETP mice

As no difference was observed in TG clearance between E3L.CETP mice with or without bexarotene treatment, it is likely that the bexarotene-induced increase in TG levels can be explained by an increased rate of VLDL-TG production. Indeed, the VLDL-TG production rate was strongly increased in mice treated with bexarotene compared to control mice (+68%, P<0.01) (Figure 5E), whereas ^{35}S-apoB production was not changed (Figure 5F). These data indicate that bexarotene increases TG levels by enhancing VLDL-TG production without affecting VLDL particle production.

Table 1. Hepatic expression levels of genes involved in VLDL production.

Gene	Control	Bexarotene
Fas	1.00 (0.29)	1.90 (0.83) *
Scd1	1.00 (0.27)	3.71 (1.31) **
Apob	1.00 (0.25)	0.84 (0.26)

Hepatic gene expression levels of control and bexarotene-treated E3L.CETP mice. Gene expression levels are normalized relative to the control group. *Fas*, fatty acid synthase; *Scd1*, stearyl-coenzyme A desaturase 1; *Apob*, apolipoprotein B. Values are means (SD). *P<0.05; **P<0.01 compared to control group.

Analysis of the hepatic expression of genes involved in VLDL production showed that bexarotene increased the expression of the lipogenic genes fatty acid synthase (*Fas*) (1.9-fold; P<0.05) and stearyl-coenzyme A desaturase 1 (*Scd1*) (3.7-fold; P<0.01), while the expression of *Apob* was not changed (Table 1).

Bexarotene increases CETP activity and mass in E3L.CETP mice

By comparing the effects of bexarotene on the plasma cholesterol distribution in E3L and E3L.CETP mice (Figures 3 and 4), it became clear that the bexarotene-induced decrease in HDL-C is dependent on CETP expression. To determine if this effect is dependent on CETP expression *per se* or if bexarotene affects CETP activity, mass and/or expression, plasma of E3L.CETP mice with or without bexarotene treatment was assayed for CETP activity (Figure 6A) and mass (Figure 6B). Furthermore, mRNA was isolated from livers of mice with or without bexarotene treatment, and expression of CETP mRNA was determined. Mice treated with bexarotene both had a higher plasma CETP activity (+41%, P<0.01) and CETP mass (+41%, P<0.01), whereas bexarotene did not change hepatic CETP expression (Figure 6C).

Discussion

Patients treated with bexarotene, a chemotherapeutic agent used for the treatment of cutaneous T-cell lymphoma, frequently develop dyslipidemia. In this study we show that bexarotene increases VLDL-TG and VLDL-C, while decreasing HDL-C in humans.

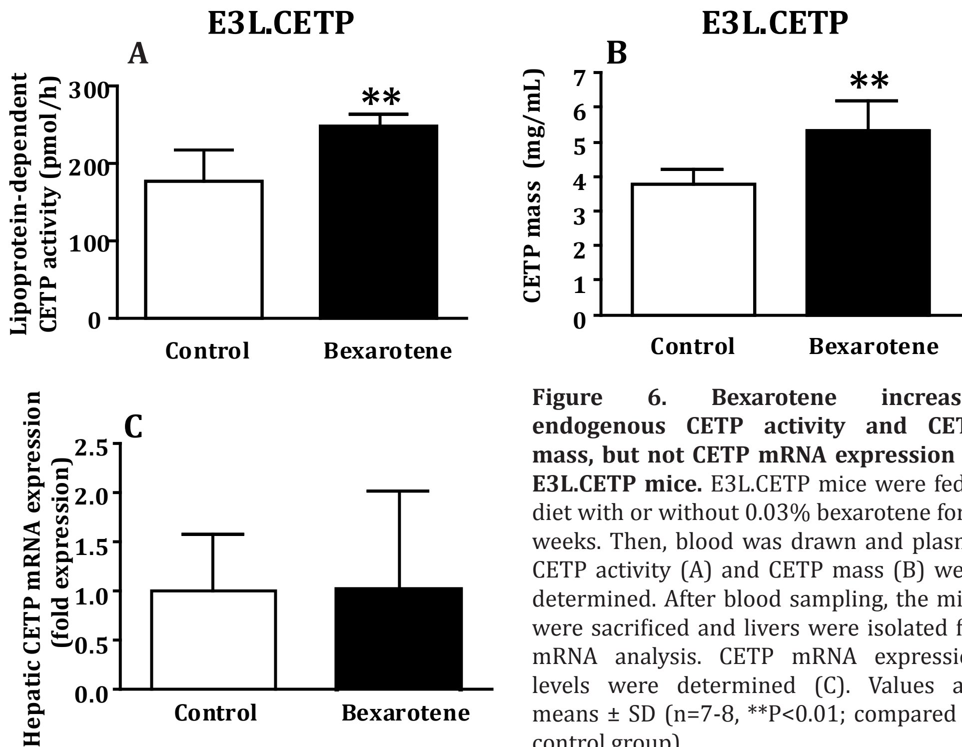


Figure 6. Bexarotene increases endogenous CETP activity and CETP mass, but not CETP mRNA expression in E3L.CETP mice. E3L.CETP mice were fed a diet with or without 0.03% bexarotene for 3 weeks. Then, blood was drawn and plasma CETP activity (A) and CETP mass (B) were determined. After blood sampling, the mice were sacrificed and livers were isolated for mRNA analysis. CETP mRNA expression levels were determined (C). Values are means ± SD (n=7-8, **P<0.01; compared to control group).

Studies in E3L and E3L.CETP mice provide evidence that increased VLDL-TG production is the cause of the bexarotene-induced hypertriglyceridemia. Furthermore, our data demonstrate that the shift in cholesterol distribution upon bexarotene treatment is a consequence of increased endogenous CETP activity.

Our present data show that bexarotene induces hypertriglyceridemia in patients with metastases of DTC. This is in accordance with the hypertriglyceridemic effect of bexarotene observed earlier in other human studies in patients with cutaneous T-cell lymphoma [8,26,27]. In addition we show by experimental studies that bexarotene also induces hypertriglyceridemia in mice, irrespective of CETP expression, by increasing VLDL-TG production rather than by reducing VLDL clearance. The observation that bexarotene did not decrease the clearance of VLDL-TG in vivo suggests that earlier findings that bexarotene increased the expression of the LPL-inhibitors apoCIII [10] and Angptl3 [9] may not be relevant for the observed hypertriglyceridemic effect of bexarotene *in vivo*.

It is interesting to speculate about the mechanism underlying the increasing effect of bexarotene on the VLDL-TG production. This increase in VLDL-production could be the result of increased expression of lipogenic LXR target genes through bexarotene-induced stimulation of LXR-RXR heterodimerisation. Indeed, in line with a previous report [9], we found that bexarotene upregulated the hepatic expression of the LXR-target

genes *Fas* and *Scd1*, which are both involved in the *de novo* production of TG in the liver. In contrast, the expression of *Apob*, a gene involved in lipid metabolism but not under control of LXR, was not changed. The hypothesis of bexarotene-induced LXR-RXR heterodimerisation is also supported by a recent report showing that the bexarotene-induced increase in TG levels is not observed in LXRa/b knock out mice [28]. In fact, LXR agonism in mice by a dual LXRa/b agonist on VLDL production showed that this LXR agonist increases the VLDL-TG production rate, without affecting apoB production [29], which is similar to our current observations with the RXR agonist bexarotene. Taken together, these data support the hypothesis that bexarotene increases the LXR-RXR mediated transcription of LXR target genes leading to increased VLDL-TG production, without affecting VLDL particle production.

We show that bexarotene not only increases plasma TG, but also increases plasma total cholesterol in humans. This is in agreement with previous observations by others [26,27], albeit that those studies did not discriminate between cholesterol associated with the various lipoproteins. We now reveal that the increased cholesterol levels are a combined result of increased VLDL-C and decreased HDL-C. Mouse studies investigating the effect of bexarotene or other RXR agonists on HDL-C have shown conflicting data [9,11]. However, it should be noted that, in contrast to humans, mice naturally lack expression of CETP, which is a crucial determinant for distribution of plasma cholesterol over the various lipoprotein fractions. In fact, we have shown previously that the HDL-C modulating effects of atorvastatin [13], fenofibrate [14], niacin [15] and torcetrapib [16] are CETP-dependent. Similarly, we show here that the HDL-C lowering effect of bexarotene is also mediated through CETP, as the HDL-C lowering effect is seen in E3L.CETP mice, but not in E3L mice.

The question arises whether the bexarotene-induced shift of cholesterol over lipoproteins in plasma results from expression of CETP *per se*, or whether an additional effect of bexarotene on CETP expression is involved. Previous studies have indicated that liver cholesterol content is positively correlated with hepatic CETP mRNA expression and plasma CETP concentration [13-15,30,31]. The lipogenic effect of bexarotene suggested a bexarotene-induced increase in liver cholesterol content and thereby of CETP expression via LXR activation. However, liver lipid analysis did not reveal an effect of bexarotene on the hepatic cholesterol content (1.09±0.14 μg cholesterol/mg liver without bexarotene treatment vs. 0.99±0.11 μg cholesterol/mg liver with bexarotene treatment in E3L.CETP mice), indicating LXR mediated gene transcription is probably not increased via increased hepatic cholesterol, but just through increased LXR/RXR heterodimerisation. In line with this hypothesis, the bexarotene-induced expression of the LXR-target genes *Fas* and *Scd1* is less pronounced than the induction observed after stimulation with a typical activator of LXR, T0901317 [32]. Apparently, the effect of bexarotene on the LXR-pathway is limited and too weak to increase hepatic CETP expression. The observation that bexarotene increases CETP mass in plasma of mice is thus not caused by increased mRNA expression but may result from either increased CETP synthesis via posttranscriptional pathways or decreased CETP clearance from

plasma. CETP gene expression is similarly regulated in E3L.CETP mice and humans, as E3L.CETP mice express human CETP under control of its own promoter and regulatory flanking regions. Despite this, bexarotene increases CETP mass in mice and not in humans by an as yet unknown mechanism.

In humans and mice bexarotene increases endogenous, i.e. lipoprotein dependent, CETP activity in plasma. TG are a driving force of CETP activity [33]. Also, our data showing that bexarotene increases TG levels in E3L mice (in absence of CETP), indicate that increased TG is rather a cause than a consequence of increased CETP activity. These data suggest that the bexarotene-induced increase in VLDL-TG induces the increase in CETP activity that we observed. This increased CETP activity would then cause a decrease in HDL-C and a concomitant increase in VLDL-C. Thus, the shift in cholesterol distribution upon bexarotene treatment is likely the effect of an increased VLDL-TG production and a resulting increase of the endogenous CETP activity.

The findings of this study support the current clinical practice to initiate lipid lowering therapy in patients using bexarotene who develop relevant hypertriglyceridemia. Statins are the preferable therapy, as no interaction between bexarotene and statins has been demonstrated. Fibrates are not recommended, because concomitant use of gemfibrozil has been shown to raise plasma bexarotene levels. It is however not clear yet if the bexarotene-induced lipid abnormalities in humans represent an adverse cardiovascular risk profile: interestingly, bexarotene prevented the development of atherosclerotic lesions in APOE2 knock-in mice, despite the induction of hypertriglyceridemia [9]. However, it should be realized that these APOE2 knock-in mice lack CETP expression.

In conclusion, we show that bexarotene causes hypertriglyceridemia by increasing the VLDL-TG production rate. Furthermore, bexarotene increases VLDL-C and decreases HDL-C through increasing the CETP-dependent transfer of cholesterol from HDL to VLDL, as a consequence of an increased VLDL-TG pool.

Acknowledgements

We thank I. Mol (Leiden University Medical Center, Leiden, The Netherlands) for excellent technical assistance. Sources of Funding: This work was performed within the framework of the Leiden Center for Cardiovascular Research LUMC-TNO and supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.), the Centre for Medical Systems Biology (CMSB115 to L.M.H.) within the framework of the Netherlands Genomics Initiative (NGI), and the Netherlands Organization for Scientific Research (NWO grant 908-02-097 to L.M.H. and NWO VIDI grant 917.36.351 to P.C.N.R.).

References

1. Gniadecki R, Assaf C, Bagot M, Dummer R, Duvic M, Knobler R, Ranki A, Schwandt P, Whittaker S. The optimal use of bexarotene in cutaneous T-cell lymphoma. *Br. J. Dermatol.* 2007; 157: 433-440.

2. Liu YY, Stokkel MP, Pereira AM, Corssmit EP, Morreau HA, Romijn JA, Smit JW. Bexarotene increases uptake of radioiodide in metastases of differentiated thyroid carcinoma. *Eur. J. Endocrinol.* 2006; 154: 525-531.
3. Esteva FJ, Glaspy J, Baidas S, Laufman L, Hutchins L, Dickler M, Tripathy D, Cohen R, DeMichele A, Yocum RC, Osborne CK, Hayes DF, Hortobagyi GN, Winer E, Demetri GD. Multicenter phase II study of oral bexarotene for patients with metastatic breast cancer. *J. Clin. Oncol.* 2003; 21: 999-1006.
4. Rigas JR and Dragnev KH. Emerging role of rexinoids in non-small cell lung cancer: focus on bexarotene. *Oncologist.* 2005; 10: 22-33.
5. Smit JV, Franssen ME, de Jong EM, Lambert J, Roseeuw DI, De WJ, Yocum RC, Stevens VJ, van De Kerkhof PC. A phase II multicenter clinical trial of systemic bexarotene in psoriasis. *J. Am. Acad. Dermatol.* 2004; 51: 249-256.
6. Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* 1995; 9: 1033-1045.
7. Hokanson JE and Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. *J. Cardiovasc. Risk*. 1996; 3: 213-219.
8. Farol LT and Hymes KB. Bexarotene: a clinical review. *Expert. Rev. Anticancer Ther.* 2004; 4: 180-188.
9. Lalloyer F, Fievet C, Lestavel S, Torpier G, van d, V, Touche V, Bultel S, Yous S, Kuipers F, Paumelle R, Fruchart JC, Staels B, Tailleux A. The RXR agonist bexarotene improves cholesterol homeostasis and inhibits atherosclerosis progression in a mouse model of mixed dyslipidemia. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2731-2737.
10. Vu-Dac N, Gervois P, Torra IP, Fruchart JC, Kosykh V, Kooistra T, Princen HM, Dallongeville J, Staels B. Retinoids increase human apo C-III expression at the transcriptional level via the retinoid X receptor. Contribution to the hypertriglyceridemic action of retinoids. *J. Clin. Invest*. 1998; 102: 625-632.
11. Mukherjee R, Strasser J, Jow L, Hoener P, Paterniti JR, Jr., Heyman RA. RXR agonists activate PPARalpha-inducible genes, lower triglycerides, and raise HDL levels in vivo. *Arterioscler. Thromb. Vasc. Biol.* 1998; 18: 272-276.
12. Ha YC, Calvert GD, Barter PJ. In vivo metabolism of esterified cholesterol and apoproteins in rabbit plasma low density lipoproteins. *Atherosclerosis*. 1979; 34: 451-455.
13. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM, Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008; 197: 57-63.
14. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM, Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *J. Lipid Res.* 2007; 48: 1763-1771.
15. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC, Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arterioscler. Thromb. Vasc. Biol.* 2008; 28: 2016-2022.
16. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM, Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory

lesions than atorvastatin. *Circulation*. 2008; 117: 2515-2522.

17. Zadelaar S, Kleemann R, Verschuren L, de Vries-van der Weij J, van der Hoorn J, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 1706-1721.
18. Liu YY, Stokkel MP, Morreau HA, Pereira AM, Romijn JA, Smit JW. Radioiodine therapy after pretreatment with bexarotene for metastases of differentiated thyroid carcinoma. *Clin. Endocrinol. (Oxf)*. 2008; 68: 605-609.
19. Smit JW, Stokkel MP, Pereira AM, Romijn JA, Visser TJ. Bexarotene-induced hypothyroidism: bexarotene stimulates the peripheral metabolism of thyroid hormones. *J. Clin. Endocrinol. Metab*. 2007; 92: 2496-2499.
20. Westerterp M, de Haan W, Berbee JF, Havekes LM, Rensen PC. Endogenous apoC-I increases hyperlipidemia in apoE-knockout mice by stimulating VLDL production and inhibiting LPL. *J. Lipid Res.* 2006; 47: 1203-1211.
21. Gautier T, Tietge UJ, Boverhof R, Perton FG, Le GN, Masson D, Rensen PC, Havekes LM, Lagrost L, Kuipers F. Hepatic lipid accumulation in apolipoprotein C-I-deficient mice is potentiated by cholesteryl ester transfer protein. *J. Lipid Res.* 2007; 48: 30-40.
22. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der Greef J, van Ommen B, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol.* 2007; 8: R200.
23. Post SM, Groenendijk M, Solaas K, Rensen PC, Princen HM. Cholesterol 7alpha-hydroxylase deficiency in mice on an APOE*3-Leiden background impairs very-low-density lipoprotein production. *Arterioscler. Thromb. Vasc. Biol.* 2004; 24: 768-774.
24. Rensen PC, van Dijk MC, Havenaar EC, Bijsterbosch MK, Kruijt JK, van Berkel TJ. Selective liver targeting of antivirals by recombinant chylomicrons--a new therapeutic approach to hepatitis B. *Nat. Med.* 1995; 1: 221-225.
25. Jong MC, Rensen PC, Dahlmans VE, van der Boom H, van Berkel TJ, Havekes LM. Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice. *J. Lipid Res.* 2001; 42: 1578-1585.
26. Duvic M, Martin AG, Kim Y, Olsen E, Wood GS, Crowley CA, Yocum RC. Phase 2 and 3 clinical trial of oral bexarotene (Targretin capsules) for the treatment of refractory or persistent early-stage cutaneous T-cell lymphoma. *Arch. Dermatol.* 2001; 137: 581-593.
27. Assaf C, Bagot M, Dummer R, Duvic M, Gniadecki R, Knobler R, Ranki A, Schwandt P, Whittaker S. Minimizing adverse side-effects of oral bexarotene in cutaneous T-cell lymphoma: an expert opinion. *Br. J. Dermatol.* 2006; 155: 261-266.
28. Lalloyer F, Pedersen TA, Gross B, Yous S, Vallez E, Gustafsson JA, Mandrup S, Fievet C, Staels B, Tailleux A. Bexarotene-activated RXR/LXR heterodimer in liver modulates triglyceride metabolism but not cholesterol homeostasis in vivo. *Atherosclerosis Supplements*. 2008; 9: 25 (Abstract).
29. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J. Biol. Chem.* 2002; 277: 34182-34190.
30. Jiang XC, Agellon LB, Walsh A, Breslow JL, Tall A. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on

natural flanking sequences. *J. Clin. Invest*. 1992; 90: 1290-1295.

31. Luo Y and Tall AR. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *J. Clin. Invest*. 2000; 105: 513-520.
32. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B. Role of LXRs in control of lipogenesis. *Genes Dev.* 2000; 14: 2831-2838.
33. Tall A. Plasma lipid transfer proteins. *Annu. Rev. Biochem.* 1995; 64: 235-257.

Chapter 8

General Discussion

Cardiovascular disease (CVD) is one of the major causes of death in the Western society and is mainly caused by atherosclerosis. Hypercholesterolemia is an important risk factor for the development of atherosclerosis, and several treatment strategies aim at reducing plasma cholesterol levels (e.g. statins) to reduce cardiovascular disease prevalence. Unfortunately, statins prevent only about one third of all cardiovascular events. Therefore, other strategies to treat patients at risk for cardiovascular disease are needed. Since statins are very efficient in lowering low density lipoprotein (LDL)-cholesterol (C), therapeutic strategies targeting risk factors other than LDL-C have the strongest potential to have an additional beneficial effect on top of statin treatment in reducing CVD. Two factors are increasingly recognized as risk factors for CVD development: inflammation and low levels of high density lipoprotein (HDL)-C. An important protein in HDL metabolism is the cholesteryl ester transfer protein (CETP), which transfers cholesteryl esters (CE) from HDL to (very) low density lipoprotein ((V)LDL) in exchange for triglycerides (TG). By facilitating this transfer, CETP thus lowers HDL-C levels and increases (V)LDL-C levels and is therefore regarded a risk factor for the development of atherosclerosis.

The studies described in this thesis evaluate the role of inflammation and of CETP, besides that of LDL-C, in atherosclerosis. In this chapter, the main conclusions and the implications of our findings are discussed.

Inflammation

Inflammation has been shown to play an important role in various stages of atherosclerosis development, starting with the activation of endothelial cells (ECs). This is followed by recruitment of leukocytes (T-cells, neutrophils, macrophages) into the vasculature, forming an early atherosclerotic lesion. Amplification of the inflammatory response by macrophages leads to progression into a more complex lesion, which may eventually rupture. The inflammatory stimuli that contribute to atherosclerosis development and progression can either have a local or a non-vascular origin. Non-vascular sources of inflammation that have been shown to be risk factors for atherosclerosis development include chronic infection, inflammatory diseases such as rheumatoid arthritis or systemic lupus erythematosus, and nutrient surplus.[1-3] This part of the general discussion will focus on metabolic inflammation (i.e. originating from nutrient surplus) and on vascular inflammation as processes that affect atherosclerosis development and that may be modulated to reduce CVD.

Metabolic inflammation

Metabolic inflammation is different from ‘classic’ acute phase inflammation in several aspects. Acute phase inflammation is a strong response of the host to fight infection. This type of inflammation has a short duration and is principally beneficial. In contrast, metabolic inflammation is a form of chronic, low-grade inflammation, which has detrimental effects as it is associated with the development of metabolic syndrome and

CVD.[4] The mechanisms underlying the development of metabolic inflammation have not been fully elucidated as yet. Since it is difficult to gain mechanistic insight in (patho) physiological processes underlying a disease from human studies, animal models have been used for this purpose. In this thesis, we used the APOE*3-Leiden (E3L) mouse model to gain insight in processes involved in the development of inflammation and in atherosclerotic disease. E3L mice develop atherosclerotic lesions that are similar in pathology to human atherosclerotic lesions, and these mice respond to lipid-lowering drugs in a similar way as humans do. Furthermore, high cholesterol diet feeding induces a hepatic inflammatory response in E3L mice,[5] similar to what has been reported in humans.[6]

A recent study showed that E3L mice fed a high cholesterol diet, accompanied with induction of hepatic inflammation, develop more atherosclerosis than E3L mice fed a low cholesterol diet, accompanied by a non-inflammatory status of the liver.[5] These data suggest that a detrimental chronic liver-derived inflammatory status can be induced by metabolic dysregulation, thus accelerating atherosclerosis. In our Western society, abundance of food leads to an increased prevalence of chronic inflammation resulting from metabolic dysregulation. Nutrients such as fatty acids and glucose, to which individuals are nowadays exposed in high quantities, can induce ER stress, leading to the induction of an inflammatory response in metabolically active tissues such as liver and adipose tissue.[4,7,8] Since it was not known how cholesterol induces inflammation, we studied this by feeding E3L mice a diet with a low dose of cholesterol (not causing inflammation) or a high dose of cholesterol (causing low-grade hepatic inflammation). By studying cholesterol homeostasis we showed that a high amount of dietary cholesterol disturbs cholesterol homeostasis, leading to increased hepatic free cholesterol (FC) levels. Increased FC then induces ER stress and a concomitant inflammatory response. (**Chapter 2**) The ER thus seems to be important in sensing metabolic surplus of different nutrients and in the translation of this metabolic surplus into an inflammatory response.

The simplest strategy to tackle metabolic inflammation is of course reducing metabolic overload by keeping a healthy diet. Since in practice this measure proves to be very difficult for a lot of people, therapeutic strategies may be developed to reduce metabolic inflammation. One approach could be the development of drugs that increase the capacity of the liver to process nutrients such as cholesterol. We showed that two drugs that modulate lipid metabolism, rosuvastatin and fenofibrate, normalize the HC-diet induced increase in hepatic FC levels and concomitantly reduce hepatic inflammation (**Chapter 2**). Normalization of hepatic FC levels may thus contribute to the anti-inflammatory nature of these drugs. Another drug that could be useful to reduce metabolic inflammation is salicylate. We showed that salicylate can partly suppress high cholesterol diet-induced inflammation in E3L mice. (**Chapter 3**) Interestingly, our data also show that adding salicylate to a high cholesterol diet strongly and acutely reduces plasma cholesterol levels. (**Chapter 3**) Although the mechanism by which salicylate reduces plasma cholesterol levels is not known yet, these data underscore the notion

that lipid metabolism and inflammation are strongly intertwined processes, although much remains to be learned about their relationship. A different approach to target metabolic inflammation may be via reduction of ER stress. Since ER stress seems to be a common denominator underlying the induction of metabolic inflammation by various nutrients,(5-7 and **Chapter 2**) drugs that reduce ER stress or inhibit downstream mediators of ER stress may prove to be attractive tools to reduce metabolic inflammation and thereby CVD.

Vascular inflammation

In the vasculature, macrophages seem an interesting target, since these cells are abundantly present in lesions, and upon accumulation of lipids they reside in the lesion where they amplify the inflammatory response. Recent evidence suggests that macrophages in the atherosclerotic lesion consist of two subtypes: M1 or 'classically' activated macrophages represent inflammatory macrophages, while M2 or 'alternatively' activated macrophages represent anti-inflammatory macrophages. The two types of macrophages can be distinguished based on the expression of specific markers, such as TNFα, MCP-1 and IL-6 (classic) or mannose receptor, CD163 and IL-10 (alternative).[9] At present, it is thought that classic macrophages have a detrimental role and that alternative macrophages have a beneficial role in atherosclerotic disease progression.

A number of ways in which the amount or the type of macrophages in atherosclerotic lesions could be influenced include: 1) reducing the influx of new macrophages, 2) changing the ratio of classic to alternative macrophages in atherosclerotic lesions, 3) increasing the turn-over rate of macrophages in the lesion, 4) stimulating the efflux of cholesterol out of macrophages and 5) stimulating emigration of macrophages out of lesions.

The influx of new macrophages may be reduced by suppressing the expression of adhesion molecules on ECs. Several studies have shown that atherosclerosis development is reduced in mice that lack the expression of one specific adhesion molecule.[10] Strategies to inhibit the function of a specific adhesion molecule, for example by administering a monoclonal antibody against this adhesion molecule, are now under development. So far, no effects of such therapies on atherosclerosis development have been reported. However, drugs targeting one specific adhesion molecule may not be very successful, as other adhesion molecules then can still bind leukocytes, which will enter the vessel wall. Therefore, reducing the general inflammatory status of ECs by targeting a central mediator of adhesion molecule expression, NF-κB, may be a more promising strategy. By studying the mechanisms underlying the atheroprotective effect of the LXR-agonist T-0901317 on atherosclerosis development in E3L mice, we showed that T-0901317 reduced the amount of active NF-κB in ECs, which was accompanied by reduced ICAM-1 and E-selectin expression, a reduction in the number of monocytes adhering to the endothelium and a reduced macrophage content of lesions. (**Chapter 4**) Reducing adhesion molecule expression thus is an important strategy, as the presence of macrophages in the vessel wall per se characterizes the early atherosclerotic lesion

and as macrophages play a central role in the progression into a lesion that is eventually prone to rupture and to cause thrombosis.

Changing the ratio of classic to alternative macrophages in atherosclerotic lesions would ideally be done by changing the phenotype of a resident macrophage from classic to alternative. However, it was recently shown that upon activation of PPARγ in macrophages, the macrophage phenotype could not be changed anymore. Alternatively, circulating monocytes can be targeted to shift their phenotype from classic to alternative, e.g. by PPARγ activation.[11] As yet, the effect of shifting the monocyte population from classic to alternative monocytes on atherosclerosis is not known. It is possible that a relative increase in alternative monocytes in the circulation may lead to an enhanced recruitment of alternative monocytes to the site of lesion development or of lesions in regression. Once having entered the vessel wall and having differentiated into macrophages, the alternative macrophages may beneficially affect processes involved in lesion remodelling. More research is needed to investigate the effects of shifting the balance between monocyte subsets on atherosclerotic lesion development and regression.

Increasing macrophage turnover could lead to a decrease in the macrophage content of lesions. Important processes determining the cell turnover rate are cell survival and apoptosis. In atherosclerosis development, apoptosis can both be beneficial and detrimental, depending on the stage of lesion development.[12] In early atherosclerotic lesions, apoptosis can reduce accumulation of macrophages and thereby reduce progression into more advanced lesions.[13-15] On the other hand, in advanced atherosclerotic lesions, apoptotic macrophages that are not efficiently cleared by efferocytosis may undergo 'secondary' necrosis, thereby increasing the inflammatory phenotype of lesions and thus the risk for plaque rupture and thrombosis.[12,16] The role of apoptosis under regressive conditions has not been studied yet. However, one could reason that apoptosis contributes to plaque regression under certain conditions. Upon plasma cholesterol lowering, lipid deposition in the vessel wall will decrease. As a result, there is less need for macrophages to clean up lipid deposits in the vessel wall. Macrophages that undergo apoptosis may then be efficiently efferocytosed by newly infiltrating macrophages that would otherwise clean up lipid deposits. In this way, apoptosis does not leave any cell residues and thus does not increase the inflammatory phenotype of the lesion, but contributes to the reduction in macrophage content that is the first step for lesion regression. Interestingly, our study evaluating the role of the NF-κB inhibitor salicylate on lesion regression in E3L mice suggests that increased apoptosis may be one of the mechanisms underlying the rapid disappearance of macrophages from pre-existing lesions. (**Chapter 3**) Although more research is needed, induction of macrophage apoptosis may provide a strategy that promotes lesion regression, at least when efferocytosis of apoptotic cells is efficient and no inflammatory residues of apoptotic cells remain.

Accumulation of free cholesterol (FC) in macrophages has been shown to activate NF-κB, leading to increased cytokine production.[17] Furthermore, macrophages

that lack the cholesterol exporters ATP binding cassette transporter (ABC)A1 and ABCG1 also accumulate FC and show concomitantly increased expression of inflammatory genes.[18] As a consequence, macrophage inflammation might be reduced by stimulating cholesterol efflux. Potential strategies to increase cholesterol efflux are activation of LXR, PPARα or PPARγ. Activation of either of these nuclear receptors was shown to increase the expression of ABCA1 and/or ABCG1, which could increase cholesterol efflux.[19,20] (**Chapter 4**) It is important to note that increasing the expression of cholesterol transporters in macrophages likely is only effective if the plasma of a subject has sufficient capacity to mediate cholesterol efflux through extracellular cholesterol acceptors (i.e. apoAI, apoE, HDL).

Another treatment strategy that involves macrophages is stimulating emigration of macrophages out of pre-existing lesions. Plasma cholesterol lowering has been shown to be an important trigger for emigration of macrophages out of lesions to lymph nodes via a C-C chemokine receptor (CCR)7-dependent mechanism.[21-23] We showed that treating E3L mice with the LXR agonist T-0901317 strongly reduced the macrophage content of pre-existing lesions. This was likely the result of increased CCR7 expression, leading to increased emigration of macrophages out of pre-existing lesions. Interestingly, the reduction in macrophages induced by T-0901317 occurred even in the presence of high plasma cholesterol levels, (**Chapter 4**) showing that plasma cholesterol lowering is not a prerequisite for macrophage emigration. As the field of macrophage emigration is just starting to be explored, other factors that can stimulate macrophage emigration may be discovered in the future. It would be interesting to find out if additional chemokine receptors besides CCR7 can also stimulate macrophage emigration.

We showed that a number of processes that affect macrophage inflammation can be positively influenced by T-0901317. Unfortunately, T-0901317 and other LXR-agonists that have been tested in *in vivo* models cause a fatty liver, hypertriglyceridemia and hypercholesterolemia by increasing *de novo* TG synthesis in the liver, which hampers further development of these compounds. Treatment with LXR-agonists thus only has potential provided that compounds are developed that do not have this detrimental side effect. Hopes are now set on the development of LXR-agonists, which have specificity for LXRβ over LXRα, or which are selective towards macrophages, without targeting the liver. Because of the multiple beneficial effects we identified, such LXR-agonists are expected to have a strong potential to both reduce the development of new atherosclerotic lesions and to induce regression of pre-existing lesions.

HDL-C

HDL-C has been found to be inversely correlated with CVD prevalence in epidemiologic studies, and is therefore thought to exert an anti-atherogenic role. Therefore, HDL-raising therapies are thought to be promising strategies to reduce CVD.

CETP inhibition
One of the first HDL-raising approaches tested in a clinical setting was CETP inhibition. Two compounds, dalcetrapib (JTT-705) and torcetrapib, were tested in humans, where they strongly increased HDL-C levels.[24,25] However, large scale clinical trials evaluating the effect of torcetrapib on CVD progression did not show a beneficial effect on atherosclerosis progression as determined by Intravascular Ultrasound (IVUS) and Intima Media Thickness (IMT) measurements [26-28], and even led to an increase in overall mortality rate and death due to CVD in subjects treated with torcetrapib.[29]

There are a number of explanations for the unexpected results obtained with torcetrapib, that relate to 1) the fact that torcetrapib was tested only in combination with atorvastatin, 2) the functionality of the HDL particles resulting from torcetrapib treatment, 3) the fact that CETP affects both (V)LDL and HDL particle levels, 4) possible effects of CETP on plaque stability and 5) compound-specific side-effects of torcetrapib.

First, in the large clinical trials in which the effect of torcetrapib on atherosclerosis progression or cardiovascular endpoints was evaluated, all subjects were treated with atorvastatin and either without or with torcetrapib.[26-29] Atorvastatin treatment resulted in low baseline LDL levels. In addition, atorvastatin treatment has been shown to reduce CETP mass and activity in plasma.[30-32] Since CETP represents a major route for the clearance of HDL-CE from the circulation in humans, it is possible that a reduced level of cholesterol acceptor ((V)LDL) in combination with strongly reduced CETP activity led to disturbed transport of cholesterol to the liver. This reverse cholesterol transport (RCT) defect could mask possible anti-atherogenic effects of torcetrapib treatment *per se*. Interestingly, by investigating the effect of torcetrapib, alone or in combination with atorvastatin, on atherosclerosis development in E3L mice expressing human CETP (E3L.CETP mice), we found that torcetrapib treatment alone reduces atherosclerosis, but that it does not have an additional effect on top of atorvastatin treatment. (**Chapter 5**)

Second, the effect of CETP (inhibition) on apoB-containing lipoprotein levels might be more important with respect to atherosclerosis development than the effect on HDL-C. Torcetrapib treatment led to an increase in HDL-C levels of up to +60%, and to a reduction in LDL-C levels of up to -20%. Therefore, torcetrapib is mainly regarded as an HDL-raising drug. However, the relative contribution of CETP-induced changes in (V)LDL-C and HDL-C levels to atherosclerosis development is unclear. In this thesis, we showed that the increase in VLDL-C levels rather than the decrease in HDL-C levels upon CETP expression in E3L mice can largely explain the atherogenic effect of CETP. (**Chapter 6**) These data are in line with post-hoc analyses of the RADIANCE 1 and 2 trials, showing that cIMT progression was related to changes in LDL-C, but not HDL-C, levels in the torcetrapib-treated group.[33] Since the changes in LDL-C were relatively modest in the torcetrapib trials, this may have contributed to the lack of efficacy of torcetrapib on atherosclerosis progression.

Third, we found indications that CETP may affect plaque stability. By studying the effect of torcetrapib, either alone or in combination with atorvastatin, on

atherosclerosis development in E3L.CETP mice, we found that torcetrapib increased the macrophage content and decreased the collagen content of lesions both when given alone, or in combination with atorvastatin, resulting in the formation of lesions with a more unstable phenotype. (**Chapter 5**) While this study could not discern whether this was a compound specific effect of torcetrapib or a more general effect of CETP inhibition, we showed in a follow-up study that the presence of CETP per se leads to an increased collagen content of atherosclerotic lesions in E3L mice. (**Chapter 6**) Together, these data suggest that CETP positively affects the stability of atherosclerotic lesions by an as yet unknown mechanism, and that CETP inhibition may thus reduce lesion stability. Although plaque rupture usually does not occur in mice, in humans the phenotype of a high lipid content and a low collagen content is considered a risk factor for plaque rupture. Extrapolation of our data to humans thus suggests that increased plaque rupture may (partly) have caused the increase in cardiovascular events and death in the ILLUMINATE trial.

Fourth, torcetrapib may have had compound-specific, adverse side effects that led to an increase in cardiovascular events and cardiovascular death in the torcetrapib-treated group. Torcetrapib was found to increase blood pressure with about 5 mm Hg in clinical trials,[26-29] which effect is compound-specific, as the CETP inhibitors anacetrapib and dalcetrapib do not increase blood pressure. This increase in blood pressure by torcetrapib was found to be related to cIMT progression in the RADIANCE 1 and 2 trials.[33] Although the increase in blood pressure is very small, and not likely to completely explain the increased death rate in the ILLUMINATE trial, it can at least have contributed to the detrimental effects observed. Other effects that were observed in subjects treated with torcetrapib were decreased serum potassium, and increased serum sodium, bicarbonate and aldosterone levels. A recent study showed that the increase in aldosterone was also a compound-specific effect of torcetrapib, as anacetrapib did not share this effect.[34] Although aldosterone can influence blood pressure, this study showed that the acute effect of torcetrapib on blood pressure is independent of aldosterone. Interestingly, aldosterone has been shown to increase atherosclerosis, inflammation and oxidative stress in animal models.[35-37] The observed increase in aldosterone may thus very well have causally contributed to the detrimental outcome of the torcetrapib trials and predominated potentially beneficial effects of torcetrapib-induced increases in HDL-C.

Having given several explanations for the failure of the torcetrapib trials, it is important to note that there are also indications that torcetrapib-treatment has beneficial effects. Subgroup analysis of the ILLUSTRATE trial revealed that there was a dose-dependent, inverse relation between change in HDL-C and change in percent atheroma volume (PAV) in combination-treated subjects, but not in subjects treated with atorvastatin alone. In this combination-treated group, subjects with the highest increases in HDL-C even showed regression of PAV, indicating that the increase in HDL-C level upon torcetrapib treatment is protective against CHD progression.[38]

Overall, it is not yet clear if CETP inhibition provides a safe strategy to increase

HDL-C levels and thereby reduce CVD. Ongoing clinical trials with anacetrapib and dalcetrapib will hopefully shed more light on this question. In the mean time, other strategies to increase HDL-C are also under development. These will be discussed below.

Other strategies to increase HDL-C levels

CETP inhibitors strongly reduce CETP activity, which may have contributed to the negative outcome of the clinical trials, e.g. by reducing the RCT pathway as outlined above. However, it is also possible to reduce hepatic CETP expression, which leads to a milder reduction of plasma CETP activity. It has been shown that the lipid-lowering drugs atorvastatin, fenofibrate and niacin all mildly increase HDL-C levels by reducing hepatic CETP expression, probably by lowering the hepatic lipid content.[31,39,40] Furthermore, we showed that the RXR-agonist bexarotene modulates CETP activity, independent of changes in hepatic lipid levels. (**Chapter 7**) This is most likely an indirect effect of LXR/RXR-mediated transcriptional regulation, suggesting that nuclear receptors could also be targeted to mildly reduce CETP activity.

Niacin currently is the most effective drug used in the clinic to raise HDL-C (with increases up to +35%), and niacin also reduces LDL-C and TG (up to -25% and -50%, respectively).[41] A frequently reported side effect of niacin treatment is flushing, which often leads to discontinuation of the treatment. Combination treatment with the prostaglandin D_2 receptor 1 antagonist laropiprant reduces the flushing, which may thus increase the use of niacin. Ongoing clinical trials evaluating the effect of niacin, when added to a statin, on cardiovascular endpoints (AIM-HIGH and HPS2-THRIVE) will hopefully show that this combination therapy is successful in further reducing CVD.

Strategies to improve HDL functionality

Although HDL-C levels are inversely correlated to CVD, a direct protective effect of increasing HDL-C on CVD has not (yet) been shown. An explanation for this may lie in the functionality of HDL particles. HDL is thought to be protective by mediating reverse cholesterol transport from macrophages to the liver, and by having anti-inflammatory, anti-oxidative and anti-thrombotic properties. Therapeutic approaches that increase HDL-C by interfering in HDL maturation or HDL clearance may negatively affect HDL-functionality. As a consequence, such therapies may not always have an atheroprotective effect. Therefore, therapies aiming at improving HDL functionality rather than at increasing HDL-C levels may prove to be more efficient in reducing CVD.

ApoAI, the most abundant apolipoprotein on HDL, is thought to exert at least part of the potentially beneficial properties of HDL. A therapeutic approach that may improve HDL functionality is thus to increase apoAI levels. One approach to do this is via upregulation of ABCA1, the transporter that mediates the lipidation of apoAI in plasma, thereby preventing the immediate clearance of apoAI by the kidneys. ABCA1 gene expression can be increased using LXR agonists. Increasing apoAI levels is thus another beneficial property of LXR agonists that can be added to the ones mentioned in the section on inflammation. However, LXR agonists may also increase hepatic

CETP expression and thereby CETP activity, which could neutralize its effects on HDL levels. This side effect may be reduced by combined treatment with a lipid-lowering drug that reduces hepatic CETP expression, such as atorvastatin. A new experimental approach to increase apoAI levels targets ABCA1 degradation: a recent report shows that spiroquinone and diphenoquinone, oxidation products of probucol, reduced ABCA1 degradation, which was accompanied by increased HDL-C levels and reduced atherosclerosis development in rabbits.[42] This strategy needs further evaluation to prove its efficacy in humans. Another method that seems promising to increase apoAI levels is upregulation of apoAI expression. The compound RVX-208 is a small molecule drug that was tested in early phase clinical trials, where it increased plasma apoAI and preβ-HDL levels.[43] So far, no side effects have been reported. Clinical trials now need to show if this compound also reduces CVD.

A different strategy to improve HDL functionality could be infusion of apoAI in complex with phosphatidylcholine (also called reconstituted HDL or rHDL). Infusion of rHDL has been shown to reduce inflammation,[44] and rHDL can induce cholesterol efflux from macrophages.[45] Furthermore, experiments have been performed with a variant of rHDL, containing apoAI(Milano), which is apoAI containing a rare point mutation that was found in subjects in a town in northern Italy, who have a reduced incidence of CVD despite low HDL levels. In one study, 5 weekly infusions of apoAI(Milano)-phosphatidylcholine disks caused a reduction of atheroma volume in patients with CVD.[46] However, long-term or follow-up studies have not been performed yet. Unfortunately, this strategy is very expensive, and it is tough for patients to undergo weekly infusions. The same holds for selective HDL delipidation, in which case large, CE-rich HDL of a patient is isolated, delipidated *ex vivo*, and infused back into the patient as small, CE-poor HDL. After the delipidation procedure, the capacity of plasma to induce cholesterol efflux *in vitro* was found to be strongly increased, and a twelve week treatment in monkeys tended to reduce atherosclerosis.[47] A method that is presumably cheaper and less incriminating for patients is the oral administration of apoAI-mimetic peptides. These are peptides that have no sequence homology to apoAI, but that mimic apoAI functionally and structurally. The most studied apoAI-mimetic peptide is D-4F. Infusion of D-4F in apoE$^{-/-}$ mice was shown to increase the capacity of HDL to mediate cholesterol efflux and to exert anti-inflammatory effects.[48] Administration of D-4F was found to be safe in clinical trials. Unfortunately, the oral bioavailability of this peptide is very low, which may hamper its further development. A new challenge thus lies in the development of apoAI-mimetic peptides with a better bioavailability.

Concluding remarks

The most important treatment strategy available to reduce CVD is lowering of LDL-C. Statins are very effective in doing this, but residual risk has led to the search for additional risk factors that can be targeted to reduce CVD. Despite the inverse correlation between HDL-C and CVD, increasing HDL-C is not atheroprotective *per se*, as exemplified by the

torcetrapib trials. There are indications that HDL-raising has anti-atherogenic effects, although we should keep in mind that improving HDL functionality likely is more important than increasing HDL levels *per se* in the search for drugs that are effective in reducing CVD.

The role of inflammation in atherosclerosis development is now well-established and therapies that suppress inflammation are under development. Macrophages especially represent an important target that may be influenced in several ways to reduce atherosclerosis development and even induce regression of pre-existing lesions.

Since atherosclerosis is a multifactorial disease, targeting multiple factors is likely the best approach to further reduce CVD. Hopefully, future studies will provide us with successful therapies that suppress inflammation and/or improve HDL functionality, and that can be safely used in combination with statins.

References

1. Lind L. Circulating markers of inflammation and atherosclerosis. *Atherosclerosis*. 2003; 169: 203-214.
2. Salmon JE and Roman MJ. Subclinical atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus. *Am. J. Med.* 2008; 121: S3-S8.
3. Valtonen VV. Role of infections in atherosclerosis. *Am. Heart J.* 1999; 138: S431-S433.
4. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006; 444: 860-867.
5. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der GJ, van OB, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol.* 2007; 8: R200.
6. Tannock LR, O'Brien KD, Knopp RH, Retzlaff B, Fish B, Wener MH, Kahn SE, Chait A. Cholesterol feeding increases C-reactive protein and serum amyloid A levels in lean insulin-sensitive subjects. *Circulation*. 2005; 111: 3058-3062.
7. Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. *PLoS. One.* 2009; 4: e4954.
8. Wang H, Kouri G, Wollheim CB. ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. *J. Cell Sci.* 2005; 118: 3905-3915.
9. Gordon S. Alternative activation of macrophages. *Nat. Rev. Immunol.* 2003; 3: 23-35.
10. Galkina E and Ley K. Vascular adhesion molecules in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 2007; 27: 2292-2301.
11. Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab*. 2007; 6: 137-143.
12. Seimon T and Tabas I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J. Lipid Res.* 2009; 50 Suppl: S382-S387.
13. Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA,

Mangelsdorf DJ, Tontonoz P, Miyazaki T. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell Metab*. 2005; 1: 201-213.

14. Liu J, Thewke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. *Arterioscler. Thromb. Vasc. Biol.* 2005; 25: 174-179.
15. van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van EM, van De WB, van Berkel TJ, Havekes LM. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. *Circ. Res.* 2001; 88: 780-786.
16. Ball RY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis*. 1995; 114: 45-54.
17. Li Y, Schwabe RF, Vries-Seimon T, Yao PM, Gerbod-Giannone MC, Tall AR, Davis RJ, Flavell R, Brenner DA, Tabas I. Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis. *J. Biol. Chem.* 2005; 280: 21763-21772.
18. Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, Lamkanfi M, Han S, Ishibashi M, Li R, Wang N, Tall AR. Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. *Circulation*. 2008; 118: 1837-1847.
19. Kooistra T, Verschuren L, de Vries-van der Weij, Koenig W, Toet K, Princen HM, Kleemann R. Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple antiatherogenic effects besides lowering plasma cholesterol. *Arterioscler. Thromb. Vasc. Biol.* 2006; 26: 2322-2330.
20. Panzenboeck U, Kratzer I, Sovic A, Wintersperger A, Bernhart E, Hammer A, Malle E, Sattler W. Regulatory effects of synthetic liver X receptor- and peroxisome-proliferator activated receptor agonists on sterol transport pathways in polarized cerebrovascular endothelial cells. *Int. J. Biochem. Cell Biol.* 2006; 38: 1314-1329.
21. Gijbels MJ, van der CM, 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.
22. Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc. Natl. Acad. Sci. U. S. A*. 2006; 103: 3781-3786.
23. Tsukamoto K, Tangirala R, Chun SH, Pure E, Rader DJ. Rapid regression of atherosclerosis induced by liver-directed gene transfer of ApoE in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 1999; 19: 2162-2170.
24. Morehouse LA, Sugarman ED, Bourassa PA, Sand TM, Zimetti F, Gao F, Rothblat GH, Milici AJ. Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits. *J. Lipid Res.* 2007; 48: 1263-1272.
25. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. 2000; 406: 203-207.
26. Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial. *Lancet*. 2007;

370: 153-160.
27. Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. *N. Engl. J. Med.* 2007; 356: 1620-1630.
28. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. *N. Engl. J. Med.* 2007; 356: 1304-1316.
29. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *N. Engl. J. Med.* 2007; 357: 2109-2122.
30. Ahnadi CE, Berthezene F, Ponsin G. Simvastatin-induced decrease in the transfer of cholesterol esters from high density lipoproteins to very low and low density lipoproteins in normolipidemic subjects. *Atherosclerosis*. 1993; 99: 219-228.
31. de Haan W, van der Hoogt CC, Westerterp M, Hoekstra M, Dallinga-Thie GM, Princen HM, Romijn JA, Jukema JW, Havekes LM, Rensen PC. Atorvastatin increases HDL cholesterol by reducing CETP expression in cholesterol-fed APOE*3-Leiden.CETP mice. *Atherosclerosis*. 2008; 197: 57-63.
32. Guerin M, Egger P, Soudant C, Le GW, van TA, Dupuis R, Chapman MJ. Dose-dependent action of atorvastatin in type IIB hyperlipidemia: preferential and progressive reduction of atherogenic apoB-containing lipoprotein subclasses (VLDL-2, IDL, small dense LDL) and stimulation of cellular cholesterol efflux. *Atherosclerosis*. 2002; 163: 287-296.
33. Vergeer M, Bots ML, van Leuven SI, Basart DC, Sijbrands EJ, Evans GW, Grobbee DE, Visseren FL, Stalenhoef AF, Stroes ES, Kastelein JJ. Cholesteryl ester transfer protein inhibitor torcetrapib and off-target toxicity: a pooled analysis of the rating atherosclerotic disease change by imaging with a new CETP inhibitor (RADIANCE) trials. *Circulation*. 2008; 118: 2515-2522.
34. Forrest MJ, Bloomfield D, Briscoe RJ, Brown PN, Cumiskey AM, Ehrhart J, Hershey JC, Keller WJ, Ma X, McPherson HE, Messina E, Peterson LB, Sharif-Rodriguez W, Siegl PK, Sinclair PJ, Sparrow CP, Stevenson AS, Sun SY, Tsai C, Vargas H, Walker M, III, West SH, White V, Woltmann RF. Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone. *Br. J. Pharmacol.* 2008; 154: 1465-1473.
35. Hashikabe Y, Suzuki K, Jojima T, Uchida K, Hattori Y. Aldosterone impairs vascular endothelial cell function. *J. Cardiovasc. Pharmacol.* 2006; 47: 609-613.
36. Hirono Y, Yoshimoto T, Suzuki N, Sugiyama T, Sakurada M, Takai S, Kobayashi N, Shichiri M, Hirata Y. Angiotensin II receptor type 1-mediated vascular oxidative stress and proinflammatory gene expression in aldosterone-induced hypertension: the possible role of local renin-angiotensin system. *Endocrinology*. 2007; 148: 1688-1696.
37. Keidar S, Kaplan M, Pavlotzky E, Coleman R, Hayek T, Hamoud S, Aviram M. Aldosterone administration to mice stimulates macrophage NADPH oxidase and increases atherosclerosis development: a possible role for angiotensin-converting enzyme and the receptors for angiotensin II and aldosterone. *Circulation*. 2004; 109: 2213-2220.
38. Nicholls SJ, Tuzcu EM, Brennan DM, Tardif JC, Nissen SE. Cholesteryl ester transfer protein inhibition, high-density lipoprotein raising, and progression of coronary atherosclerosis: insights from ILLUSTRATE (Investigation of Lipid Level Management Using Coronary

Ultrasound to Assess Reduction of Atherosclerosis by CETP Inhibition and HDL Elevation). *Circulation*. 2008; 118: 2506-2514.

39. van der Hoogt CC, de Haan W, Westerterp M, Hoekstra M, Dallinga-Thie GM, Romijn JA, Princen HM, Jukema JW, Havekes LM, Rensen PC. Fenofibrate increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression. *J. Lipid Res.* 2007; 48: 1763-1771.
40. van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC, Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. *Arterioscler. Thromb. Vasc. Biol.* 2008; 28: 2016-2022.
41. Bodor ET and Offermanns S. Nicotinic acid: an old drug with a promising future. *Br. J. Pharmacol.* 2008; 153 Suppl 1: S68-S75.
42. Arakawa R, Tsujita M, Iwamoto N, Ito-Ohsumi C, Lu R, Wu CA, Shimizu K, Aotsuka T, Kanazawa H, be-Dohmae S, Yokoyama S. Pharmacological inhibition of ABCA1 degradation increases HDL biogenesis and exhibits antiatherogenesis. *J. Lipid Res.* 2009;
43. Gordon, Jahagirdar, Johansson, Chiacchia, Hansen, Wagner, Bailey, Krimbou, Genest, and Wong. RVX-208 A small molecule increases apoAI, preB-HDL, and functionality of HDL in humans. Atherosclerosis supplements 10(2). 2009. Ref Type: Abstract
44. van Leuven SI, Birjmohun RS, Franssen R, Bisoendial RJ, de KH, Levels JH, Basser RL, Meijers JC, Kuivenhoven JA, Kastelein JJ, Stroes ES. ApoAI-phosphatidylcholine infusion neutralizes the atherothrombotic effects of C-reactive protein in humans. *J. Thromb. Haemost.* 2009; 7: 347-354.
45. Westman J, Roobol C, Carlson LA, Wulfert E. In vitro reverse cholesterol transport from THP-1-derived macrophage-like cells with synthetic HDL particles consisting of proapolipoprotein A1 or apolipoprotein A1 and phosphatidylcholine. *Scand. J. Clin. Lab Invest*. 1995; 55: 23-33.
46. Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC, Kerensky R. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA*. 2003; 290: 2292-2300.
47. Sacks FM, Rudel LL, Conner A, Akeefe H, Kostner G, Baki T, Rothblat G, de lL-M, Asztalos B, Perlman T, Zheng C, Alaupovic P, Maltais JA, Brewer HB. Selective delipidation of plasma HDL enhances reverse cholesterol transport in vivo. *J. Lipid Res.* 2009; 50: 894-907.
48. Navab M, Anantharamaiah GM, Reddy ST, Hama S, Hough G, Grijalva VR, Wagner AC, Frank JS, Datta G, Garber D, Fogelman AM. Oral D-4F causes formation of pre-beta high-density lipoprotein and improves high-density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice. *Circulation*. 2004; 109: 3215-3220.

Summary

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the western world and is mainly caused by atherosclerosis. One of the major risk factors for atherosclerosis development, high levels of low density lipoprotein (LDL)-cholesterol (C), can be efficiently treated with cholesterol-lowering drugs such as statins. However, treatment with statins prevents only about 30% of all cardiovascular events, thus a significant residual risk remains. Therefore, therapeutic strategies targeting other risk factors than high LDL-C levels may be useful to further reduce CVD. Two other risk factors for CVD have been identified: inflammation and low levels of high density lipoprotein (HDL)-C. In the first part of this thesis, we focused on cholesterol as an inducer of inflammation, and on the role of inflammation in atherosclerosis. In the second part of this thesis we studied the role of CETP, an important protein in HDL metabolism, in lipid metabolism and atherosclerosis. For these studies we used the APOE*3-Leiden (E3L) and the E3L.CETP transgenic mouse models. These mice respond to a high cholesterol diet with induction of hyperlipidemia and hepatic inflammation and respond to lipid-modifying drugs in a similar way as humans do. Furthermore, these mice develop atherosclerotic lesions that are similar in pathology to atherosclerotic lesions in humans.

The first part of this thesis focused on inflammation. Studies in humans and in mice have shown that cholesterol can induce a hepatic inflammatory response. Since the molecular mechanism underlying the induction of inflammation by cholesterol is not known, we aimed to elucidate this mechanism in **Chapter 2**. To this end, we fed E3L mice a diet without cholesterol (control) or with low cholesterol (LC) or high cholesterol (HC) concentrations, which caused the liver to switch from an adaptive state (control and LC diet) to an inflammatory state (HC diet). Cholesterol feeding dose-dependently increased plasma cholesterol levels and hepatic cholesteryl ester (CE) content and dose-dependently decreased cholesterol synthesis in the liver. In contrast, the hepatic free cholesterol (FC) concentration and plasma levels of the hepatic inflammation marker serum amyloid A (SAA) increased only with HC diet feeding and were found to be significantly correlated. Microarray analysis of livers suggested that HC, but not LC, evokes endoplasmic reticulum (ER) stress. Furthermore, the activity of ER stress-inducible transcription factors and positive regulators of SAA expression, NF-κB and STAT3, were found to be enhanced upon HC, but not LC feeding. We concluded that HC diet feeding induces hepatic inflammation and SAA gene expression in the liver through an increase of hepatic NF-κB and STAT3 activity, putatively as a result of an FC-induced ER stress response.

The stimulatory role of inflammation in atherosclerosis development is now widely established. It is not known if inflammation also plays a role in atherosclerosis regression. In **Chapter 3** we wondered if suppressing inflammation using the NF-κB inhibitor salicylate leads to atherosclerosis regression on top of cholesterol-lowering. To this end, ApoE*3-Leiden (E3L) mice were fed a high cholesterol diet to induce the formation of mild atherosclerotic lesions. Subsequently, one group of mice was

sacrificed to determine lesion area and lesion severity at the start of different regression treatments (reference group). A second group of mice was then fed a high cholesterol diet supplemented with salicylate (HC+SAL) to suppress NF-κB activity. As salicylate not only quenched inflammation, but also reduced plasma cholesterol levels (- ~49%), a third group of mice was fed a low cholesterol (LC) diet to establish similar plasma cholesterol levels as obtained by salicylate treatment. Compared to the reference group, HC+SAL suppressed hepatic NF-κB activity, tended to suppress hepatic STAT3 activity and reduced plasma levels of the hepatic inflammation marker SAA. Compared to HC+SAL, LC diet feeding suppressed hepatic NF-κB activity to a lesser extent, similarly suppressed STAT3 activity, and more strongly reduced plasma SAA levels; HC+SAL and LC feeding similarly suppressed aortic NF-κB activity. At 16 weeks after starting the regression treatments, neither HC+SAL or LC treatment showed an effect on lesion area (compared to the reference group), but HC+SAL had reduced the macrophage area of lesions and increased the plaque stability index (ratio of collagen to macrophage area) more strongly than LC diet feeding. These data led us to conclude that the combined effect of suppressing NF-κB activity and reducing plasma cholesterol with SAL improves lesion severity and promotes lesion regression more efficiently than cholesterol lowering alone.

LXR agonists are a class of compounds that regulate various cellular processes, including inflammation. LXR agonists can both prevent atherosclerosis development and induce regression of pre-existing lesions. However, the mechanisms underlying their atheroprotective effects have not been fully explored. In **Chapter 4** we therefore investigated the mechanisms underlying the anti-atherogenic potency of the LXR agonist T0901317 under both lesion-progressive and lesion-regressive conditions, in a time-dependent manner. Using E3L mice, we showed that T0901317 strongly suppresses lesion evolution and promotes lesion regression. We found that under progressive (high cholesterol diet) as well as regressive (cholesterol-free diet) conditions T0901317 (i) significantly increased plasma triglyceride and total cholesterol levels; (ii) did not affect the systemic inflammation marker SAA; (iii) suppressed endothelial monocyte adhesion; and (iv) induced the expression of the cholesterol efflux-related genes apoE, ABCA1 and ABCG1. Furthermore, under progressive conditions, T0901317 suppressed the vascular inflammatory status, lowered lesional macrophage accumulation, and blocked the transition from lesional stage II to III. Under regressive conditions, T0901317 induced lesional macrophage disappearance and increased the expression of the C-C chemokine receptor CCR7, a factor functionally required for regression. Taken together, we showed that the LXR-agonist T0901317 retards atherosclerotic lesion development and promotes lesion regression by exerting several atheroprotective effects on the vasculature. Furthermore, our findings support that vascular LXR is a potential anti-atherosclerotic target.

The second part of this thesis addressed the role of CETP in lipid metabolism and atherosclerosis. As CETP decreases HDL-C levels, CETP inhibition is being regarded a

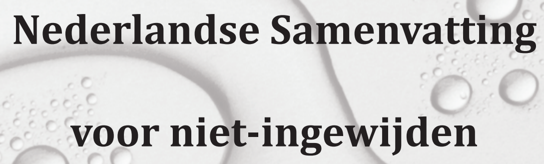
promising strategy to increase HDL-C levels and thereby reduce CVD. The first CETP inhibitor tested in large clinical trials was torcetrapib. Although torcetrapib increased HDL-C levels with about 60% in humans on a background of atorvastatin, torcetrapib did not reduce atherosclerosis progression, and even increased cardiovascular death rate. Importantly, the effect of torcetrapib on atherosclerosis progression and cardiovascular endpoints in humans was only studied in combination with atorvastatin. Therefore, we evaluated the anti-atherogenic potential of torcetrapib with and without atorvastatin in E3L.CETP mice in **Chapter 5**. Furthermore, we aimed to gain insight in the adverse effects underlying the increase in cardiovascular death rate in humans. To this end, E3L.CETP mice were fed a cholesterol-rich without drugs or with torcetrapib, atorvastatin or both. Torcetrapib decreased plasma cholesterol, albeit to a lesser extent than atorvastatin or the combination of torcetrapib and atorvastatin. Torcetrapib similarly increased HDL-C in the absence and in the presence of atorvastatin. Torcetrapib and atorvastatin alone reduced atherosclerotic lesion size to a similar extent, and combination therapy did not further reduce atherosclerosis. Remarkably, as compared to atorvastatin, torcetrapib induced enhanced monocyte recruitment and expression of monocyte chemoattractant protein-1 and resulted in lesions of a more inflammatory phenotype, as reflected by an increased macrophage content and a reduced collagen content. In conclusion, we showed that CETP inhibition by torcetrapib *per se* reduces atherosclerotic lesion size but does not enhance the anti-atherogenic potential of atorvastatin. However, as compared to atorvastatin, torcetrapib introduces lesions of a less stable phenotype.

CETP adversely affects the plasma lipoprotein profile by increasing VLDL-C and decreasing HDL-C. However, the relative contribution of either of these changes to atherosclerosis development is not known. Therefore, we investigated in **Chapter 6** to what extent the increase in VLDL-C can explain the atherogenic action of human CETP expression in E3L mice. For this purpose, E3L and E3L.CETP mice were fed a low cholesterol (LC) diet, resulting in an increased VLDL-C level as well as an increased atherosclerotic lesion area in the aortic root in E3L.CETP mice compared to E3L-LC mice. E3L mice fed a high cholesterol (HC) diet to match for the increased VLDL-C levels in E3L.CETP mice, displayed a similar atherosclerotic lesion area as observed in E3L.CETP mice. Despite similar atherosclerosis development, E3L.CETP mice had lower HDL-C as compared to E3L-HC mice. Remarkably, atherosclerotic lesions in CETP-expressing mice were enriched in collagen, suggesting a role of CETP or the diet in modifying the collagen content of lesions. These data led us to conclude that, in this experimental setting, the pro-atherogenic effect of CETP is largely explained by increased VLDL-C.

The drug bexarotene, an RXR agonist, is being used as a strategy to treat patients with different types of cancer. A common dose-limiting side effect of treatment with bexarotene is dyslipidemia. In **Chapter 7**, we evaluated the effects of bexarotene on plasma lipid metabolism in patients with metastatic differentiated thyroid carcinoma (DTC). To this end, ten patients with metastatic DTC were treated with bexarotene. Bexarotene increased plasma TG, primarily in VLDL, and raised plasma total cholesterol (TC). However, while bexarotene increased VLDL-C and LDL-C, it decreased HDL-C and

tended to decrease apoAI concomitant with an increase in endogenous CETP activity. To evaluate the cause of the bexarotene-induced hypertriglyceridemia and the role of CETP in the bexarotene-induced shift in cholesterol distribution, E3L and E3L.CETP mice were treated with bexarotene. Bexarotene increased VLDL-TG in both E3L and E3L.CETP mice, by increasing VLDL-TG production. Bexarotene did not affect the TC levels or distribution in E3L mice, but increased VLDL-C and decreased HDL-C as well as apoAI in E3L.CETP mice, concomitant with increased endogenous CETP activity. This increased CETP activity by bexarotene treatment is likely due to the increase in VLDL-TG, a CETP substrate that drives CETP activity. We concluded that bexarotene causes combined dyslipidemia as reflected by increased TG, VLDL-C and LDL-C and decreased HDL-C, which is the result of an increased VLDL-TG production that causes an increase of the endogenous CETP activity.

Taken together, the studies described in this thesis show that inflammation and CETP are both important factors in lipid metabolism and atherosclerosis. In the first part of this thesis we showed that high dietary cholesterol can induce hepatic inflammation via disturbed cholesterol homeostasis and ER stress, revealing new targets for the treatment of metabolic inflammation. Next, we demonstrated that intervention in both systemic and vascular inflammation can reduce atherosclerosis progression and/or induce regression, highlighting the importance of developing drugs targeting the inflammatory component of atherosclerotic disease. In the second part of this thesis we showed that CETP inhibition per se may be anti-atherogenic, but that combination therapy of the CETP inhibitor torcetrapib with atorvastatin may have obscured its atheroprotective effect. Furthermore, we showed that the VLDL-increasing effect of CETP largely explains its atherogenic effect, at least in APOE*3-Leiden.CETP mice, and that CETP inhibition may negatively affect lesion stability. Our data suggest that CETP inhibition may not be the most optimal strategy to increase HDL-C levels and thereby reduce atherosclerosis. We anticipate that strategies improving HDL functionality, rather than raising the HDL level, are more likely to effectively reduce CVD.



Nederlandse Samenvatting

voor niet-ingewijden

Hart- en vaatziekten zijn een zeer belangrijke doodsoorzaak in de westerse wereld. Een belangrijke oorzaak van hart- en vaatziekten is aderverkalking, ofwel **atherosclerose**. Atherosclerose is een proces dat gekenmerkt wordt door ophoping van vetten (waaronder **cholesterol**) en cellen van het immuunsysteem in de vaatwand. De belangrijkste cel van het immuunsysteem betrokken bij atherosclerose is de **macrofaag**, die naar de vaatwand toe komt om cholesterol dat zich daar ophoopt als een soort stofzuiger op te ruimen, maar die er daarna voor kan zorgen dat het proces van atherosclerose verergert. Ophoping van cholesterol en macrofagen in de vaatwand leidt namelijk tot de vorming van een zogenaamde **atherosclerotische lesie**. Dit is een verdikking van de vaatwand (met onder andere cholesterol en macrofagen erin) die het bloedvat vernauwt. Een dergelijke lesie kan ervoor zorgen dat er minder bloed door het vat stroomt, waardoor er een tekort aan zuurstof en aan voedingsstoffen kan ontstaan in weefsel dat door dit bloedvat wordt voorzien van deze stoffen. Ook kan het gebeuren dat een lesie openscheurt, waarbij een soort wond ontstaat. Vetten, macrofagen en materialen uit de vaatwand die zich in de lesie hebben opgehoopt komen dan in direct contact met het bloed. Dit zorgt ervoor dat het bloed stolt en een bloedprop, ofwel **trombus**, vormt. Dit wordt trombose genoemd. Een bloedprop kan een bloedvat zo ernstig verstoppen dat dit bijvoorbeeld tot een hart- of een herseninfarct leidt.

Er zijn verschillende factoren die het risico op het ontstaan van atherosclerose verhogen. Dit zijn bijvoorbeeld een te hoge bloeddruk en een te hoog niveau van het cholesterol in bepaalde deeltjes die cholesterol via het bloed door het lichaam transporteren: het lage dichtheids lipoproteïne (LDL) en het zeer lage dichtheids lipoproteïne (VLDL). **VLDL-cholesterol** en **LDL-cholesterol** kunnen ervoor zorgen dat cholesterol zich ophoopt in de vaatwand en staan hierdoor ook wel bekend als 'slecht' cholesterol. Er zijn wel medicijnen waarmee het VLDL-cholesterol en LDL-cholesterol efficiënt verlaagd kunnen worden. De klasse medicijnen die hiervoor het meest voorgeschreven wordt, is de statine. Helaas leidt behandeling met statines slechts tot een vermindering van ongeveer 30% in het aantal incidenten van hart- en vaatziekten. Het lijkt er dus op dat er ook nog andere belangrijke factoren zijn die een rol spelen in deze ziekten. In de voorbije jaren zijn ook andere risicofactoren voor hart- en vaatziekten geïdentificeerd. Dit zijn onder andere ontsteking en een laag niveau van cholesterol in het hoge dichtheids lipoproteïne (HDL) in het bloed.

Ontsteking kennen we over het algemeen als een proces dat het lichaam beschermt tegen infecties van bijvoorbeeld bacteriën of virussen. Hoewel zo'n ontsteking vervelend kan zijn door de pijn die ermee gepaard gaat, heeft deze vorm van ontsteking een beschermende werking, omdat deze in staat is de ziekteverwekkers op te ruimen. Verder is een dergelijke ontstekingsreactie hevig van intensiteit en van korte duur. Er is echter ook een andere vorm van ontsteking, die juist matig van intensiteit en langdurig is. Deze chronische vorm van ontsteking vormt een risico voor het ontstaan van hart- en vaatziekten.

Hoge **HDL-cholesterol** niveaus in het bloed zijn geassocieerd met een laag risico op hart- en vaatziekten, en daarom staat HDL-cholesterol ook wel bekend als

'goed' cholesterol. Mensen hebben in hun bloed een eiwit (proteïne) dat er voor zorgt dat cholesterol uit het HDL terechtkomt in het LDL. Dit eiwit, dat cholesteryl ester transfer proteïne (**CETP**) heet, zorgt er dus voor dat het HDL-cholesterol niveau wordt verlaagd, terwijl het LDL-cholesterol niveau omhoog gaat. Dit zijn twee effecten die allebei het risico op hart- en vaatziekten kunnen verhogen.

De studies die beschreven zijn in dit proefschrift zijn uitgevoerd om meer te weten te komen over de rol van ontsteking en van HDL-cholesterol in het ontstaan van hart- en vaatziekten, en de rol van deze factoren bij de mogelijke behandeling van hart- en vaatziekten. In mensen is het niet goed mogelijk om deze factoren te bestuderen, omdat een proces zoals het ontwikkelen van atherosclerose het beste onderzocht kan worden door organen zoals het hart in verschillende stadia van het ziekteproces op moleculair niveau te bestuderen. Weefsels van mensen zijn doorgaans alleen beschikbaar na een chirurgische ingreep, en vaak zelfs pas als iemand overleden is. Bovendien is het dan meestal alleen mogelijk om het eindstadium van de ziekte te onderzoeken en niet de processen die hieraan vooraf zijn gegaan. Daarom hebben we voor dit onderzoek gebruik gemaakt van speciale muismodellen, namelijk de APOE*3-Leiden (E3L) muizen met en zonder het eiwit CETP. In deze **E3L muizen** en **E3L.CETP muizen** verloopt het ontstaan van hart- en vaatziekten via vergelijkbare stappen als in mensen. Ook reageren deze muizen op een vergelijkbare manier als mensen op medicijnen tegen hart- en vaatziekten.

De studies in het eerste deel van dit proefschrift waren gericht op ontsteking. Studies in mensen en in muizen hebben laten zien dat de inname van grote hoeveelheden cholesterol kan leiden tot een chronische ontstekingsreactie van de lever, een belangrijk orgaan voor het verwerken van cholesterol en andere vetten. Omdat het niet op moleculair niveau bekend was hoe te veel cholesterol in het voedsel zorgt voor ontsteking van de lever, hebben wij dit mechanisme onderzocht in **Hoofdstuk 2**. Wij hebben E3L muizen een dieet gegeven dat geen cholesterol (controle groep), een lage hoeveelheid cholesterol (**LC** groep) of een hoge hoeveelheid cholesterol (**HC** groep) bevatte. Dit zorgde ervoor dat de lever omschakelde van een status waarin deze zich nog goed kan aanpassen (onder de controle en LC omstandigheden) naar een status waarin zij zich niet meer kan aanpassen en ontstoken raakt (onder HC omstandigheden). Door de productie, verwerking en afvoer van cholesterol te bestuderen lieten wij zien dat cholesterol in de voeding op een dosisafhankelijke manier het cholesterol niveau in het bloed, de hoeveelheid veresterd cholesterol (opgeslagen cholesterol) in de lever en de aanmaak van cholesterol in de lever beïnvloedt. Dit in tegenstelling tot het niveau van vrij cholesterol (de direct beschikbare voorraad) in de lever en van de indicator voor leverontsteking die in het bloed gemeten kan worden: serum amyloid A (**SAA**). De niveaus van deze twee parameters gingen alleen omhoog in muizen die HC voeding kregen en niet in de muizen die LC voeding kregen vergeleken met de controle groep, en er was een onderling verband tussen de stijging van elk van deze parameters. Uit geavanceerde analyse (zogenaamde microarray analyse) van de 'activiteit' van genen

in de lever bleek dat in de HC groep, en niet in de LC groep, sprake was van stress in het endoplasmatisch reticulum (ER), een onderdeel van een cel waar veranderingen in cholesterolniveaus waargenomen worden. Factoren die geactiveerd worden door stress in het ER, en die kunnen leiden tot leverontsteking (**NF-κB en STAT3**), waren inderdaad geactiveerd in de HC groep, en niet in de LC groep. Hieruit trokken wij de conclusie dat een grote hoeveelheid cholesterol in de voeding leidt tot leverontsteking doordat het niveau van vrij cholesterol in de lever verhoogd wordt, wat leidt tot stress in het ER en een verhoging van factoren die leverontsteking veroorzaken.

Het is tegenwoordig duidelijk dat ontsteking belangrijk is voor het ontstaan van hart- en vaatziekten, maar het is nog niet bekend of remming van ontsteking ook een rol speelt in de genezing hiervan. Het doel van het onderzoek in **Hoofdstuk 3** was daarom om te bepalen of onderdrukking van ontsteking met de NF-κB remmer **salicylaat** bijdraagt aan de genezing van atherosclerose bovenop verlaging van cholesterol. Om dit te onderzoeken gaven wij E3L muizen een hoog cholesterol dieet, waardoor deze muizen milde atherosclerotische lesies ontwikkelden. Vervolgens werd de ontwikkeling van atherosclerose geanalyseerd in één groep muizen die diende als referentiegroep. Een tweede groep muizen kreeg vervolgens een hoog cholesterol dieet waar salicylaat aan was toegevoegd (HC+salicylaat), om de activiteit van de ontstekingsmediator NF-κB te remmen. Het HC+salicylaat dieet verlaagde het cholesterol in het bloed met -49%. Daarom kreeg een derde groep muizen een dieet met een lage hoeveelheid cholesterol (LC), wat resulteerde in een vergelijkbare hoeveelheid cholesterol in het bloed als bij de muizen die met HC+salicylaat behandeld werden. De activiteit van NF-κB was onderdrukt in de levers van muizen die het HC+salicylaat dieet kregen in vergelijking tot de referentiegroep. Ook waren de niveaus van de indicator voor leverontsteking SAA in het bloed waren verlaagd. In vergelijking tot de HC+salicylaat behandelde groep onderdrukte het LC dieet de activiteit van NF-κB in de lever minder sterk en was het niveau van SAA in het bloed verder verlaagd. Verder onderdrukten het HC+salicylaat dieet en het LC dieet de activiteit van NF-κB in de aorta in dezelfde mate. Beide diëten hadden geen effect op de grootte van de ontwikkelde atherosclerotische lesies. Daarentegen verminderde het HC+salicylaat dieet de hoeveelheid macrofagen en verbeterde dit dieet de verhouding tussen **collageen** (stabiliserend materiaal) en macrofagen, wat een indicatie is voor de stabiliteit van de lesies en dus voor het risico op het scheuren van een lesie en op trombose. Deze effecten waren sterker bij het HC+salicylaat dieet dan bij het LC dieet. Op grond van deze gegevens concludeerden wij dat de combinatie van onderdrukking van de activiteit van NF-κB en verlaging van het cholesterol in het bloed met salicylaat een betere behandeling van bestaande atherosclerotische lesies is dan het verlagen van het cholesterol in het bloed alleen. Het is belangrijk om te onderzoeken of behandeling met salicylaat ook een goede behandeling is voor mensen met hart- en vaatziekten.

Activatoren (agonisten) van de lever X receptor (LXR) behoren tot een klasse van stoffen die verschillende belangrijke processen in lichaamscellen reguleren, waaronder ontsteking. Het is al bekend dat **LXR agonisten** in diermodellen zowel het ontstaan

van hart- en vaatziekten kunnen voorkomen als genezing van hart- en vaatziekten kunnen bewerkstelligen. De mechanismen die aan de beschermende werking van LXR agonisten ten grondslag liggen zijn echter niet uitgebreid bestudeerd. Daarom hebben wij in **Hoofdstuk 4** bestudeerd via welke mechanismen één bepaalde LXR agonist, **T0901317**, beschermt tegen het ontstaan van hart- en vaatziekten, en hoe deze stof kan leiden tot genezing van hart- en vaatziekten. Uit ons onderzoek, dat werd uitgevoerd in E3L muizen, bleek dat een aantal effecten zowel optreedt tijdens het voorkomen van de ontwikkeling als bij het genezen van atherosclerotische lesies. Ten eerste veroorzaakte T0901317 een aanzienlijke verhoging van de hoeveelheid vetten (cholesterol en **triglyceriden**) in het bloed. Dit is een effect dat in principe ongunstig is voor de ontwikkeling en genezing van atherosclerose. Ten tweede zorgde T0901317 niet voor een verlaging van de indicator voor leverontsteking SAA in het bloed. De beschermende werking van T0901317 kon hierdoor dus niet worden verklaard. Er werden ook een aantal gunstige effecten van T0901317 ontdekt. Het eerste gunstige effect was dat T0901317 ervoor zorgde dat er minder macrofagen aangetrokken werden naar de plek waar een atherosclerotische lesie is. Het tweede gunstige effect was dat T0901317 de activiteit van genen (apoE, ABCA1 en ABCG1) die ervoor zorgen dat cholesterol weer uit macrofagen in een atherosclerotische lesie kan verdwijnen verhoogde, zodat het cholesterol daar minder ophoopt. Het derde gunstige effect was dat T0901317 het ontstaan van atherosclerose kan verminderen door de ontstekingsgraad in de vaatwand te verlagen. T0901317 doet dit laatste door ervoor te zorgen dat er minder macrofagen ophopen in de vaatwand en door ervoor te zorgen dat er geen vermenigvuldiging van spiercellen vanuit de vaatwand in de lesie plaatsvindt. Ook droeg T0901317 bij aan genezing van atherosclerose door ervoor te zorgen dat macrofagen die al in de lesie aanwezig waren hieruit verdwenen. Ondanks het feit dat T0901317 dus een ongunstig effect heeft op de niveaus van vetten in het bloed, zorgen de gunstige effecten op ontsteking er uiteindelijk voor dat de E3L muizen die met T0901317 behandeld werden, minder atherosclerose ontwikkelden. Samengevat hebben wij laten zien dat T0901317 op een aantal manieren een direct beschermend effect op de vaatwand heeft, waardoor zowel het ontstaan van atherosclerose geremd, als de genezing van atherosclerose gestimuleerd wordt. Omdat verschillende onderzoeken in diermodellen hebben laten zien dat T0901317 ook ongunstige bijwerkingen heeft op de lever, onder andere door de hoeveelheden van vetten in de lever heel sterk te verhogen, wordt dit medicijn niet gebruikt om in mensen hart- en vaatziekten te verminderen. Onze bevindingen wijzen erop dat LXR in de vaatwand een belangrijk eiwit kan zijn om aan te pakken met nieuw te ontwikkelen medicijnen, die de hoeveelheden van vetten in de lever en in het bloed niet mogen verhogen.

In het tweede deel van dit proefschrift is onderzoek beschreven dat was gericht op de rol van HDL-cholesterol, en met name van het eiwit CETP in de verwerking van cholesterol en triglyceriden (vetten) uit HDL, in het ontstaan van atherosclerose. Aangezien CETP het HDL-cholesterol verlaagt, wordt remming van CETP gezien als een veelbelovende

therapie om HDL-cholesterol te verhogen en daardoor hart- en vaatziekten te verlagen. Daarom worden er medicijnen ontwikkeld om specifiek dit ene eiwit, CETP, te remmen. **Torcetrapib** is ontwikkeld als potentieel medicijn dat het eiwit CETP remt en torcetrapib was de eerste CETP remmer die getest werd in grote groepen patiënten. Alle patiënten in dit onderzoek werden behandeld met de LDL-cholesterolverlager atorvastatine, en de helft van de mensen kreeg daarbij nog torcetrapib. Zoals gehoopt zorgde torcetrapib voor een sterke stijging van het HDL-cholesterol. Maar onverwacht bleek dat torcetrapib de ontwikkeling van hart- en vaatziekten niet verminderde, en dat in de groep mensen die met de combinatie van torcetrapib en atorvastatine behandeld werd zelfs meer mensen stierven aan hart- en vaatziekten dan in de groep die alleen behandeld werd met atorvastatine. Dit betekende het einde van de ontwikkeling van torcetrapib als nieuw medicijn om HDL-cholesterol te verhogen. Een belangrijke opmerking hierbij is dat het effect van torcetrapib op hart- en vaatziekten alleen is bestudeerd in combinatie met atorvastatine. Omdat het effect van torcetrapib in combinatie met atorvastatine anders kan zijn dan het effect van torcetrapib alleen, hebben wij in **Hoofdstuk 5** onderzocht wat het effect van torcetrapib alleen en in combinatie met atorvastatine is op de ontwikkeling van atherosclerose. Verder hebben we geprobeerd inzicht te krijgen in de manier waarop torcetrapib heeft geleid tot een toename in sterfte aan hart- en vaatziekten. Om dit te onderzoeken hebben we E3L. CETP muizen een dieet met daarin veel cholesterol te eten gegeven. We verdeelden de muizen in vier groepen. Er was één groep die geen medicijn kreeg, één groep kreeg alleen torcetrapib, één groep kreeg alleen atorvastatine en één groep kreeg deze twee in combinatie. Torcetrapib verlaagde het plasma cholesterol, hoewel dit effect iets minder sterk was dan dat van atorvastatine of van de combinatietherapie. Torcetrapib verhoogde het HDL-cholesterol als monotherapie en als combinatietherapie in gelijke mate. Behandeling met torcetrapib of atorvastatine alleen verminderde de grootte van atherosclerotische lesies even sterk. De combinatietherapie was niet effectiever dan elk van de afzonderlijke behandelingen in het verminderen van lesie grootte. Het viel op dat behandeling met torcetrapib, vergeleken met atorvastatine behandeling, leidde tot het aantrekken van meer macrofagen naar de plaats van de lesies. Omdat de lesies van muizen die met torcetrapib behandeld waren meer macrofagen bevatten en minder stabiliserend materiaal, werden deze lesies als meer ontstoken en mogelijk minder stabiel gekarakteriseerd. Onze conclusie was dat remming van CETP met torcetrapib op zich leidt tot minder ontwikkeling van atherosclerose, maar dat torcetrapib in combinatie met atorvastatine geen extra effect heeft op het voorkomen van atherosclerose. Verder leidt behandeling met torcetrapib in vergelijking tot behandeling met atorvastatine tot lesies die een groter risico hebben om te scheuren en zo trombose en zelfs de dood te veroorzaken.

Recent hebben we aangetoond dat CETP expressie in E3L muizen tot meer atherosclerose leidde, wat gepaard ging met verlaging van HDL-cholesterol en verhoging van VLDL-cholesterol. Deze beide tegenovergestelde veranderingen kunnen het risico op hart- en vaatziekten verhogen. Het is echter niet bekend wat het relatieve effect

van elk van deze veranderingen op het ontstaan van atherosclerose is. In **Hoofdstuk 6** hebben wij onderzocht in hoeverre het verschil in VLDL-cholesterol tussen E3L muizen met en zonder CETP kan verklaren dat CETP leidt tot meer atherosclerose. Om dit te bestuderen hebben we E3L en E3L.CETP muizen een dieet met een lage hoeveelheid cholesterol (LC dieet) te eten gegeven. De E3L.CETP muizen hadden op dit dieet een hoger VLDL-cholesterol dan de E3L muizen en ook meer atherosclerose. Om te bestuderen in hoeverre dit verhoogde VLDL-cholesterol kon verklaren dat de E3L.CETP muizen meer atherosclerose hadden, onderzochten we ook een derde groep met E3L muizen die een dieet met een hoge hoeveelheid cholesterol (HC) te eten kregen. De hoeveelheid cholesterol in het HC dieet was zo gekozen dat de E3L muizen op dit HC dieet evenveel VLDL-cholesterol hadden als de E3L.CETP muizen. Verder zagen we dat deze muizen atherosclerotische lesies hadden die vergelijkbaar in grootte waren met de lesies van de E3L.CETP muizen. Deze gegevens wijzen erop dat het verhoogde VLDL-cholesterol grotendeels verklaart waarom de aanwezigheid van CETP meer atherosclerose veroorzaakt. Hoewel de E3L muizen en E3L.CETP muizen met evenveel VLDL-cholesterol ook in vergelijkbare mate atherosclerose ontwikkeld hadden, hadden de E3L.CETP muizen lagere HDL-cholesterol niveaus. Het lijkt er dus op dat het verschil in HDL-cholesterol niveaus in dit geval geen effect had op de ontwikkeling van atherosclerose. Iets anders dat opviel was dat de atherosclerotische lesies in de muizen met CETP meer collageen bevatten. Dit suggereert dat CETP de hoeveelheid collageen in atherosclerotische lesies, en daarmee het risico op scheuren van een lesie en trombose, kan beïnvloeden. Wij concludeerden op grond van deze gegevens dat, onder de omstandigheden die wij hebben onderzocht, CETP voornamelijk tot meer atherosclerose leidt doordat CETP het VLDL-cholesterol niveau in bloed verhoogt.

In **Hoofdstuk 7** hebben we eerst een medicijn bestudeerd dat gebruikt wordt bij patiënten met verschillende types kanker. Dit medicijn, **bexaroteen**, is een activator van het eiwit retinoide X receptor (RXR) en heeft als bijwerking dat de niveaus van verschillende vetten (cholesterol en triglyceriden) in het bloed omhoog gaan. Wij onderzochten meer specifiek welke veranderingen er optraden in de niveaus van vetten in het bloed van mensen die met bexaroteen behandeld werden. Daarnaast onderzochten we in E3L en E3L.CETP muizen de oorza(a)k(en) van deze veranderingen. Voor dit onderzoek behandelden we tien patiënten met uitzaaiingen van schildklierkanker met bexaroteen. Bexaroteen veroorzaakte een stijging van het niveau van verschillende vetten (triglyceriden en cholesterol) in het VLDL en LDL, maar juist een daling van het HDL-cholesterol. Om de onderliggende oorzaak te achterhalen behandelden we E3L en E3L.CETP muizen met bexaroteen. Ook in deze muizen leidde behandeling met bexaroteen tot een stijging van de triglyceridenniveaus in VLDL. Wij toonden aan dat dit werd veroorzaakt door een verhoogde productie van triglyceriden in de lever. Opvallend was dat het effect van bexaroteen op cholesterol in de E3L en E3L.CETP muizen verschillend was. Bexaroteen had geen effect op het cholesterol in E3L muizen, terwijl bexaroteen in E3L.CETP muizen het VLDL-cholesterol verhoogde en het HDL-cholesterol verlaagde, wat vergelijkbaar is met wat we in mensen zagen.

Hierbij zagen wij ook dat de activiteit van het eiwit CETP, dat cholesterol van HDL naar VLDL en LDL kan transporteren, hoger was na behandeling met bexaroteen. Aangezien triglyceriden de activiteit van CETP kunnen beïnvloeden (meer triglyceriden zorgen voor een hogere CETP activiteit), is de verhoogde CETP activiteit die wij waarnamen hoogstwaarschijnlijk het gevolg van de toename van de triglyceridenniveaus in het bloed. Wij concludeerden uit deze gegevens dat bexaroteen een ongunstige verandering in de hoeveelheid vet in het bloed (dyslipidemie) veroorzaakt (zowel een verhoging van triglyceriden, VLDL-cholesterol en LDL-cholesterol als een verlaging van HDL-cholesterol), die veroorzaakt wordt door een verhoogde productie van triglyceriden in de lever, wat weer leidt tot een hogere CETP activiteit.

Samengevat laten de onderzoeken die in dit proefschrift beschreven zijn, zien dat ontsteking en CETP twee belangrijke factoren zijn bij het verwerken van cholesterol en voor de ontwikkeling van hart- en vaatziekten. In het eerste deel van dit proefschrift lieten we zien dat een grote hoeveelheid cholesterol in het voedsel leverontsteking kan veroorzaken door ophoping van vrij cholesterol, wat leidt tot stress in een bepaald onderdeel van de cel, het ER. De ophoping van vrij cholesterol en het ontstaan van ER stress zijn processen waar mogelijk nieuwe medicijnen tegen ontwikkeld kunnen worden. Vervolgens lieten we zien dat het de moeite waard is om zowel ontsteking in de vaatwand als ontsteking op andere plaatsen in het lichaam (zoals de lever) te remmen, omdat dit de ontwikkeling van hart- en vaatziekten kan remmen, en zelfs tot genezing van hart- en vaatziekten kan leiden in E3L muizen. Dit is veelbelovend voor de ontwikkeling van ontstekingsremmende medicijnen die uiteindelijk in mensen zullen moeten worden getest om hart- en vaatziekten te verminderen. In het tweede deel van dit proefschrift lieten we zien dat het remmen van CETP op zich hart- en vaatziekten kan verminderen, maar dat de combinatietherapie van de CETP-remmer torcetrapib samen met de LDL-cholesterolverlager atorvastatine dit gunstige effect mogelijk tegengewerkt heeft. Ook lieten we zien dat het feit dat CETP het VLDL-cholesterol verhoogt grotendeels verklaart waarom CETP tot meer atherosclerose leidt in E3L.CETP muizen. Bovendien bleek dat remming van CETP mogelijk het risico op het scheuren van een atherosclerotisch lesie, en dus op trombose, verhoogt. De uitkomsten van ons onderzoek suggereren dat het remmen van CETP mogelijk niet de beste manier is om HDL-cholesterol niveaus te verhogen, en daardoor hart- en vaatziekten te verminderen. Het is waarschijnlijk beter om therapieën te ontwikkelen die er voor zorgen dat HDL zijn beschermende functie beter kan uitvoeren, dan therapieën die er uitsluitend op gericht zijn om HDL-cholesterol niveaus te verhogen. Wij denken dat therapieën die rekening houden met de beschermende functie van HDL uiteindelijk dus een grotere kans maken om hart- en vaatziekten succesvol te verminderen.

List of Publications

List of Publications

Kooistra T, Verschuren L, de Vries-van der Weij J, Koenig W, Toet K, Princen HM, Kleemann R.
Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple antiatherogenic effects besides lowering plasma cholesterol.
Arterioscler Thromb Vasc Biol. 2006:2322-30.

Zadelaar S, Kleemann R, Verschuren L, de Vries-van der Weij J, van der Hoorn J, Princen HM, Kooistra T.
Mouse models for atherosclerosis and pharmaceutical modifiers.
Arterioscler Thromb Vasc Biol. 2007;27:1706-21.

de Vries-van der Weij J*, de Haan W*, van der Hoorn JWA, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM, Rensen PC.
Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more pro-inflammatory lesions than atorvastatin.
Circulation 2008;117:2515-2522. *Both authors contributed equally.

Bobeldijk I, Hekman M, de Vries-van der Weij J, Coulier L, Ramaker R, Kleemann R, Kooistra T, Rubingh C, Freidig A, Verheij E.
Quantitative profiling of bile acids in biofluids and tissues based on accurate mass high resolution LC-FT-MS: compound class targeting in a metabolomics workflow.
J Chromatogr B Analyt Technol Biomed Life Sci. 2008;871:306-313.

Verschuren L, de Vries-van der Weij J, Zadelaar ASM, Kleemann R and Kooistra T.
LXR agonist T0901317 suppresses atherosclerotic lesion initiation and progression and promotes lesion regression in APOE*3Leiden transgenic mice.
J Lipid Res. 2009;50:301-311.

de Vries-van der Weij J, de Haan W, Hu L, Kuif M, Oei HLDW, van der Hoorn JWA, Havekes LM, Princen HM, Romijn JA, Smit JWA, Rensen PC.
Bexarotene induces dyslipidemia by increased VLDL production and cholesteryl ester transfer protein (CETP)-mediated reduction of HDL.
Endocrinology. 2009;150:2368-2375.

de Haan W, de Vries-van der Weij J, Mol IM, Hoekstra M, Romijn JA, Jukema JW, Havekes LM, Princen HMG, Rensen PC.
PXR agonism decreases plasma HDL levels in ApoE*3Leiden.CETP mice.
Biochim Biophys Acta. 2009;1791:191-197.

de Vries-van der Weij J, Toet K, Zadelaar S, Havekes LM, Kooistra T, Rensen PC.
Human CETP aggravates atherosclerosis by increasing VLDL-cholesterol rather than by decreasing HDL-cholesterol in APOE*3-Leiden mice.
Atherosclerosis 2009; 206: 153-158.

Verschuren L, Kooistra T, Bernhagen J, Voshol PJ, Ouwens DM, van Erk M, de Vries-van der Weij J, Leng L, Van Bockel JH, Van Dijk KW, Fingerle-Rowson G, Bucala R, Kleemann R.
MIF Deficiency Reduces Chronic Inflammation in White Adipose Tissue and Impairs the Development of Insulin Resistance, Glucose Intolerance, and Associated Atherosclerotic Disease.
Circ Res. 2009; 105: 99-107.

Den Boer AM, Westerterp M, De Vries-van der Weij J, Hu L, Espirito Santo SM, Kooistra T, Reiss P, Romijn JA, Havekes LM, Rensen PC.
Ritonavir protects against the development of atherosclerosis in APOE*3-Leiden mice
In press.

De Vries-van der Weij J, Verschuren L, Bobeldijk I, Van Duijvenvoorde W, Toet K, Van Erk M, Kleemann R, Kooistra T.
High cholesterol feeding induces hepatic inflammation through disturbed cholesterol homeostasis in APOE*3-Leiden mice.
Submitted.

De Vries-van der Weij J, Toet K, Zadelaar S, Kleemann R, Rensen PC, Kooistra T.
Combined suppression of NF-κB activity and cholesterol lowering by salicylate induces regression of pre-existing atherosclerotic lesions beyond cholesterol lowering alone
Submitted.

Curriculum Vitae

Curriculum Vitae

Annette Jessica van der Weij (roepnaam Jitske) werd geboren op 20 mei 1982 in Enkhuizen. Zij behaalde haar VWO diploma in juni 2000 aan scholengemeenschap Werenfridus in Hoorn. In september van dat jaar begon zij met haar studie Scheikunde aan de Vrije Universiteit van Amsterdam. Zij behaalde in 2003 haar Bachelor diploma's van de studies Scheikunde en Farmacochemie, waarna zij zich voor de masterfase van haar studie Scheikunde specialiseerde in de richting Biochemie en Moleculaire Biologie. Haar eerste van twee stages voerde zij uit bij de sectie Biochemie en Moleculaire Biologie van de afdeling Scheikunde aan de Vrije Universiteit, onder begeleiding van Dr. H.R. Vos en Dr. J.C. Vos. Tijdens deze stage deed zij onderzoek naar ribosoombiogenese in de giststam *Saccharomyces cerevisiae*. Haar tweede stage voerde zij uit bij TNO Kwaliteit van Leven in Leiden onder begeleiding van Dr. R. Kleemann, waar haar onderzoek zich richtte op de rol van het cytokine MIF in de ontwikkeling van insuline resistentie. In augustus 2005 behaalde zij haar Master diploma. Aansluitend startte zij als promovenda met haar promotieonderzoek bij de afdeling Humane Genetica van het Leids Universitair Medisch Centrum (LUMC). Het onderzoek werd uitgevoerd bij de afdeling Endocrinologie van het LUMC en bij TNO Kwaliteit van Leven in Leiden, onder supervisie van Prof. Dr. L.M. Havekes, Prof. Dr. R.R. Frants, Dr. P.C.N. Rensen en Dr. T. Kooistra. Voor een presentatie van haar onderzoek won zij in 2008 de DAS fellowship op het 11e symposium van de Dutch Atherosclerosis Society. Het promotieonderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, werd afgerond in augustus 2009. Jitske werkt momenteel als Regulatory Project Leader bij het College ter Beoordeling van Geneesmiddelen in Den Haag.