Modulation of HDL metabolism : studies in APOE*3-Leiden.CETP mice

Haan, W. de

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Modulation of HDL Metabolism - Studies in APOE*3-Leiden.CETP mice

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Chapter 1

GENERAL INTRODUCTION

Contents General Introduction

1. Lipids and lipoprotein metabolism

Triglycerides (TG) and cholesterol are the most common dietary lipids. TG serve as an important energy source for muscle tissue and can be stored in adipose tissue. Cholesterol is an important component of the cell membrane and the precursor of vitamin D, bile acids and steroid hormones. Since TG and cholesterol are hydrophobic molecules, they are transported in the blood in specialized particles called lipoproteins. Lipoproteins consist of a hydrophobic core containing cholesteryl esters (CE) and TG, which is surrounded by a polar surface of phospholipids, unesterified cholesterol and apolipoproteins. Apolipoproteins stabilize the lipid particle. In addition, they regulate the transport and redistribution of lipids by modulation of enzyme activities in plasma and by serving as ligands for cell surface receptors. Lipoproteins are divided in five main classes according to their density, namely (in order with increasing density): chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).[1,2] A schematic representation of lipoprotein metabolism is depicted in Figure 1, and explained in sections 1.1-1.3.

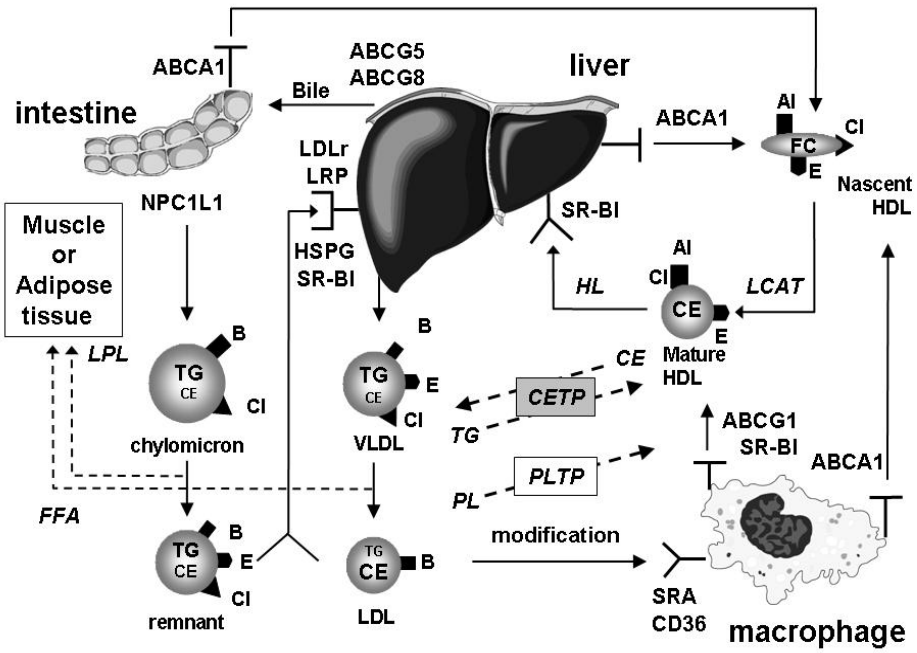

Figure 1. Schematic overview of lipoprotein metabolism. See text for explanation.

1.1 Chylomicrons

TG are the major dietary lipids, and other dietary lipids include phospholipids and cholesterol. In the intestine, lipids are lipolyzed, the lipolysis products are taken up by the enterocyte, and lipids are re-synthesized.[3-6] Enterocytes synthesize apoB48 which is lipidated in the endoplasmatic reticulum (ER) by the microsomal TG transfer protein (MTP), resulting in the formation of a small apoB48 containing particle which fuses with large apoB48-free lipid droplets to form a prechylomicron which moves to the Golgi, and is subsequently excreted via exocytosis.[3,5] The chylomicron which than contains apoB48, apoAI and apoAIV enters the lymph and subsequently the blood where chylomicrons acquire exchangeable apolipoproteins apoCI, apoCII, apoCIII and apoE whereas apoAI and apoAIV can dissociate from the particle and stay in the plasma in a free form or become associated with HDL.[5,7] Chylomicrons can exchange lipids with other lipoproteins. TG and CE are exchanged via the cholesteryl ester transfer protein (CETP), phospholipids via the phospholipid transfer protein (PLTP) and cholesterol via passive diffusion.[8,9,10,11] Chylomicrons transport dietary TG and cholesterol to various parts of the body where TG are hydrolyzed by lipoprotein lipase (LPL), an enzyme attached to the endothelium. Since LPL expression is highest on adipose tissue in the postprandial state, fatty acids (FA) released by LPL are predominantly taken up by adipose tissue, where they are stored as TG, and the remainder is taken up by muscle (skeletal muscle and heart) for use as energy source.[12,13] Chylomicron remnants which are relatively rich in cholesterol and apoE can then be taken up by the liver mainly via the apoE-recognizing receptors LDL receptor (LDLr) and LDLr-related protein (LRP). In addition, uptake can occur via interaction of apoE with cell surface-bound heparin sulfate proteoglycans (HSPG) or scavenger receptor class B type I (SR-BI).[14-18] During fasting, the intestine can produce smaller, less TG-rich, VLDL particles via a similar pathway.[5,6]

1.2 VLDL and LDL

The liver thus takes up chylomicron remnants, but can also synthesize new lipids as the liver contains several lipogenic enzymes including FA synthase (FAS) and stearoyl CoA desaturase (SCD1) for TG synthesis and HMG-CoA reductase for cholesterol synthesis. Hepatic lipids can be stored as TG and CE or can be excreted as VLDL particles. Assembly of VLDL involves a similar pathway as chylomicron synthesis including the transfer of lipids to apoB-100 (or, in some animal species including mice also apoB-48) by MTP and subsequent fusion with protein-free lipid droplets formed in the ER to form mature VLDL which is secreted into the circulation and then is enriched with apoCI, apoCII, apoCIII and apoE.[17,19,20] Similarly to chylomicrons, VLDL can exchange TG, CE and phospholipids via CETP and PLTP, and cholesterol via

passive diffusion.[8,9,10,11] VLDL particles undergo similar LPL-mediated degradation as chylomicrons, resulting in the uptake of liberated FA by tissues. In contrast to chylomicrons which are synthesized in the postprandial state, VLDL ensures a supply of FA as energy source for muscle tissue in the fasted state as LPL is highest on muscle tissue during fasting.[12,13] During lipolysis, VLDL becomes depleted of TG leading to conversion of VLDL via IDL to LDL and eventually to small dense LDL (sdLDL).[2,17] Just like chylomicron remnants, VLDL remnants are taken up mainly via apoE by the LDLr and LRP, with additional roles for HSPG and SR-BI.[1,2,16-18,21,22] The lipolytic end product LDL is virtually depleted of TG, has lost most of its apolipoproteins except for apoB-100 and is taken up mainly via apoB-100 by the LDLr in the liver and by tissues involved in hormone synthesis such as the adrenals and gonads for cholesterol supply.

1.3 HDL

In contrast to apoB-containing lipoproteins, HDL is generally believed to be anti-atherogenic, mainly because of its involvement in the reverse cholesterol transport (RCT). This process describes the transport of cholesterol from the periphery back to the liver, after which cholesterol is secreted via the bile into the feces. The liver and intestine both synthesize the major HDL apolipoprotein, apoAI, and release it into the circulation. Subsequently, apoAI is lipidated via the ATP binding cassette transporter A1 (ABCA1), which is also mainly expressed in the liver and in the intestine. This is a crucial step in HDL formation as subjects with ABCA1 gene mutations as well as mice lacking ABCA1 have very low HDL levels.[23-26] During this process nascent discoidal HDL (HDL-3) is formed, a small lipoprotein particle mainly consisting of apoAI and phospholipids. While the liver and intestine are essential for the initial lipidation of apoAI, nascent HDL can take up additional cholesterol and other lipids from the periphery via ABCAI or from other lipoproteins via PLTP.[8,9] The acquired cholesterol is esterified by lecitin:cholesterol acyltransferase (LCAT), allowing the resulting CE to accumulate in the core. Esterification of cholesterol by LCAT is also essential in HDL metabolism, as patients or mice lacking LCAT have low HDL levels.[27,28] Following cholesterol esterifcation by LCAT, HDL becomes a spherical HDL-2 particle which contains not only apoAI but can also acquire apoAII, apoAIV, apoAV, apoCI, apoCII, apoCIII and apoE. It has been suggested that more cholesterol from the periphery, including from macrophages in the vessel wall can be taken up in the mature particle via ABCG1.[29] Cholesteryl esters can be transferred from HDL to TG-rich lipoprotein particles by CETP,[10,11] or selectively taken up by the liver via SR-BI.[30,31] TG and phospholipids in HDL can be lipolyzed by hepatic lipase (HL) and endothelial lipase (EL).[32-34] Once taken up by the liver, HDL-derived cholesterol can be stored, used for the assembly of new lipoproteins, or

converted into bile acids (initiated by Cyp7A1 or Cyp27A1) or neutral sterols. In the liver, ABCG5 and ABCG8 are involved in secretion of sterols into the bile, after which sterols enter the intestine and are reabsorbed (so-called enterohepatic circulation) or excreted into the feces.[33,35-38] In addition to its role in reverse cholesterol transport, HDL has anti-oxidative, anti-inflammatory and anti-thrombotic properties.

2. Atherosclerosis

2.1 Dyslipidemia

The apoB-containing lipoproteins (*i.e.* chylomicrons, VLDL, LDL) are considered to be atherogenic since these particles can enter the arterial wall, become modified by *e.g.* oxidation and aggregation, after which they can be taken up by arterial macrophages that subsequently turn into foam cells and initiate the atherosclerotic process (see section 2).[1,2,39,40]
HDL is protective in atherosclerosis because of its role in RCT as described above. In addition HDL also has antioxidative, anti-inflammatory and anti-thrombotic properties. HDL inhibits the oxidation of LDL by transition metal ions and 12/15-lipooxygenase-mediated formation of lipid hydroperoxides. HDL can scavenge oxygen-derived free radicals and carries antioxidative proteins including paraoxonase, platelet-activation factor acetylhydrolase (PAF-AH) and glutathione peroxidase. In addition, apoAI may also have anti-oxidative functions. HDL is anti-inflammatory as it can repress induction of cell adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which reduces leukocyte attachment to the vessel wall. Other anti-inflammatory properties probably also result from its ability to remove cholesterol and oxysterols from the vessel wall and because of its anti-oxidative actions.[35,41,42] HDL improves vasorelaxation via stimulation of nitric oxide (NO) and prostacyclin synthesis which are both stimulators of vasorelaxation. Its stimulatory effects on NO and the ability of HDL to inhibit tissue factors render HDL anti-thrombotic as well.[41,42]
Dyslipidemia as characterized by high plasma levels of cholesterol and TG in VLDL and LDL particles and low plasma levels of HDL-C, is thus an important risk factor for the development of atherosclerosis. Dyslidemia can be caused by genetic disorders (*i.e.* primary dyslipidemia). Impaired lipoprotein clearance by defects in the LDLr or genes that interact with the LDLr, such as apoE and apoB, results in familial hypercholesterolemia (FH). Defects in LPL or genes interacting with LPL, for example deficiency of apoCII, the cofactor for LPL, are main causes of familial hypertriglyceridemia. Hypoalphalipoproteinemia (low HDL) can be caused by mutations in apoAI, ABCA1 and LCAT. Secondary dyslipidemias are not caused by a monogenetic disorder but by other

diseases, life style or medication. These include obesity, diabetes, hypothyroidism, exercise, diets rich in saturated fat, glucocorticoids, retinoic acid derivates, and HIV protease inhibitors.[43,44] Dyslipidemia is a major risk factor for coronary heart disease (CHD) (see section 2.2). In addition to dyslipidema, other lipid-unrelated factors can also increase CHD risk. These lipid unrelated risk factors for CHD include homocysteinemia, hypertension and infection.[45]

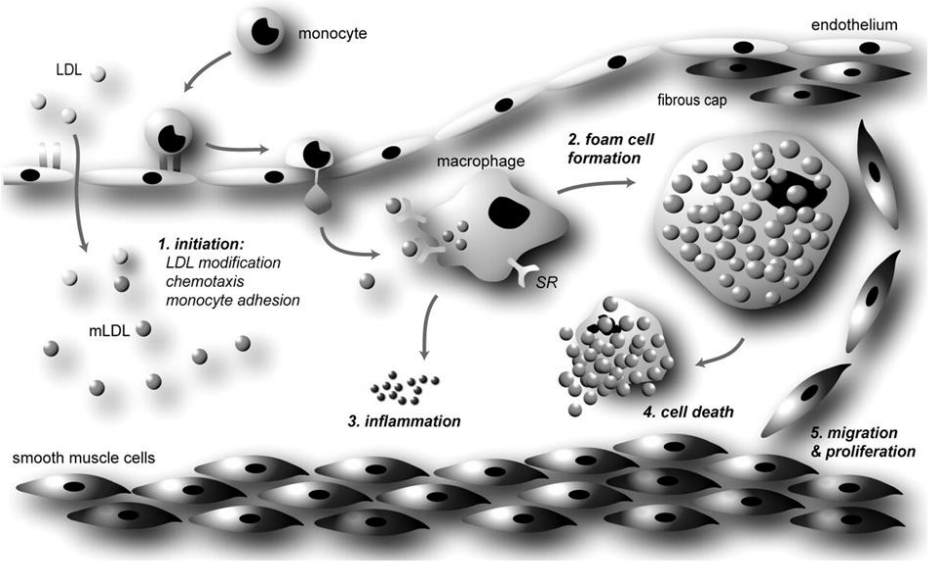

Figure 2. pathogenesis of atherosclerosis development. Adapted from de Winther *et al*.[46] See text for explanation.

2.2 Atherosclerosis

Atherosclerosis is a complex disorder in which lipids and fibrous elements accumulate in the vessel wall (Fig. 2). The innermost layer of a healthy vessel is the thin intima, consisting of a monolayer of endothelial cells (EC) on the luminal side and the internal elastic lamina consisting of elastic fibers. The second layer, the media, consists of smooth muscle cells (SMC) and the third layer, adventitia, consists of connective tissue with fibroblasts and SMC.[39] Atherosclerosis starts with the infiltration of atherogenic lipoproteins such as LDL into the vessel wall where LDL-apoB can interact with proteoglycans which can lead to retention of LDL in the vessel wall. Once trapped, LDL can be modified (*e.g.* by oxidation or aggregation). Accumulation of oxidized LDL stimulates EC to produce inflammatory cytokines, such as chemoattractant

molecules (*e.g.* monocyte chemoattractant protein-1, MCP-1) and growth factors (e.g. macrophage colony-stimulation factor M-CSF) and they begin to express adhesion molecules such as VCAM-1, ICAM, E-selectin, P-selectin, ß2 integrin, Very late antigen-4 (VLA-4) and platelet cell adhesion molecule (PCAM-1). Leukocytes such as monocytes and T cells are attracted and adhere to and migrate into the vessel wall via these adhesion molecules. Activation of leukocyte Toll-like receptors (TLRs) in the vessel wall leads to production of more pro-inflammatory molecules including cytokines and proteases. Within the plaque, monocytes become macrophages that take up LDL after extensive oxidation by reactive oxygen produced by EC and several enzymes including myeloperoxidase, sphingomyelinase and secretory phospholipase. The uptake of oxidized LDL occurs mainly via the scavenger receptors scavenger receptor-A (SR-A), cluster designation (CD)36 and CD68 which are upregulated by cytokines present in the plaque. Internalized cholesterol is esterified by acyl CoA:cholesterol acyltransferase-1 (ACAT-1) and is stored in lipid droplets, and the macrophage becomes a lipid-rich foam cell. In the macrophage foam cell, CE can be hydrolyzed again and the cell can dispose cholesterol via efflux via ABCA1 and ABCG1 to apoE produced by the macrophage and HDL. Early lesions consisting of macrophage foam cells and T cells are called fatty streaks and give no symptoms but are precursors of more advanced lesions.[39,40,45,47]
When the lesion becomes more advanced, interactions between inflammatory cells, ECs and SMCs evoke a chronic inflammatory state, more cytokines are expressed and SMCs migrate into the plaque. SMCs and macrophage foam cells accumulate and die leaving their lipid content behind leading to accumulation of a lipid-rich necrotic debris which is usually covered by a fibrous cap consisting of SMCs and extracellular matrix excreted by these SMCs. The plaques can become more complex e.g. by calcification. The large plaque can lead to ischemic symptoms when it blocks blood flow. In addition, the plaque can lead to an acute block of blood flow when it ruptures. The stability of a plaque depends on its composition. Vunerable plaques have thin fibrous caps and a large number of leukocytes, mainly as macrophages produce proteases that degrade the extracellular matrix of the fibrous cap. Plaque rupture exposes pro-thrombotic material which activates a coagulation cascade leading to thrombosis and an acute blockage of blood flow and infarction.[39,40,45,47]

2.3 Animal models to study lipid metabolism and atherosclerosis

To study atherosclerosis *in vivo*, several animal models have been used. Non-human primates develop atherosclerosis very similar to humans but are a less suitable model because of ethical issues, high costs and because it takes very long to develop atherosclerosis.[48] Therefore, in early atherosclerosis studies, birds, especially pigeons, chickens and quails[49-51] were used, as they are relatively hypercholesterolemic and atherosclerosis prone.[49-52] Another

atherosclerosis-susceptible model used in early studies is the swine,[48,53,54] a major drawback is however the size of the animals leading to high costs and the need for large amounts of experimental agents.[48] Dogs and rats are resistant to atherosclerosis and cats develop no human-like lesions and are, therefore, also not suitable models.[48,52,55] The hamster is sensitive for cholesterol-enriched diets and develops mild fatty streak-like atherosclerotic lesions and is, therefore, used in some studies.[48,55] Rabbits, a widely used animal model, have as herbivores naturally low cholesterol levels and no atherosclerosis development. On atherogenic diets, however, rabbits are atherosclerosis prone but lesions are macrophage-rich and have a fatty streak-like appearance.[52] The Watanabe heritable hyperlipidemic (WHHL) rabbit has a defect in the LDLr and is therefore hyperlipidemic, susceptible to atherosclerosis and able to develop more advanced lesions.[56,57] A drawback of rabbits is that atherosclerosis development is dependent on infections as pathogen-free rabbits develop no atherosclerosis.[48] Another drawback is that they have no HL which is important in HDL metabolism.[58]

The mouse is a widely used model for atherosclerosis studies because of low costs and availability of several strains and genetically modified mice. Wild-type mice have a plasma cholesterol of approximately 2 mM, which is almost all confined to the HDL fraction, while VLDL and LDL are virtually absent. These animals require an extreme atherogenic diet to develop atherosclerosis.[59,60] Since mice are exceptionally suitable for genetic modification, several atherosclerosis-prone mouse models have been generated, such as apoE-knockout ($apoE^{-/-}$), LDLr-knockout ($LDLr^{-/-}$) and APOE*3-Leiden transgenic (E3L) mice. Nowadays these mice are widely used as animal model in atherosclerosis research.

The apoE-knockout mouse has highly impaired VLDL and LDL clearance, as apoE is important in the uptake of lipoprotein remnants by the liver. Therefore, plasma cholesterol is increased in these mice (approx. 8 mM on chow and up to 70 mM on a high cholesterol-containing diet) and present mainly in VLDL and LDL. Therefore, these animals already develop atherosclerosis on a chow diet[61-64] and heterozygote apoE-knockout mice develop atherosclerosis on an atherogenic diet.[65] Lack of macrophage apoE also contributes substantially to the atherosclerosis susceptibility of these mice as mice lacking apoE specifically in the macrophage have increased foam cell formation and atherosclerosis.[66]

LDLr knockout mice have also highly impaired VLDL and LDL clearance and increased plasma cholesterol levels, as the LDLr is important in uptake of these lipoproteins by the liver. However, their phenotype is milder than that of apoE-knockout mice and they therefore need an atherogenic diet to develop atherosclerosis.[67,68] A drawback of both $apoE^{-/-}$ and $LDLr^{-/-}$ mice is that they do not respond in a human-like manner to pharmacotherapeutic interventions like statins and fibrates, with respect to their lipid-lowering properties.[69]

E3L mice carry a construct containing apoE*3-Leiden, a mutation of apoE characterized by a tandem duplication of codons 120-126 that causes hyperlipidemia in humans,[70,71] together with apoCI that elevates plasma TG. As a result, E3L mice have somewhat increased levels of plasma cholesterol and TG on a chow diet,[70] but their phenotype with respect to plasma lipids is milder than that of apoE-knockout and LDLr-knockout mice, and the mice need an atherogenic diet for inducing atherosclerosis development.[72,73] A major advantage of E3L mice is that they respond in a human-like manner to pharmaceutical interventions including statins and fibrates with respect to lipid lowering.[69] Probably because mice lack CETP, an important protein in HDL metabolism, E3L mice do not properly respond to HDL modulating therapy. Therefore, we have crossbred E3L mice with CETP transgenic mice.[74-77]

3. Factors regulating lipid metabolism

3.1 Apolipoproteins

Apolipoproteins stabilize the lipoprotein particle and have functions in lipid and lipoprotein metabolism. Several apolipoproteins are known and they all have their own functions.
ApoAI and apoAII are the two major HDL-associated apolipoproteins and are both required for normal HDL synthesis. ApoAI is present on most HDL particles and constitutes 70% of HDL protein. ApoAII is present on two third of the HDL particles and constitutes 20% of HDL protein content.[33,36] ApoAI is synthesized in the liver and intestine and is lipidated to form HDL. In humans and mice, apoAI deficiency leads to a large decrease in HDL[78,79] and increase in atherosclerosis.[80] ApoAII is synthesized in the liver, and apoAII deficiency also reduces HDL levels,[81] indicating that both apoAI and apoAII are needed for HDL synthesis. ApoAIV is expressed in the intestine, increases HDL levels and protects against atherosclerosis in mice.[82,83] It may also be involved in chylomicron synthesis[84,85] and is important in regulation of food intake.[86] ApoAV is a more recently discovered apolipoprotein that reduces TG levels.[87] ApoAV stimulates LPL-mediated TG lipolysis and inhibits VLDL production which may explain the effect of apoAV on TG levels.[88]
ApoB is present on chylomicrons, VLDL, IDL and LDL. ApoB consists in two forms: apoB48 is expressed by the intestine and is present on chylomicrons, whereas apoB100 is expressed in the liver and present on VLDL. In several species including mice both apoB forms are expressed by the liver. ApoB is required in the assembly of chylomicrons and VLDL and serves as ligand for lipoprotein clearance by the LDLr in liver and other tissues.[89] Mutations in the apoB gene can lead to hypolipidemia or, when the LDLr binding domain is affected, to hyperlipidemia.[90]

ApoE is present on chylomicrons, VLDL, IDL and HDL. ApoE is crucial for the efficient uptake of lipoprotein remnants by the liver.[91] However, at high concentrations, apoE inhibits LPL that may lead to hypertriglyceridemia.[92] Lack of apoE in mice severely increases atherosclerosis development[61-64] and apoE deletion in macrophage increases foam cell formation and atherosclerosis.[66]
An interesting apolipoprotein which, despite its small size, has many functions in lipid metabolism but also in inflammation is apoCI. ApoCI is mainly synthesized in the liver but also in macrophages. ApoCI is released into the circulation, is present on chylomicrons, VLDL, LDL and HDL and is highly exchangeable between these lipoproteins. ApoCI is the smallest of the apolipoproteins (57 amino acids, 6.6 kDa) and highly positively charged. The apoCI peptide forms 2 α-helices which are separated by a flexible linker. ApoCI has many functions in lipoprotein metabolism. Overexpression of apoCI leads to highly increased TG levels and mildly elevated total cholesterol levels in mice,[93] while apoCI knockout mice have decreased TG levels and decreased HDL levels at least on an apoE-knockout background. The elevated TG and cholesterol levels in apoCI-overexpressing mice are mainly confined to VLDL and were initially explained by an inhibitory effect of apoCI on lipoprotein clearance via the LDLr and other classical apoE-recognizing receptors.[94-98] Later, apoCI was found to be an inhibitor of LPL[93] which explains the relative large elevation in TG as compared to cholesterol in apoCI overexpressing mice. In addition, apoCI affects HDL metabolism by stimulation of LCAT,[99,100] inhibition of HL,[101,102] and inhibition of CETP.[103] These effects on HDL thus suggest that apoCI may causally increase HDL levels. Overall, apoCI expression is atherogenic, at least in absence of CETP, probably because of the induction of hyperlipidemia.[104] In addition to affecting plasma cholesterol and TG levels, apoCI has also a role in endodermal lipid metabolism as mice overexpressing high levels of apoCI have skin abnormalities.[105] In addition, apoCI is involved in regulation of inflammation as it enhances the early response to LPS.[106]
Other members of the apoC family are also involved in regulating TG lipolysis. ApoCII is the cofactor for LPL, is essential for lipolysis of TG, and apoCII deficiency thus leads to severe hyperlipidemia.[107,108] ApoCIII, on the other hand, is the main endogenous LPL inhibitor. Overexpression of apoCIII thus leads to elevated TG levels and apoCIII-deficiency to decreased TG levels.[109,110] ApoCIII may also increase intestinal lipid uptake and VLDL production.[111]

3.2 Cholesteryl ester transfer protein

The human CETP gene is located on the long arm of chromosome 16 (16q12-16q21).[112] The gene is 25 kb and consist of 16 exons between 32 to 250 bp which account for 8% of the total gene sequence.[113,114] CETP gene expression is regulated by several factors, including the zinc finger proteins SP1 and

SP3,[115,116] ARP-1,[117] C/EBP,[118] and lipids, directly, or indirectly via SREBP[119,120] and LXR.[11,121-124] In addition to the normal full-length mRNA, an alternatively spliced mRNA can be expressed in which exon 9 is removed. The exon 9-deleted protein is inactive and inhibits secretion of the normal CETP protein[125,126] and may also be involved in the regulation of plasma CETP activity. CETP is expressed mainly in the liver, adipose tissue and in macrophage-rich tissues and is a 74 kDa (476 aa) glycoprotein.[114] CETP is highly hydrophobic as it consists of 45% hydrophobic amino acids which form a hydrophobic pocket for the binding of neutral lipids.[127] CETP circulates in the plasma at a concentration of approx. 1-3 μg/mL and is mainly bound to HDL (74%).[11,128] CETP transfers neutral lipids (*i.e.* TG and CE) between plasma lipoproteins leading to a net transfer of CE from HDL to VLDL and a reciprocal transfer of TG from VLDL to HDL.[11]

As CETP transfers CE out of HDL, CETP activity leads to decreased HDL-C levels. Since HDL-C is associated with reduced cardiovascular disease (CVD) risk, CETP has been suggested to be atherogenic. Albeit that CETP-deficiency thus was expected to be atheroprotective, studies involving CETP-deficient subjects showed controversial results. In Japanese subjects a CETP mutation has been identified that leads to complete CETP deficiency (Intron14+1 G>A)[129] and another mutation that leads to a marked reduction of CETP (D442G).[130] Both mutations indeed increase HDL levels, especially those of large HDL. The HDL of CETP-deficient subjects is enriched in CE and poor in TG. TG and LDL-C levels are not or only mildly affected and LDL particles are smaller and more heterogeneous compared to LDL of normal subjects.[130-133] Although their high HDL levels suggest protection from atherosclerosis, CETP deficient subjects are susceptible to atherosclerosis development.[134] Remarkably, prevalence of CETP-deficiency in people over 80 years is reduced indicating that CETP deficiency does not reduce overall mortality.[135] Zong *et al.*[136] showed even an increase of CHD in carriers of a CETP mutation with HDL-C levels between 1-1.5 mM. However, CHD prevalence was similar in patients with higher HDL levels with and without CETP mutations. Another study showed that high HDL (>2 mM) protects against CHD independent of CETP mutations.[137] The relation between CETP deficiency and atherosclerosis thus remains controversial. However, these mutations are often linked to HL deficiency.[134] Together with the low number of subjects, this makes it difficult to study the effect of CETP on atherosclerosis.[133,138]

Besides the Intron14+1 G>A and D442G mutations, some other CETP gene polymorphisms have been identified. These polymorphisms affect plasma CETP activity to a milder extent, but these mutations are more common and, therefore, easier to study in large patient groups. The TaqIB polymorphism which is in strong linkage disequilibrium with -C629A[139] is the most widely studied CETP polymorphism. The B2 allele is clearly associated with reduced CETP levels and higher HDL levels. However, again literature is inconsistent

about effect of the TaqIB phenotype on CHD risk. The B2 allele has either been associated with reduced CHD risk,[140-143] no effect on CHD risk,[144-146] or even an increased CHD risk.[147,148] A recent review showed that the odds ratio for CVD risk was 1.45 in B2B2 carriers versus B1B1 carriers in population based studies, while the odds ratio in high risk populations was 0.84 for B2B2 carriers versus B1B1. This difference could possibly be explained by selection for a lower frequency of B2B2 carriers in high risk populations. However the effect of this common CETP gene variant on CVD was only modest.[149] Mutations in the CETP gene that cause low CETP mass thus clearly cause higher HDL-C levels, but the effect of CETP mutations on atherosclerosis is controversial.

In addition to human studies, animal models have been used to experimentally address the effect of CETP on atherosclerosis further. Since wild-type mice are naturally deficient for CETP, CETP transgenic mice have been created.[121,150,151] These mice have reduced HDL-C levels in plasma.[150,151] Simian CETP expression in wild-type mice increases atherosclerosis.[151] Since LDLr$^{-/-}$ and apoE$^{-/-}$ mice are hyperlipidemic and have a more human like lipoprotein profile, they have been considered as relevant mouse models to study atherosclerosis. Similarly to wild-type mice, CETP expression in both hyperlipidemic mice increases atherosclerosis.[152] Also, in E3L mice, CETP expression leads to a higher VLDL, lower HDL and increases atherosclerosis by 7-fold.[75]

CETP thus increases atherosclerosis in wild-type mice and in hyperlipidemic mice in which VLDL clearance is impaired to some extent. However, CETP has been shown to be anti-atherogenic in other mouse models. LCAT overexpressing mice have a high increase of plasma levels of large HDL, CETP expression in these mice reduces HDL levels and atherosclerosis.[153] Similarly, SR-BI-deficient mice accumulate large HDL. In this model, CETP expression reduces atherosclerosis[154] as explained by normalization of dysfunctional HDL. However, CETP expression did not reduce atherosclerosis in SR-BI mice in another study despite of HDL normalization (Van Eck *et al.* unpublished results). The latter may be related to the finding that SR-BI-deficiency not only results in dysfunctional HDL, but also increases oxidative stress,[155] which is not relieved upon CETP expression. In mice with hypertriglyceridemia due to apoCIII overexpression and in diabetic mice, CETP expression protects against atherosclerosis development, probably by reduction of total cholesterol levels.[156-158]

CETP is thus protective in mouse models of diabetes and hypertriglyceridemia, possibly related to a plasma cholesterol lowering effect of CETP in these models. Also when HDL accumulates CETP is protective by reducing HDL via an alternative route. In animal models with a more humanized lipoprotein profile however, CETP is atherogenic.

3.3 Nuclear receptors

Since several nuclear receptors are important in regulating expression of genes in lipid metabolism, they are potential targets in drug development.
Peroxisome proliferator-activated receptors (PPARs) are important regulators of expression of genes involved in lipid and glucose metabolism. PPARs are activated by FA and ecosanoids and heterodimerize with RXR to affect gene expression by binding to DR-1 responsive elements. PPARα is expressed in several metabolically active tissues including liver and muscle where it is important for regulation of genes involved in lipid metabolism such as apoCIII, LPL and apoAI (the latter in humans, but not in mice). PPARγ is expressed in adipose tissue, macrophages, colon and placenta and is important in regulation of lipid and glucose metabolism and adipocyte differentiation. PPARγ activation makes tissues more insulin sensitive and agonists are therefore applied in diabetes. PPARδ is expressed at low levels in a variety of tissues, is involved in lipid and glucose metabolism[159-162] and is regarded as novel target in the treatment of dyslipidemia and insulin resistance.[163]
Sterol Regulatory Element Binding Proteins (SREBPs) are other important regulators in lipid metabolism. SREBPs are activated when cells are depleted of cholesterol. Three SREBP isoforms exist, namely SREBP1a, SREBP1c and SREBP2. SREBP1a and SEBP1c are derived from the same gene by use of alternative transcription start sites. SREBP1a is a potent activator of all SREBP responsive genes, these genes are involved in cholesterol, FA and TG synthesis and include HMG-CoA reductase, FAS and SCD. SREBP1c mainly activates genes for FA synthesis and SREBP2 activates genes in cholesterol synthesis.[164] SREBP1c and SREBP2 also induce genes important for the synthesis of NADPH which is used in lipid biosynthesis.
Another group of receptors important in regulation of lipid metabolism include LXR, FXR, PXR and RXR. LXR consists in 2 forms, LXRα and LXRß which are both activated by oxysterols (*i.e.* cholesterol derivates). LXRα is mainly expressed in liver and macrophages while LXRß is more ubiquitously expressed. Upon activation, LXR forms a heterodimer with RXR which binds to LXR-responsive elements to affect expression of genes in lipid metabolism such as apoE, CETP, ABCA1 and SREBP1c.[165,166] In mouse models, LXR agonism shows protection against atherosclerosis, but leads also to hypertriglyceridemia due to increased VLDL production.[167,168] FXR and PXR also form heterodimers with RXR to regulate gene expression. FXR is activated by bile acids and target genes include Cyp7a1 and PLTP. FXR plays an important role in the regulation of synthesis, excretion and reuptake of bile acids from the intestine but also reduces plasma lipid levels.[166] PXR is activated by xenobiotics and increases expression of the Cyp3a enzymes to increase removal of xenobiotics by the body. PXR increases hepatic TG synthesis and may affect HDL metabolism but the effect of PXR on overall lipid metabolism is unknown.[162,166,169]

4. Pharmacological interventions in dyslipidemia

High LDL-C and low HDL-C are associated with increased CVD risk. Several anti-atherogenic drugs have been developed that mainly aim at reducing (V)LDL levels, including statins, fibrates, bile acid binding resins and cholesterol uptake inhibitors.

4.1 Statins

Statins are the most widely used drugs to reduce plasma (V)LDL-C levels. The first statins were fungal derivatives (*e.g.* pravastatin and simvastatin) but later more potent fully synthetic statins including atorvastatin and rosuvastatin were developed. Statins show structural similarities to the cholesterol precursor hydroxymethylglutaryl-coenzyme A (HMG-CoA) and, therefore, they block entry of HMG-CoA to HMG-CoA reductase, an enzyme important in cholesterol synthesis.[170,171] Via this action, statins inhibit cholesterol synthesis in the liver and its subsequent release in the plasma within VLDL particles.[172] In addition, to compensate for hepatic cholesterol depletion, the LDLr is upregulated and uptake of lipoproteins from the plasma is increased, which contributes to the reduction in plasma cholesterol levels.[173] Statins reduce not only cholesterol levels but also TG which may contribute to their anti-atherogenic effects.[174] In addition, statins mildly increase HDL-C levels (up to +10%).[175] Atorvastatin treatment has been associated with a decrease in CETP mass[176-178] and activity.[177-180] however whether a reduction in CETP is the causal factor for the observed HDL increase has not been established yet. In addition to lipid lowering, statins improve endothelial function, are anti-oxidative and are anti-inflammatory contributing to its atheroprotective actions. Statins also inhibit cell proliferation and are, therefore, anticarcinogenic and statins may inhibit kidney graft rejection.[181] Statins decrease plasma (V)LDL-C efficiently up to -40%[182] and the combined actions of statins lead to a reduction of cardiovascular events of about -20% per mM cholesterol reduction.[183]

4.2 Fibrates

Fibrates are PPARα agonists, and therefore affect transcription of many genes in lipid metabolism leading to a net reduction of mainly plasma TG (up to -50%) and a mild reduction in plasma cholesterol.[160,184,185] The reduction in plasma TG may be a consequence of increased TG lipolysis caused by upregulation of LPL[186] and downregulation of the LPL inhibitor apoCIII.[187-189] TG may also be reduced by increased hepatic ß-oxidation and reduced FA synthesis.[189-192] In addition, fibrates mildly increase HDL-C (up to +20%).[175,193] Fibrates induce apoAI expression in humans but not in mice which may contribute to the observed HDL increase in humans.[194] Another difference

between humans and mice is that mice do not express CETP. CETP activity in humans is reduced upon treatment with fibrates[195] but if this contributes to the HDL increase in humans is still unknown. The clinical benefit of fibrates is uncertain.[184,185] A recent meta analysis shows a reduction of non fatal MI (-22%) but not of other cardiovascular events including cardiovascular mortality.[184]

4.3 Bile acid binding resins and cholesterol uptake inhibitors

Other cholesterol lowering drugs available are bile acid binding resins and cholesterol uptake inhibitors. Resins bind bile acids in the intestine, which interrupts the enterohepatic circulation of bile acids and results in an increased excretion of bile acids via the feces. This results in an increased production of new bile acids from cholesterol in the liver and therefore lowers plasma cholesterol levels. Resins reduce plasma cholesterol levels up to -25%.[196-198] Cholesterol uptake inhibitors such as ezetimibe reduce intestinal cholesterol absorption via Niemann-Pick C1 Like 1 (NPC1L1), a protein essential in cholesterol uptake from the intestine.[199] On top of statin treatment, ezetimibe reduces cholesterol by an additional -16%, but does not affect IMT, possibly related to a low baseline IMT of the study subjects. The effect of ezetimibe on clinical endpoints is still uncertain.[200]

Via effective plasma cholesterol lowering, statins, fibrates, bile acid binding resins and cholesterol uptake inhibitors prevent up to 40% of cardiovascular events, a significant residual risk thus remains. Therefore several new drugs to prevent CVD further are under development. As HDL has been suggested to be a more important predictor of CVD development,[201] one group of these new drugs are aimed to increase HDL and include niacin and CETP inhibitors.

4.4 Niacin

Niacin (nicotinic acid, vitamin B3) is the most potent HDL-raising drug used in the clinic. In addition to raising HDL-C (up to +35%),[196,202-204] niacin decreases plasma LDL-C and TG levels (up to -25 and -50% respectively). The reduction in TG and cholesterol may be explained as niacin decreases hormone sensitive lipase activity via the GRP109A. This rapidly decreases plasma FA. Therefore, less FA are available for TG synthesis in the liver and subsequent VLDL production.[204] Niacin may also decrease TG synthesis via a direct effect on TG production in hepatocytes.[205,206] The underlying mechanism of the HDL increase is also not fully understood but is possibly related to CETP.[207] Niacin reduces IMT progression[38] and overall mortality (-11%)[208] but is not well tolerated because it causes severe flushing via increasing plasma prostaglandins.

Addition of the prostaglandin D_2 receptor 1 blocker laropiprant reduces niacin mediated flushing and makes niacin therefore a better tolerated drug.[203]

4.5 CETP inhibitors

As CETP decreases HDL, various strategies have been developed to inhibit CETP activity to increase HDL levels. Two natural CETP inhibitors are known. The lipid transfer inhibitory protein (LTIP), also called apoF, has been detected in LDL and inhibits the involvement of LDL in the actions of CETP.[209,210] In addition, apoCI has been discovered as the main endogenous inhibitor of CETP activity on HDL.[103]
The first experiments to evaluate the effect of inhibition of CETP were performed with antibodies against CETP. These antibodies were indeed able to increase HDL in hamsters and rabbits.[211-213] Antisense oligodeoxynucleotides (ODNs) against CETP also reduced CETP mRNA and increased HDL in rabbits,[214] and were also able to reduce the aortic cholesterol content and lesion area.[215] Atherosclerosis could also be reduced in rabbits by vaccination to generate auto-antibodies against CETP.[216] The first chemical compound designed to inhibit CETP tested in rabbits was JTT-705. JTT-705 inhibits CETP by the formation of a disulphide bond with CETP. In rabbits, JTT-705 indeed increased HDL, decreased non HDL-C and reduced atherosclerosis.[217] In a second study in which rabbits were fed a high cholesterol diet, JTT-705 failed to reduce atherosclerosis despite of an increase in HDL.[218] A second CETP inhibitor is torcetrapib, which inhibits CETP via the formation of an inactive complex with CETP and HDL.[219] Torcetrapib increases HDL and reduces atherosclerosis in rabbits.[220] In humans, both JTT-705 and torcetrapib are well tolerated in short term studies, despite of a small increase in blood pressure in torcetrapib treated subjects. Both compounds raise HDL-C in humans.[221-224] JTT-705 is only tested in short term studies in humans, and therefore its effect on atherosclerosis and cardiovascular events in humans is still unknown. Torcetrapib is tested in long term studies in combination with atorvastatin. Despite of a HDL increase of about 60% however, torcetrapib in combination with atorvastatin treatment failed to reduce atherosclerosis, as assessed by Intima Media Thickness (IMT) an Intravascular Ultrasound (IVUS), compared to atorvastatin alone.[225-227] Moreover, more people died in the torcetrapib treated group as compared to the atorvastatin alone group and cardiovascular event rates were increased rather than decreased by torcetrapib.[228] These adverse effects may well be compound-specific, but further studies into the mechanism of the adverse effects are necessary to evaluate if CETP inhibition is still a promising strategy in the search for new anti-atherogenic drugs. In addition, it is still unknown if the combination with atorvastatin extinguished a protective effect of torcetrapib. Therefore further studies are needed to evaluate if torcetrapib or other CETP inhibitors alone are able to reduce atherosclerosis. A

new CETP inhibitor is anacetrapib, a torcetrapib-like compound that increases HDL in humans without affecting blood pressure.[229] If anacetrapib will decrease atherosclerosis is however still unknown.

5. Outline of this thesis

Statins, fibrates and cholesterol absorption inhibitors lower plasma cholesterol very efficiently (up to 40%). However, efficient cholesterol lowering only prevents a fraction of cardiovascular events. Therefore new therapeutic strategies to further reduce cardiovascular events are necessary. HDL-raising therapy may be such a new strategy, and CETP is an important factor in regulating HDL levels. In this thesis we evaluate the mechanism underlying the effects of pharmaceutical intervention on HDL metabolism in E3L.CETP mice.
In humans statins and fibrates mildly increase HDL. This effect is not observed in E3L mice, despite a human-like cholesterol lowering effect. To evaluate whether the HDL increase as seen in humans depends on CETP expression, we treated E3L.CETP and E3L mice with a diet rich in fat and cholesterol, and added fenofibrate (**chapter 2**) or atorvastatin (**chapter 3**). The most potent HDL raising drug available is niacin, but the mechanism underlying the HDL increase is still unknown. In **chapter 4** we treated E3L.CETP mice with niacin to evaluate the involvement of CETP in niacin's HDL raising properties.
Torcetrapib has been the first CETP inhibitor tested in large clinical trials, and is able to increase HDL by about 60%. However, despite the large increase in HDL, humans treated with atorvastatin and torcetrapib showed no reduction in atherosclerosis (measured by IMT and IVUS) as compared to patients treated with atorvastatin only. Moreover, torcetrapib treatment led to adverse effects including an increase in cardiovascular events and increased death rate. To study the effects of torcetrapib with and without atorvastatin and to study the adverse effects of torcetrapib, in **chapter 5** we treated E3L.CETP mice with torcetrapib and atorvastatin.
In **chapter 6** we studied another mechanism to interfere with HDL metabolism. In literature, PXR agonists are shown to increase HDL levels in wild type mice. However, other studies suggest that PXR activation decreases rather than increases HDL. In addition, the effect of PXR on HDL in the presence of CETP is not known. To evaluate the effect of PXR in a model with a human like lipoprotein profile, we treated E3L and E3L.CETP mice with a high fat/cholesterol diet with and without the PXR agonist pregnenolone-16α-carbonitrile (PCN).
ApoCI has several functions in HDL metabolism. ApoCI is the main endogenous HDL associated CETP inhibitor, the second LCAT activator, and apoCI inhibits HL. The effect of apoCI on HDL clearance and overall HDL levels is however not known. Therefore, we studied the effect of apoCI on SR-BI *in vitro* and overall effect of apoCI in HDL metabolism *in vivo* in **chapter 7**.

In **chapter 8** we focused on the CETP-inhibitory effect of apoCI. As full length apoCI increases VLDL levels by LPL reduction, full length apoCI is not a good agent to increase HDL by CETP inhibition. Therefore we used an array of apoCI peptides to identify a peptide that inhibits CETP but does not inhibit LPL efficiently.
Chapter 9 gives an overview of animal models that are used to study HDL metabolism. The results of the studies described in this thesis and the future perspectives are discussed in **chapter 10**.

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Chapter 2

FENOFIBRATE INCREASES HDL-CHOLESTEROL BY REDUCING CHOLESTERYL ESTER TRANSFER PROTEIN EXPRESSION

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Abstract

In addition to efficiently decreasing VLDL-triglycerides (TG), fenofibrate increases HDL-cholesterol levels in humans. We investigated whether the fenofibrate-induced increase in HDL-cholesterol is dependent on the expression of the cholesteryl ester transfer protein (CETP). To this end, *APOE*3-Leiden* (*E3L*) transgenic mice without and with the human CETP transgene, under control of its natural regulatory flanking regions, were fed a Western-type diet with or without fenofibrate. Fenofibrate (0.04% in the diet) decreased plasma TG in *E3L* and *E3L.CETP* mice (-59% and -60%; $P<0.001$), caused by a strong reduction in VLDL. Whereas fenofibrate did not affect HDL-cholesterol in *E3L* mice, fenofibrate dose-dependently increased HDL-cholesterol in *E3L.CETP* mice (up to +91%). Fenofibrate did not affect the turnover of HDL-cholesteryl esters (CE), indicating that fenofibrate causes a higher steady-state HDL-cholesterol level without altering the HDL-cholesterol flux through plasma. Analysis of the hepatic gene expression profile showed that fenofibrate did not differentially affect the main players in HDL metabolism in *E3L.CETP* mice compared with *E3L* mice. However, in *E3L.CETP* mice, fenofibrate reduced hepatic *CETP* mRNA (-72%; $P<0.01$) as well as the CE transfer activity in plasma (-73%; $P<0.01$). We conclude that fenofibrate increases HDL-cholesterol by reducing the CETP-dependent transfer of cholesterol from HDL to (V)LDL, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool.

Introduction

High plasma TG levels are correlated with an increased risk for cardiovascular disease.[1] Fibrates are widely used to reduce hypertriglyceridemia, thereby generating a less atherogenic lipid phenotype. Fibrates perform their actions through activation of peroxisome proliferator-activated receptor alpha (PPARα).[2,3] Activated PPARα heterodimerizes with retinoid X receptor (RXR) and subsequently binds to specific peroxisome proliferator response elements (PPREs) in target genes to alter their transcription.[2,4] Fibrates thus decrease TG levels by inhibiting hepatic TG production through increased hepatic ß-oxidation and inhibition of *de novo* fatty acid synthesis, increasing LPL-mediated lipolysis, and providing a higher affinity of remnants for the LDL receptor (LDLr).[3]

A meta-analysis of 53 clinical studies using fibrates enrolling 16,802 subjects indicated that apart from a 36% reduction in plasma TG, fibrates increase HDL-cholesterol levels by ~10% in humans.[5] Studies *in vitro* and in (transgenic) mice showed that fibrates increase the hepatic transcription of human *APOA1*[6] and *APOA2*,[7] decrease hepatic receptor B type I (SR-BI) protein,[8] increase the SR-BI-mediated[9] and ABCA1-mediated[10] cholesterol efflux from human macrophages, and increase plasma phospholipid transfer protein (PLTP) activity.[11,12] All of these effects may thus potentially contribute to the increase in HDL-cholesterol as observed in humans.

In contrast to humans, fibrates decrease HDL-cholesterol levels in apolipoprotein E (apoE)-deficient mice[13] and do not affect HDL-cholesterol levels but increase the HDL particle size in wild-type mice and human APOA1 transgenic mice by downregulation of SR-BI[8] and/ or induction of PLTP.[11] The fact that fibrates do not increase the level of regularly sized HDLs in mice may be attributed to the fact that, in contrast to the human *APOA1* promoter, which contains a functional positive PPRE leading to increased *APOA1* transcription, the murine *apoa1* promoter contains a nonfunctional PPRE.[6] However, another major difference between both species is that, in contrast to humans,[14] mice do not express the cholesteryl ester transfer protein (CETP).[15] CETP is a hydrophobic plasma glycoprotein that is involved in the exchange of cholesteryl esters (CE) and TG between HDL and apoB-containing lipoproteins (*e.g.* VLDL and LDL), resulting in the net transfer of CE from HDL to apoB-containing lipoproteins.[16] CETP deficiency in humans is associated with increased HDL-cholesterol levels[17] and inhibition of CETP activity by small-molecule inhibitors increases HDL-cholesterol levels.[18-21] Interestingly, bezafibrate,[22,23] fenofibrate,[24] and ciprofibrate[25] increase HDL-cholesterol in subjects with hyperlipidemia with a concomitant reduction in plasma CETP activity. In the latter study, plasma apoAI levels were not affected, which indicates that fibrates may increase HDL-cholesterol levels via apoAI-independent mechanisms, including a potential effect of fibrates on CETP expression.

Therefore, our aim was to investigate whether the fibrate-induced increase in HDL-cholesterol depends on CETP expression. To this end, we used, *APOE*3-Leiden* (*E3L*) mice that express a natural mutation of the human APOE3 gene (i.e., tandem repeat of codons 120-126, yieling a protein of 306 amino acids) in addition to the human APOC1 gene. Introdution of these genes results in an attenuated clearance of apoB-containing lipoproteins via the LDL receptor pathway. Therefore these mice show modlerately increased cholesterol and TG levels on a chow diet and a human-like lipoprotein profile an a cholesterol rich diet.[26,27] E3L mice were crossbred with mice expressing human *CETP* under control of its natural flanking regions,[28] resulting in *E3L.CETP* mice.[29] *E3L.CETP* and *E3L* littermates were fed a cholesterol-rich (0.25%, w/w) diet with or without fenofibrate. After 2 weeks of administration, fenofibrate dose-dependently increased HDL-cholesterol in *E3L.CETP* mice, but did not affect HDL levels in *E3L* mice. In addition, in *E3L.CETP* mice fenofibrate reduced hepatic *CETP* mRNA expression, as well as CE transfer activity in plasma. From these studies, we conclude that fenofibrate increases HDL-cholesterol by reducing CETP-dependent transfer of CE from HDL to apoB-containing lipoproteins.

Materials and Methods

Animals

Hemizygous human CETP transgenic (*CETP*) mice, expressing a human CETP minigene under the control of its natural flanking sequences[28] were purchased from the Jackson Laboratory (Bar Harbor, ME) and cross-bred with hemizygous *E3L* mice[30] at our Institutional Animal Facility to obtain *E3L* and *E3L.CETP* littermates.[29] In this study, male mice were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 8 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 0.25% (w/w) cholesterol and 15% (w/w) fat (Western-type diet) (Hope Farms, Woerden, The Netherlands) for 3 weeks. Upon randomization according to total plasma cholesterol (TC) levels, mice received Western-type diet without or with 0.004%, 0.012%, or 0.04% (w/w) fenofibrate (Sigma, St. Louis, MO). Experiments were performed after 4 h of fasting at 12:00 PM with food withdrawn at 8:00 AM, unless indicated otherwise. The institutional Ethical Committee on Animal Care and Experimentation approved all experiments.

Plasma lipid and lipoprotein analysis

Plasma was obtained via tail vein bleeding as described[31] and assayed for TC and TG, using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN), respectively. The distribution of lipids over plasma lipoproteins was determined by fast-

performance liquid chromatography using a Superose 6 column as described previously.[31]

Cholesteryl ester transfer activity in plasma
The transfer of newly synthesized CE in plasma was assayed by a radioisotope method as described previously.[32] In short, [^{3}H]cholesterol was complexed with BSA and incubated overnight at 4°C with mouse plasma to equilibrate with plasma free cholesterol. Subsequently, the plasma samples were incubated for 3 h at 37°C. VLDL and LDL were then precipitated by addition of sodium phophotungstate/$MgCl_2$. Lipids were extracted from the precipitate by methanol-hexane (1:2, v/v) and [^{3}H]CE was separated from [^{3}H]cholesterol on silica columns, followed by counting of radioactivity.

Plasma apoAI concentration
Plasma apoAI concentrations were determined using a sandwich ELISA. Rabbit anti-mouse apoAI polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (3 µg/ml) at 4°C and incubated with diluted mouse plasma (dilution 1:400,000) for 90 min at 37°C. Subsequently, goat anti-mouse apoAI antibody (600-101-196; Rockland Immunochemicals, Inc., Gilbertsville, PA; dilution 1:3000) was added and incubated for 90 min at 37°C. Finally, HRP-conjugated rabbit anti-goat IgG antibody (605-4313; Rockland Immunochemicals; dilution 1:15,000) was added and incubated for 90 min at 37°C. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, ME) was used as a standard.

Radiolabeling of autologous HDL
One mouse from each experimental group was euthanized by cervical dislocation, and blood was drawn from the retro-orbital vein. Sera were collected and HDL was isolated after density ultracentrifugation in a SW 40 Ti rotor (Beckman Instruments, Geneva, Switzerland) (4°C; 40,000 rpm; overnight).[33] HDL (0.4 µmol HDL-cholesterol) was radiolabeled by incubation (37°C; 24 h) with [^{3}H]cholesteryl oleyl ether ([^{3}H]COEth)-labeled egg yolk phosphatidylcholine vesicles (40 µCi; 0.5 mg phospholipid) in the presence of lipoprotein deficient serum (1 ml) from *E3L.CETP* mice. Subsequently, HDL was reisolated after density ultracentrifugation (12°C; 40,000 rpm; 24 h).

In vivo clearance of autologous HDL
After 6 weeks of diet, mice were injected via the tail vein with a trace of autologous [^{3}H]COEth-labeled HDL ($0.2x10^6$ cpm in PBS) at 8:00 am. At the indicated time points after injection, blood was collected to determine the plasma decay of [^{3}H]COEth by scintillation counting (Packard Instruments,

Dowers Grove, IL). The total plasma volumes of the mice were calculated from the equation V (ml) = 0.04706 x body weight (g), as determined from previous ^{125}I-BSA clearance studies.[34] The fractional catabolic rate (FCR) was calculated as pools of HDL-CE cleared per h from the plasma decay curves as described previously.[35] Briefly, curves were fitted using GraphPad Prism software, giving the best fit for one-phase exponential decay curves, described by the formula Y=span*exp(-k*x)+plateau. Subsequently the FCR was calculated as span/(area under the curve). Taking into account the fact that the plasma level of HDL was altered by the expression of CETP and fenofibrate treatment, the FCR was also calculated from these data as millimolar HDL-CE cleared per hour, based on the actual level of HDL-CE in the various mouse groups.

Hepatic mRNA expression, SR-BI protein analysis, and lipid analysis

Livers were isolated after cervical dislocation. Total RNA was isolated using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. RNA expression was determined in duplicate by real-time PCR on a MyiQ Single-Color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Primers for *CETP*[36] and *sr-b1*[37] have been described previously. Primers for *abca1*, *apoa1*, *cyp7A1,* and *pltp* are listed in Table 1. Expression levels were normalized using hypoxanthine-guanine phosphoribosyl transferase (HPRT) and cyclophilin as housekeeping genes.[37,38] Hepatic SR-BI protein was determined by immunoblot analysis as described previously.[39] Liver lipids were determined by homogenizing liver samples in water (~10% wet w/v) using a mini-bead beater (Biospec Products, Inc., Bartlesville, OK; 20 sec; 5,000 rpm), followed by lipid extraction as described by Bligh and Dyer.[40] Extracts were assayed for TC as described above. Protein was determined according to the method of Lowry et al.[41]

Table 1. Primers for quantitative real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
abca1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
apoa1	GGAGCTGCAAGGGAGACTGT	TGCGCAGAGAGTCTACGTGTGT
cyp7a1	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
pltp	TCAGTCTGCGCTGGAGTCTCT	AAGGCATCACTCCGATTTGC

Abca1, ATP-binding cassette transporter a1; *apoa1*, apolipoprotein a1; *cyp7a1*, cholesterol 7α-hydroxylase; *pltp*, phospholipid transfer protein.

Statistical analysis

All data are presented as means ± SD unless indicated otherwise. Data were analyzed using the unpaired Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Fenofibrate increases HDL-cholesterol in E3L.CETP mice

To study the dose-dependent effect of fenofibrate on plasma lipid levels on a hyperlipidemic background, *E3L.CETP* mice were fed a cholesterol-rich diet with increasing doses of fenofibrate in the diet (0%, 0.004%, 0.012%, and 0.04%) for 2 weeks each (Fig. 1). Fenofibrate caused a dose-dependent decrease in plasma TG levels (up to -62% at the highest dose; $P<0.05$) (Fig. 1A), and only tended to reduce plasma cholesterol levels (up to -35%; NS) (Fig. 1B). However, fenofibrate had a great impact on the distribution of cholesterol over the various lipoproteins. Whereas on a cholesterol-rich diet, most cholesterol in *E3L.CETP* mice is carried in (V)LDL, fenofibrate resulted in a dose-dependent shift of cholesterol from (V)LDL to HDL (Fig. 1C).

Subsequently, we compared the effect of fenofibrate on plasma lipid levels in *E3L.CETP* mice with those in *E3L* mice by using the highest dose of fenofibrate (0.04%) (Fig. 2). In *E3L* mice, fenofibrate decreased plasma TG levels (-59%; $P<0.001$) (Fig. 2A) to a similar extent as in *E3L.CETP* mice (-60%; $P<0.01$)

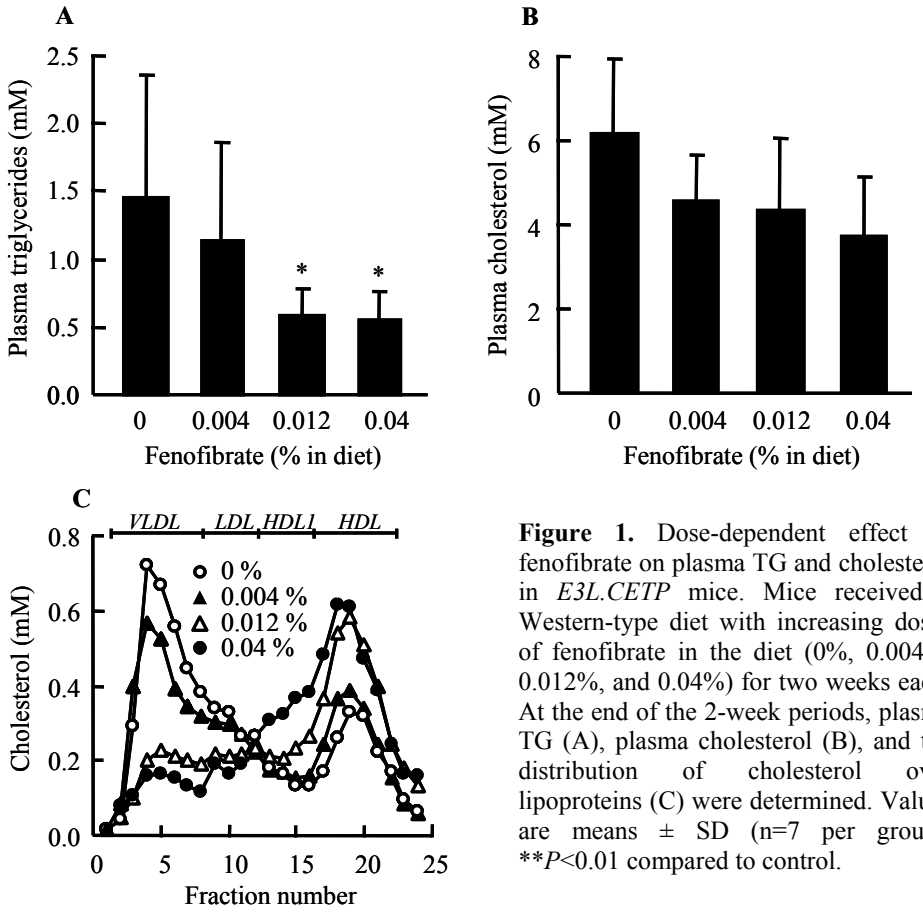

Figure 1. Dose-dependent effect of fenofibrate on plasma TG and cholesterol in *E3L.CETP* mice. Mice received a Western-type diet with increasing doses of fenofibrate in the diet (0%, 0.004%, 0.012%, and 0.04%) for two weeks each. At the end of the 2-week periods, plasma TG (A), plasma cholesterol (B), and the distribution of cholesterol over lipoproteins (C) were determined. Values are means ± SD (n=7 per group). **$P<0.01$ compared to control.

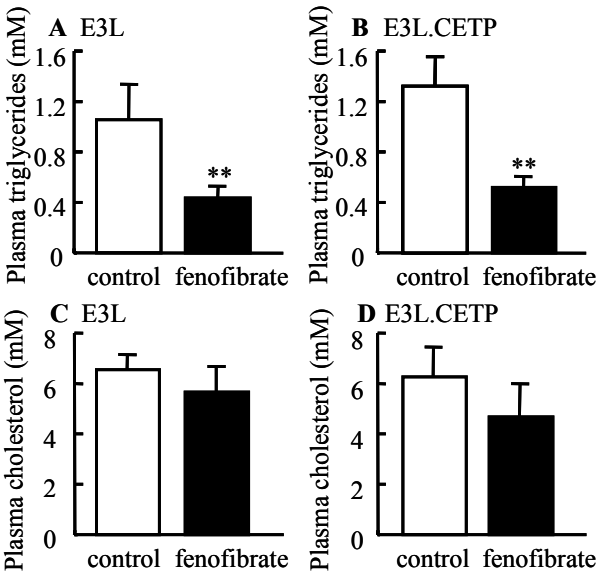

Figure 2. Effect of fenofibrate on plasma TG and cholesterol in *E3L* and *E3L.CETP* mice. *E3L* mice (A, C) and *E3L.CETP* mice (B, D) received a Western-type diet without (white bars) or with (black bars) fenofibrate for 2 weeks, and plasma TG (A, B) and cholesterol (C, D) were determined. Values are means ± SD (n=6 per group). ***P*<0.01 compared with controls.

(Fig. 2B). In both *E3L* mice and *E3L.CETP* mice, these effects of fenofibrate on plasma TG levels were reflected by a strong reduction in VLDL-TG (not shown). Fenofibrate also caused small trends towards lower plasma cholesterol levels in both *E3L* and *E3L.CETP* mice (Fig. 2C,D). Fenofibrate similarly decreased (V)LDL-cholesterol in both *E3L* mice (-91%) and *E3L.CETP* mice (-93%). However, whereas fenofibrate did not affect HDL-cholesterol levels in *E3L* mice, fenofibrate increased HDL-cholesterol in *E3L.CETP* mice (+91%) (Fig. 3), consistent with the dose-escalating study (Fig. 1).

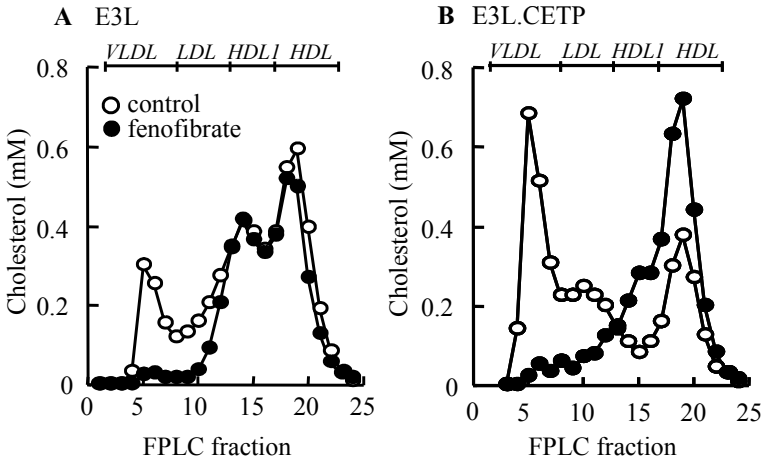

Figure 3. Effect of fenofibrate on the distribution of cholesterol over lipoproteins in *E3L* and *E3L.CETP* mice. *E3L* mice (A) and *E3L.CETP* mice (B) received a Western-type diet without (white circles) or with (black circles) fenofibrate. Plasmas of the various mouse groups were pooled (n=6 per group). Lipoproteins were separated by FPLC, and fractions were analyzed for cholesterol.

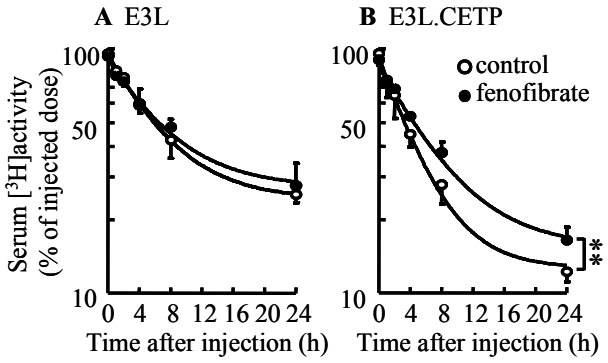

Figure 4. Effect of fenofibrate on the plasma clearance of HDL in *E3L* and *E3L.CETP* mice. *E3L* mice (A) and *E3L.CETP* mice (B) received a Western-type diet without (white circles) or with (black circles) fenofibrate. Mice were injected with autologous [^{3}H]COEth-labeled HDL and plasma ^{3}H-activity was determined at the indicated time points. Values are means ± SD (n=5 per group). ***P*<0.01 compared with controls.

Fenofibrate increases the steady-state plasma HDL level without affecting net HDL-CE output in E3L.CETP mice

To examine the mechanism underlying the fenofibrate-induced increased HDL-cholesterol in *E3L.CETP* mice, *E3L* and *E3L.CETP* mice were injected with autologous [^{3}H]COEth-labeled HDL and the plasma decay was determined (Fig. 4). The expression of CETP *per se* appeared to accelerate the plasma decay, reflected by an increased fractional catabolic rate (FCR) as calculated pools of HDL-CE cleared per hour (+65%; *P*<0.01; Table 2). In *E3L* mice, fenofibrate administration did not affect the clearance of HDL-CE (Fig. 4A; Table 1). In contrast, fenofibrate decreased the FCR of HDL in *E3L.CETP* mice (-27%; *P*<0.01). However, taking into account the fact that CETP expression and fenofibrate treatment influence plasma HDL levels (Fig. 3), the FCR was also calculated as millimolar HDL-CE cleared per hour. In fact, CETP expression in E3L mice, or fenofibrate feeding of either *E3L* or *E3L.CETP* mice, did not affect the amount (mM) of HDL-CE cleared per hour (Table 2). This indicates

Table 2. Effect of fenofibrate on the FCR of HDL-CE in E3L and E3L.CETP mice

	Control	Fenofibrate
FCR (pools HDL-CE per h)		
E3L	0.067±0.003	0.057±0.004
E3L.CETP	0.111±0.006	0.081±0.003*
FCR (mM HDL-CE per h)		
E3L	0.166±0.008	0.162±0.011
E3L.CETP	0.142±0.008	0.162±0.007

E3L and *E3L.CETP* mice were fed a Western-type diet with or without fenofibrate, and mice were injected with autologous [^{3}H]COEth-labeled HDL. The data from Figure 4 were used to calculate the FCR as pools of HDL-CE cleared per hour or millimolar HDL-CE cleared per hour. Values are expressed as means ± SEM relative to control mice (n=5 mice per group). **P*<0.01 compared with controls.

that CETP expression and fenofibrate feeding alter the steady-state plasma HDL-cholesterol level without affecting the net HDL-cholesterol flux through plasma. These data indicate that the residual CETP activity in E3L.CETP mice on fenofibrate is sufficient to maintain net HDL-CE output.

Fenofibrate does not differentially affect hepatic mRNA expression of genes involved in plasma HDL metabolism

Because differences in genes encoding proteins that are crucially involved in HDL metabolism may account for the increase in HDL-cholesterol in *E3L.CETP* mice upon fenofibrate treatment, we examined the effect of fenofibrate on their hepatic expression (Fig. 5). The expression of these genes was not substantially different in E3L.CETP mice compared with E3L mice. Fenofibrate increased *Pltp* in *E3L* (3.5-fold; $P<0.01$) and *E3L.CETP* mice (2.7-fold; $P<0.05$), consistent with previously reported effects of fenofibrate.[11,12] The expression of *abca1*, which is involved in HDL formation, was similarly decreased in *E3L* (-50%; $P<0.05$) and *E3L.CETP* (-33%; $P<0.05$) mice. Likewise, s*r-b1* was decreased in *E3L* (-48%; $P<0.05$) and *E3L.CETP* (-42%; $P<0.05$) mice to a similar extent, as reflected by similar reductions in hepatic SR-BI protein levels (~-25%) for *E3L* ($P=0.06$) and *E3L.CETP* mice ($P<0.05$) (data not shown). *Apoa1* expression was decreased in *E3L* (-49%; $P<0.05$) and *E3L.CETP* (-41%; $P<0.05$) mice, without substantially affecting the plasma apoAI level (~80 mg/dL in all groups). The expression of *cyp7a1*, *pltp*, *abca1*, *sr-b1*, and *apoa1* is thus similarly affected by fenofibrate in *E3L* and *E3L.CETP* mice, and cannot explain the differentially raised HDL in *E3L.CETP* mice as compared to *E3L* mice.

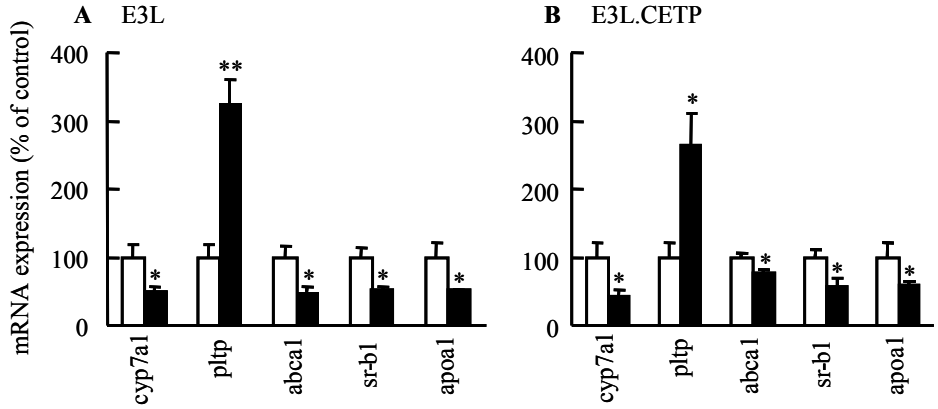

Figure 5. Effect of fenofibrate on hepatic mRNA expression in *E3L* and *E3L.CETP* mice. *E3L* mice (A) and *E3L.CETP* mice (B) were fed a Western-type diet with or without fenofibrate. Mice were euthanized, and livers were collected to determine mRNA expression. Values are expressed as means ± S.E. relative to control mice (n=4 per group). *$P<0.05$; **$P<0.01$ compared with controls.

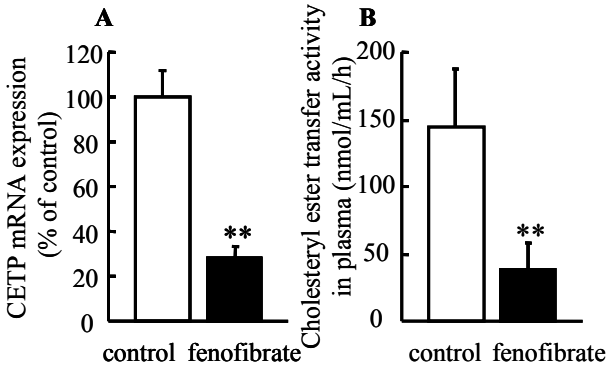

Fig. 6. Effect of fenofibrate on hepatic CETP mRNA expression and cholesteryl ester transfer activity in plasma of *E3L.CETP* mice. *E3L.CETP* mice received a Western-type diet with or without fenofibrate, and mice were euthanized. Livers were collected to determine CETP mRNA expression (A), and plasma was assayed for CE transfer activity (B). Values are means ± SD (n=4-6 per group). ***P*<0.01 compared with controls.

Fenofibrate decreases hepatic CETP mRNA expression and CE transfer activity in plasma

To investigate whether the effect of fenofibrate on increasing HDL-cholesterol in *E3L.CETP* mice is caused by reduction of CETP activity, we determined the hepatic *CETP* expression and CE transfer activity in plasma (Fig. 6). Indeed, fenofibrate markedly decreased *CETP* expression in *E3L.CETP* mice (-72%; *P*<0.01) (Fig. 6A). Because the liver X receptor (LXR) strongly regulates the expression of *CETP*,[42] we determined whether fenofibrate feeding would decrease the cholesterol content in the liver. Indeed, fenofibrate reduced the hepatic cholesterol content in *E3L* mice (4.9±2.6 vs 9.6±3.7 μg TC/mg protein) and *E3L.CETP* mice (3.6±1.0 vs 13.0±3.7 μg TC/mg protein; *P*<0.05). The fenofibrate-induced reduction in hepatic *CETP* expression was accompanied by a similar reduction in the CE transfer activity in plasma of *E3L.CETP* mice (-73%; *P*<0.01) (Fig. 6B). Therefore, the HDL-raising effect of fenofibrate in *E3L.CETP* mice is thus likely to be a direct consequence of lower CETP expression.

Discussion

In this study, we investigated whether CETP might play a role in the fenofibrate-induced increase in HDL-cholesterol. Here we show that fenofibrate increases HDL-cholesterol in *E3L.CETP* mice, as paralleled by a reduction in hepatic *CETP* mRNA and plasma CE transfer activity, whereas fenofibrate does not increase HDL in *E3L* mice.

We showed previously that *E3L* mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels, and that these mice show a human-like response to drug interventions aimed at reducing plasma levels of apoB-containing lipoproteins, including statins (atorvastatin[43] and rosuvastatin)[44] and fibrates (gemfibrozil).[45] This is in sheer contrast with wild-

type mice[6,13] and more conventional hyperlipidemic mice such as apoE-deficient[13,46] or LDL receptor-deficient[47] mice, which show either an adverse or no response to such interventions. In particular, administration of fenofibrate to wild-type[13] and apoE-deficient[13,48] mice showed an unexpected increase in plasma TG and TC levels caused by elevated levels of lipoprotein remnants, with a concomitant reduction in HDL-cholesterol. Here we demonstrate that *E3L* mice also show a human-like response to fenofibrate with respect to decreasing TG and cholesterol in apoB-containing particles, although HDL-cholesterol was not increased after 2 weeks of intervention (Fig. 3A). We reasoned that introduction of human CETP in these *E3L* mice, which permits CE exchange between HDL and apoB-containing lipoproteins, would result in an excellent mouse model to study the effects of fenofibrate on HDL metabolism.

Indeed, we demonstrate that although *E3L.CETP* mice retain their ability to respond to fenofibrate with respect to a similar reduction of VLDL-TG and VLDL-cholesterol as compared with *E3L* mice, they also respond with an increase in HDL-cholesterol level. Apparently, the fact that mice normally do not express CETP prevents a human-like response to HDL-modulating drug interventions, such as fibrate treatment. In agreement with this hypothesis, we observed previously that treatment of *E3L* mice with statins also did not increase HDL-cholesterol even though VLDL reductions of as much as 60% were achieved.[43,45,49]

HDL-cholesterol levels can theoretically be modulated by several key proteins involved in HDL metabolism, including ABCA1,[10] SR-BI,[9] PLTP,[11,23] apoAI,[3,6,48,50,51] and CETP.[22,24,25] Therefore, we examined the potential contribution of each of these factors in the fenofibrate-induced increase of HDL-cholesterol in *E3L.CETP* mice. The HDL-cholesterol level in mice is largely determined by the hepatic expression of ABCA1, which plays an important role in HDL formation by mediating hepatic cholesterol efflux to apoAI.[52] In fact, it has been reported that treatment of chow-fed rats with ciprofibrate increased their hepatic *abca1* expression, concomitant with an increase in plasma HDL-cholesterol levels.[53] However, fenofibrate did not increase hepatic *abca1* expression in either *E3L* or *E3L.CETP* mice. On the contrary, fenofibrate decreased *abca1* mRNA in both genotypes and thus can not explain the selective increase of HDL-cholesterol in E3L.CETP mice.

Whereas bezafibrate did not increase the plasma PLTP mass and activity levels in humans,[23] fenofibrate has been shown to increase the hepatic *pltp* expression in mice, which was associated with increased plasma PLTP activity and HDL size, at least in human apoAI transgenic mice.[11] Accordingly, we found that fenofibrate induced the hepatic *pltp* expression both in *E3L* and *E3L.CETP* mice. However, the relative increase was even more pronounced in *E3L* mice as compared to *E3L.CETP* mice, whereas HDL-cholesterol was not affected in *E3L* mice. It is also of note that adenovirus-mediated hepatic expression of

PLTP results in a dose-dependent reduction of HDL-cholesterol levels, instead of increasing HDL-cholesterol, in both wild-type and human apoAI-transgenic mice.[54] It is thus unlikely that the induction of PLTP is the cause of the increase in HDL-cholesterol as observed in *E3L.CETP* mice.

In mice, hepatic SR-BI represents the most important pathway for the selective clearance of HDL-associated cholesteryl ester from plasma.[55] It has been shown that fenofibrate can downregulate hepatic SR-BI protein in wild-type mice, independent of *sr-b1* expression, via a posttranscriptional mechanism. This was correlated with a substantially increased HDL size, based on fast-performance liquid chromatography profiling.[8] We found that fenofibrate treatment did result in a similar reduction of *sr-b1* expression in *E3L* mice (-48%) and *E3L.CETP* mice (-42%), with a concomitant reduction in hepatic SR-BI protein levels (~-25%). Although fenofibrate did not increase in large HDL-1 in E3L mice after only 2 weeks of fenofibrate intervention (Fig 3A), cholesterol within large HDL-1 was indeed increased (+69%) after prolonged treatment of *E3L* mice (i.e., 6 weeks), as has also been shown for wild-type mice,[8] In *E3L.CETP* mice fenofibrate treatment for 2 weeks increased the levels of cholesterol in regularly sized HDLs but also increased the levels of HDL-1 to some extent (Figs. 1C, 3B). Therefore, the reduction in hepatic SR-BI levels may contribute to the appearance of HDL-1 in both *E3L* and *E3L.CETP* mice but does not explain the increase of regularly sized HDL in E3L.CETP mice.

In *APOA1* transgenic mice, human apoAI hepatic mRNA and plasma protein levels were increased after fenofibrate treatment,[6] probably by the binding of PPARα to a positive PPRE in the human apoAI gene promoter.[51] Given the tight relation between HDL-cholesterol and apoAI levels in humans, it could be expected that upregulation of apoAI expression would be the main causal factor for increasing HDL-cholesterol levels in humans. Fenofibrate treatment has an opposite effect on murine apoAI (i.e., reduction of expression and plasma levels),[6] which theoretically could easily explain why fenofibrate does not increase HDL-cholesterol in mice. However, although we do observe a reduction in hepatic *apoa1* expression upon fenofibrate treatment of *E3L* (-49%) and *E3L.CETP* (-41%) mice, HDL-cholesterol was nevertheless markedly increased in *E3L.CETP* mice. The fact that plasma apoAI was not affected by fenofibrate treatment may thus be explained by increased lipidation of apoAI, thereby preventing the clearance of apoAI.

Collectively, these data thus suggest that downregulation of CETP expression is the predominant cause of the fenofibrate-induced elevation of HDL-cholesterol. Expression of CETP in *E3L* mice decreased the HDL-cholesterol level (~-35%) but did not affect the HDL turnover, calculated as millimolar HDL-CE cleared per hour. Likewise CETP inhibition in rabbits, although increasing HDL-cholesterol, does not compromise the HDL-CE clearance from plasma.[56] Treatment of *E3L.CETP* mice with fenofibrate resulted in an increased HDL-cholesterol level, strongly decreased hepatic CETP expression levels, and

reduced cholesteryl ester transfer activity in plasma. Thus the increase in HDL-cholesterol may be caused by the combination of reduced hepatic *CETP* expression and reduced levels of apoB-containing lipoproteins as CE acceptors, thereby inhibiting the CETP-mediated transfer of CE from HDL to (V)LDL.

It is tempting to speculate about the mechanism(s) underlying the effect of fenofibrate on hepatic *CETP* expression. Dietary cholesterol has been shown to increase *CETP* mRNA expression in CETP transgenic mice,[28,29] presumably via an LXR responsive element in the CETP promoter.[42] Conversely, a decrease in hepatic *CETP* mRNA expression might thus be the consequence of a reduction in LXR signaling. Fenofibrate treatment indeed decreased hepatic cholesterol, which is likely to reduce the level of oxysterols, the natural ligands of LXRα. Down-regulation of LXRα is supported by a concomitant decrease in the expression of *cyp7a1*, another LXR-target gene.[57] This is in accordance with the observation that administration of ciprofibrate to wild-type mice caused a 65% reduction in hepatic *cyp7a1* mRNA.[58] Nevertheless it should be mentioned that *cyp7a1* is also regulated directly by fibrates via a negative PPRE in its promoter sequence.[59] A reduction in LXRα might also explain the reduction of *abca1* expression.[60] In addition to these mechanisms explaining reduced CETP expression by fenofibrate, a potential PPRE in the promoter region of CETP was recently identified,[61] which provides the possibility for direct regulation of CETP by PPARα agonists, although it is unclear whether this potential PPRE is functional.

Our finding that fenofibrate reduced CETP activity in E3L.CETP mice corroborates the outcome of two human studies. Although one study failed to detect an effect of fenofibrate on plasma CETP activity,[62] fenofibrate treatment did decrease CETP activity by 26% in subjects with combined hyperlipidemia[24] and by 18% in subjects with the metabolic syndrome.[63] Based on our experimental study, the fenofibrate-induced decrease in CETP activity in humans is likely also a causal factor for the generally observed increase in HDL-cholesterol.

Fibrate treatment has been associated with a reduction of cardiovascular disease.[5] The recent FIELD study, which assessed the effects of fenofibrate on cardiovascular risk in subjects with type 2 diabetes mellitus in a long-term, controlled trial, showed a reduction in total cardiovascular events, but did not reveal a reduced risk of the primary outcome of coronary events.[64] Nevertheless, the authors suggested that a more beneficial outcome might have been masked by a larger portion of statin treatment in the placebo group as compared with the fenofibrate group. Even though the benefit of an increase in HDL-cholesterol by CETP inhibition is still under debate,[65-68] and despite the recent failure of the CETP inhibitor torcetrapib in the ILLUMINATE study,[69] increasing HDL-cholesterol levels is still generally considered anti-atherogenic. Besides the ability of fibrates to potently reduce plasma TG, their concomitant effect on increasing HDL by reducing CETP expression may thus be an additional

advantageous anti-atherogenic property. We speculate that combination therapies of fibrates (i.e., reducing CETP expression) with small molecule CETP inhibitors (i.e., reducing plasma CETP activity) may help to further reduce cardiovascular risk.
Together, our data show that fenofibrate increases HDL-cholesterol by reducing CETP expression and plasma CE transfer activity in *E3L.CETP* mice. Therefore, we postulate that reduction of CETP expression also contributes to the increase in HDL that is found in human subjects treated with fibrates. Furthermore, we anticipate that the *E3L.CETP* mouse is a valuable model in which to test the effect of combination therapies (i.e., fibrates and CETP inhibitors) on plasma lipid metabolism and atherosclerosis.

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Chapter 3

ATORVASTATIN INCREASES HDL CHOLESTEROL BY REDUCING CETP EXPRESSION IN CHOLESTEROL-FED *APOE*3-LEIDEN.CETP* MICE

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Abstract

Objective: In addition to lowering low-density lipoprotein (LDL)-cholesterol, statins modestly increase high-density lipoprotein (HDL)-cholesterol in humans and decrease cholesteryl ester transfer protein (CETP) mass and activity. Our aim was to determine whether the increase in HDL depends on CETP expression.

*Methods and results: APOE*3-Leiden* (*E3L*) mice, with a human-like lipoprotein profile and a human-like responsiveness to statin treatment, were crossbred with mice expressing human CETP under control of its natural flanking regions resulting in *E3L.CETP* mice. *E3L* and *E3L.CETP* mice were fed a Western-type diet with or without atorvastatin. Atorvastatin (0.01% in the diet) reduced plasma cholesterol in both *E3L* and *E3L.CETP* mice (-26% and -33%, $P<0.05$), mainly in VLDL, but increased HDL-cholesterol only in *E3L.CETP* mice (+52%). Hepatic mRNA expression levels of genes involved in HDL metabolism, such as phospholipid transfer protein (*Pltp*), ATP-binding cassette transporter A1 (*Abca1*), scavenger receptor class B type I (*Sr-b1*), and apolipoprotein AI (*Apoa1*), were not differently affected by atorvastatin in *E3L.CETP* mice as compared to *E3L* mice. However, in *E3L.CETP* mice, atorvastatin down-regulated the hepatic CETP mRNA expression (-57%; $P<0.01$) as well as the total CETP level (-29%) and CE transfer activity (-36%; $P<0.05$) in plasma.

Conclusions: Atorvastatin increases HDL-cholesterol in *E3L.CETP* mice by reducing the CETP-dependent transfer of cholesterol from HDL to (V)LDL, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool.

Introduction

Epidemiological studies have established that a high level of low-density lipoprotein (LDL)-cholesterol is a major cardiovascular risk factor. In the past decades, statins have been successfully used to reduce LDL-cholesterol. Statins inhibit the rate-limiting enzyme of cholesterol synthesis, *i.e.* 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, resulting in hepatic depletion of cholesterol. As a consequence, VLDL production is reduced and the hepatic expression of the LDL receptor (LDLr) is upregulated, leading to decreased plasma cholesterol levels in apoB-containing lipoproteins (i.e., VLDL and LDL).[1] Indeed, a meta-analysis of 25 studies indicated that statins reduce LDL-cholesterol levels by 20-40%.[2] In addition, statins elevate high-density lipoprotein (HDL)-cholesterol levels by typically 5-15%.[3-5] This effect is already observed at a low dose (20 mg/day) while higher doses (40 and 80 mg/day) have no additional elevating effects on HDL levels.[3-5]

Low HDL-cholesterol has been confirmed as a strong and independent risk factor for cardiovascular disease. An increase in HDL-cholesterol of 1 mg/dL results in a 2-3% decrease in cardiovascular risk.[6] One of the key players in HDL-metabolism is cholesteryl ester transfer protein (CETP). CETP is involved in the exchange of triglycerides (TG) and cholesteryl esters (CE) between lipoproteins, resulting in the net flux of CE from HDL towards apoB-containing lipoproteins (*e.g.* VLDL and LDL) in exchange for TG.[7] Treatment of patients with combined hyperlipidemia with atorvastatin resulted in increased levels of relatively CE-rich large HDL2a with a concomitant decrease in CE-poor small HDL3c,[8] as associated with a reduction in CETP mass.[8] Likewise, in type 2 diabetic subjects carrying the CETP *Taq*IB polymorphism, the increase in HDL-cholesterol (+7.2%) after atorvastatin treatment was correlated with a reduction in CETP mass (-32%).[9] These data suggest that the effects of statin treatment on HDL-cholesterol levels may actually be caused by a reduction in the CETP-mediated transfer of CE.

Therefore, the aim of this study was to evaluate whether the statin-induced increase in HDL-cholesterol would depend on CETP expression. Previously, we demonstrated that *APOE*3-Leiden* (*E3L*) mice, with a human-like lipoprotein profile[10] show a human-like response to atorvastatin with reduced (V)LDL-cholesterol levels accompanied by reduced VLDL production.[11] In the current study, these mice were crossbred with mice expressing human CETP under control of the natural flanking regions, resulting in *E3L.CETP* mice.[12] We treated *E3L* and *E3L.CETP* mice with atorvastatin to investigate whether CETP expression contributes to the HDL-raising effect of atorvastatin.

Methods

Animals
Hemizygous human CETP transgenic (*CETP*) mice, expressing a human CETP minigene under the control of natural flanking sequences were crossbred with hemizygous *E3L* mice[10] at our Institutional Animal Facility to obtain *E3L* and *E3L.CETP* littermates (C57Bl/6J background).[12] In this study, mice were housed under standard conditions in conventional cages with free access to food and water. Male mice were fed a semi-synthetic diet containing 15% (w/w) fat (Hope Farms, Woerden, The Netherlands), supplemented with 0.25% (w/w) cholesterol (Sigma, St. Louis, MO) for two weeks. Subsequently, the mice received the same diet without or with 0.01% (w/w) atorvastatin (Lipitor®20, Pfizer B.V., Capelle a/d IJssel, The Netherlands) for 6 weeks (*i.e.* approx. 10 mg/kg/day, which corresponds to a dose of 70 mg/day for an average 70 kg person, assuming a 10-fold higher metabolic rate in mice as compared to humans). To study whether atorvastatin sorts similar effects in female mice, and to evaluate the dose-response relationship, female *E3L.CETP* mice were fed a diet containing 15% (w/w) fat, supplemented with 0.1% (w/w) cholesterol and 0.001% or 0.01% of atorvastatin for two weeks successively. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am, unless indicated otherwise. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

Plasma lipid and lipoprotein analysis
Plasma was obtained via tail vein bleeding and assayed for total cholesterol (TC) using the enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) as described previously.[12]

Hepatic liver lipid levels
Livers were isolated from control-treated and atorvastatin-treated mice after cervical dislocation. A small piece of liver was homogenated in 400 µL PBS and 1.5 mL CH_3OH:$CHCl_3$ (2:1, v/v) was added. After centrifugation, lipids were extracted from the supernatant with $CHCl_3$ and H_2O (1:1, v/v) and the $CHCl_3$ phase was dried. Lipids were dissolved in H_2O with 2% Triton-X100. TC levels were assayed as described above. Free cholesterol (FC) was analyzed with the Free Cholesterol C kit (WAKO, Neuss, Germany), and cholesteryl esters (CE) were determined as the difference between TC and FC. Phospholipids (PL) and TG were analyzed with the, phospholipids B kits (Wako, Neuss Germany) and the enzymatic kit 1488872 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.), respectively.

Plasma CETP level
The total CETP level in plasma was measured as the transfer of [^{3}H]cholesteryl oleate from exogenous human LDL to HDL as described.[12]

Plasma cholesteryl ester transfer activity
The transfer of newly synthesized CE in plasma was assayed by a radioisotope method as previously described.[13] In short, [^{3}H]cholesterol was complexed with BSA and incubated overnight at 4°C with mouse plasma to equilibrate with plasma free cholesterol. Subsequently, the plasma samples were incubated for 3 h at 37°C. VLDL and LDL were then precipitated by addition of sodium phophotungstate/$MgCl_2$. Lipids were extracted from the precipitate by methanol: hexane (1:2, v/v) and [^{3}H]CE was separated from [^{3}H]cholesterol on silica columns, followed by counting of radioactivity.

Plasma apoAI concentration
Plasma apoAI concentrations were determined using a sandwich ELISA. Hereto, rabbit anti-mouse apoAI polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (3 µg/ml) at 4°C and incubated with diluted mouse plasma (dilution 1:400,000) for 90 min at 37°C. Subsequently, goat anti-mouse apoAI antibody (600-101-196; Rockland Immunochemicals, Inc., Gilbertsville, PA; dilution 1:3000) was added and incubated for 90 min at 37°C. Finally, horse radish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (605-4313; Rockland; dilution 1:15000) was added and incubated for 90 min at 37°C, and HRP was detected by incubation with tetramethylbenzidine (Organon Teknika,

Table 1. Primers for quantitative real-time PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Hmgcoa red.	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Pltp	TCAGTCTGCGCTGGAGTCTCT	AAGGCATCACTCCGATTTGC
Abca1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Sr-b1	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA
Apoa1	GGAGCTGCAAGGGAGACTGT	TGCGCAGAGAGTCTACGTGTGT
CETP	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA
Abcg5	TGTCCTACAGCGTCAGCAACC	GGCCACTCTCGATGTACAAGG
Abcg8	GACAGCTTCACAGCCCACAA	GCCTGAAGATGTCAGAGCGA
Lpl	GTGGCCGAGAGCGAGAAC	TCCACCTCCGTGTAAATCAAGA
Srebp-1c	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA
Ldlr	GCATCAGCTTGGACAAGGTGT	GGGAACAGCCACCATTGTTG

Abca1, ATP-binding cassette transporter A1; *Abcg5/8*, ATP-binding cassette transporter G5/G8, *Apoa1*, apolipoprotein AI; *CETP*, cholesteryl ester transfer protein; *Hmgcoa reductase*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *Ldlr*, low density lipoprotein receptor; *Lpl*, lipoprotein lipase; *Pltp*, phospholipid transfer protein; *Sr-b1*, scavenger receptor class B type I; *Srepb-1c*, sterol regulatory element-binding protein-1c.

Boxtel, The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, Maine, USA) was used as a standard.

Hepatic mRNA expression and SR-BI protein analysis
Livers were isolated after cervical dislocation. Total RNA was isolated using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. RNA expression was determined in duplicate by real-time PCR on a MyiQ Single-Color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Primers are listed in online Table 1. Expression levels were normalized, using hypoxanthine-guanine phosphoribosyl transferase (HPRT) and cyclophilin as housekeeping genes.[14] Hepatic SR-BI protein was determined by immunoblot analysis exactly as described previously.[15]

Statistical analysis
All data are presented as means ± SD unless indicated otherwise. Data were analyzed using the unpaired Student's *t* test unless indicated otherwise. *P*-values less than 0.05 were considered statistically significant. SPSS 12.0.01 was used for statistical analysis.

Results

Atorvastatin increases HDL-cholesterol in E3L.CETP mice
On a diet containing 0.25% (w/w) cholesterol, atorvastatin (0.01%, w/w) reduced plasma total cholesterol in both *E3L* mice from 5.1 ± 0.9 mM to 3.8 ± 1.2 mM (-26%; $P<0.05$) and *E3L.CETP* mice from 4.3 ± 0.8 mM to 2.9 ± 1.0 mM (-33%; $P<0.05$) (Fig. 1), without substantially affecting TG levels (not shown). These effects were reflected by a strong decrease in (V)LDL-cholesterol in *E3L* mice (-86%) and *E3L.CETP* mice (-88%) (Fig. 2). However, whereas atorvastatin did not affect HDL-cholesterol *E3L* mice (3.2 mM vs 2.9 mM) (Fig. 2A), it did raise HDL-cholesterol (+52%) in *E3L.CETP* mice (2.1 mM vs 1.4 mM) (Fig. 2B).

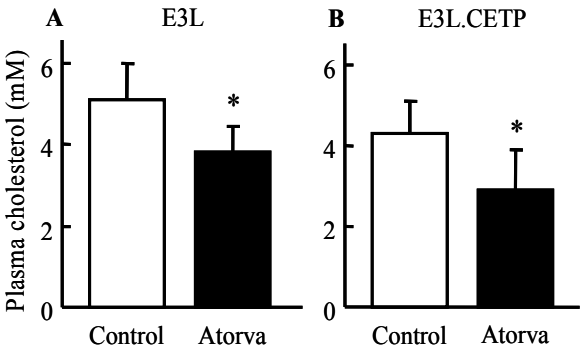

Figure 1. Effect of atorvastatin on plasma total cholesterol levels. *E3L* (A) and *E3L.CETP* (B) mice received a diet containing 0.25% (w/w) cholesterol without (white bars) or with (black bars) 0.01% (w/w) atorvastatin for 6 weeks. Plasma was obtained, and assayed for total cholesterol. Values are means ± SD (n=6 per group). *$P<0.05$ compared to control.

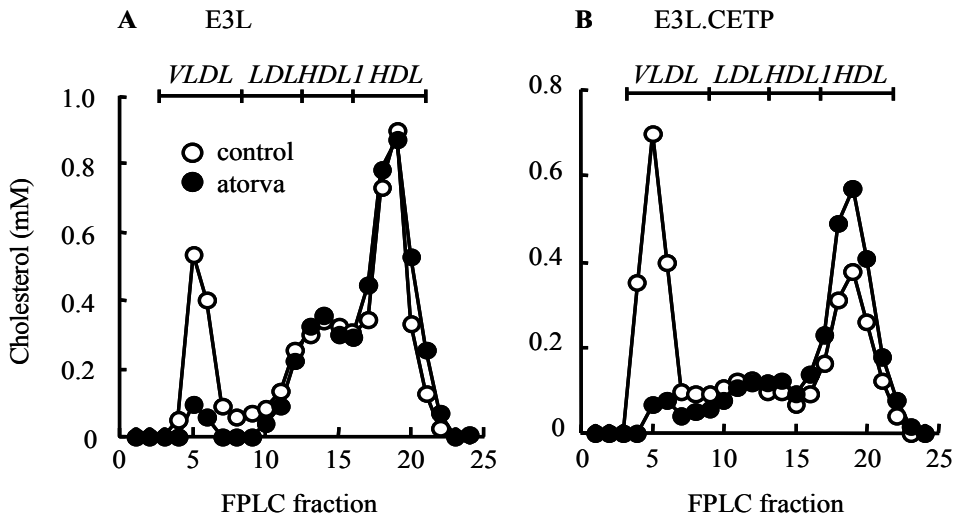

Figure 2. Effect of atorvastatin on the distribution of cholesterol over lipoproteins. *E3L* (A) and *E3L.CETP* (B) mice received a cholesterol-containing diet without (white circles) or with (black circles) atorvastatin for 6 weeks. Plasmas of the various mouse groups were pooled (n=6 per group). Lipoproteins were separated by FPLC, and fractions were analyzed for cholesterol.

Atorvastatin also reduced cholesterol levels in the liver of *E3L* mice (-24%) and *E3L.CETP* mice (-32%) ($P<0.05$). This decrease in hepatic cholesterol was mainly confined to the cholesteryl ester content in *E3L* mice (-38%) and *E3L.CETP* mice (-60%) ($P<0.05$)(Fig. 3).

Atorvastatin does not differentially affect hepatic mRNA expression of genes involved in HDL metabolism in E3L versus E3L.CETP mice

Atorvastatin increased the hepatic expression of *Hmgcoa reductase* both in *E3L* mice (2.5-fold; $P<0.05$) and in *E3L.CETP* mice (2.8-fold; $P<0.05$) (Fig. 4). Concomitantly, *Ldlr* expression was increased in *E3L* mice (+22%) and *E3L.CETP* mice (+24%) (not shown). These effects are in line with previous observations in *E3L* mice,[16] and likely reflect an attempt of the liver to maintain its cholesterol balance.

Since atorvastatin may affect PLTP, ABCA1, SR-BI, and apoAI, which are crucially involved in HDL metabolism, and may account for the increase in HDL-cholesterol in *E3L.CETP* mice upon atorvastatin treatment, we examined the effect of atorvastatin on their hepatic mRNA expression (Fig. 4). The expression of these genes was not substantially different in *E3L.CETP* mice as compared to *E3L* mice (<16%, not significant). Atorvastatin tended to increase the expression of *Pltp*, involved in remodeling of HDL by mediating transfer of phospholipids between lipoproteins, in *E3L* mice (+34%) and *E3L.CETP* mice (+69%), which did not reach statistical significance. The expression of *Abca1*, which is an important determinant for HDL formation, was decreased by

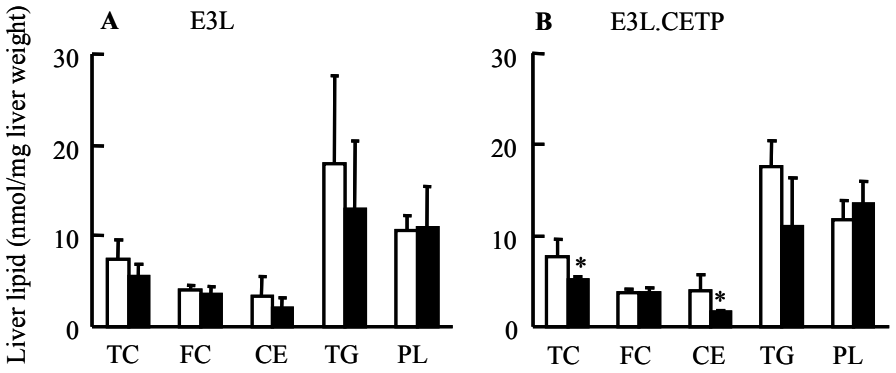

Figure 3. Effect of atorvastatin on hepatic lipid levels. *E3L* (A) and *E3L.CETP* (B) mice were fed a cholesterol-containing diet without (white bars) or with (black bars) atorvastatin. After 6 weeks, livers were collected and lipids were extracted. Total cholesterol (TC), free cholesterol (FC), cholesteryl esters (CE), triglycerides (TG) and phospholipids (PL) were quantified. Values are means ± SD (n=3-5 per group). **P*<0.05 compared to control.

atorvastatin in *E3L* mice (-59%; *P*<0.05) and *E3L.CETP* mice (-45%; *P*<0.05) to a similar extent. The expression of *Sr-b1*, which is largely involved in the selective uptake of HDL-CE in mice, tended to be decreased in *E3L* (-30%) and *E3L.CETP* (-27%) mice, but hepatic SR-BI protein levels were unaffected in both mouse groups (not shown). Also, in both types of mice, atorvastatin did not increase hepatic *Apoa1* expression or the plasma apoAI levels (not shown). Atorvastatin thus affects the mRNA expression of *Pltp*, *Abca1*, *Sr-b1*, and *Apoa1* to a similar extent in *E3L* and *E3L.CETP* mice, and is thus unlikely to explain the differentially raised HDL in *E3L.CETP* mice as compared to *E3L* mice.

In general, atorvastatin tended to decrease the expression of LXR target genes, including *Abcg5* (-2% and -38%), *Abcg8* (-26% and -46%), *Lpl* (-85% and -77%) and *Srebp-1c* (-31% and -32%) in *E3L* and *E3L.CETP* mice, respectively.

Atorvastatin decreases hepatic CETP mRNA expression and cholesteryl ester transfer activity in plasma of E3L.CETP mice

To investigate whether atorvastatin increases HDL-cholesterol in *E3L.CETP* mice by reduction of CETP activity, we determined the hepatic *CETP* mRNA expression, the total plasma CETP level, and the CE transfer activity in plasma (Fig. 5). Indeed, atorvastatin markedly decreased *CETP* expression in *E3L.CETP* mice (-57%; *P*<0.01) (Fig. 5A). This effect was accompanied by a trend towards a reduction in the total plasma CETP level (-29%), which did not reach statistical significance, probably related to the relatively high variation in combination with the limited group size (Fig. 5B). Additionally, the CE transfer activity in plasma of *E3L.CETP* mice was reduced (-36%; *P*<0.05) (Fig. 5C). Taken together, the HDL-raising effect of atorvastatin in *E3L.CETP* mice appears a direct consequence of reduced CETP expression.

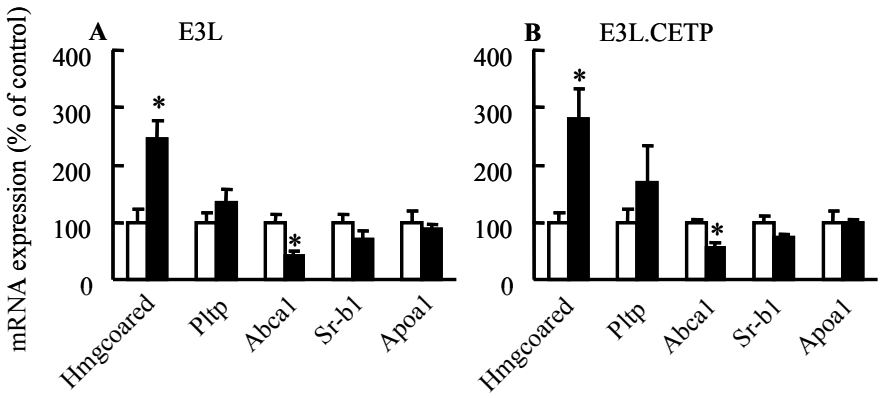

Figure 4. Effect of atorvastatin on hepatic mRNA expression of genes. *E3L* (A) and *E3L.CETP* (B) mice were fed a cholesterol-containing diet without (white bars) or with (black bars) atorvastatin. After 6 weeks, livers were collected to determine mRNA expression. Values are expressed as means ± S.E. relative to control mice (n=4 per group). **P*<0.05 compared to control.

Atorvastatin dose-dependently decreases CETP and increases HDL

To determine whether atorvastatin also reduces CETP and increases HDL-cholesterol in female mice, and to evaluate whether these effects would be dose-dependent, female *E3L.CETP* mice were fed a cholesterol-containing diet that successively contained 0.001% and 0.01% of atorvastatin (w/w) for two weeks each. Atorvastatin dose-dependently decreased plasma cholesterol (-34% and -71%, *P*<0.01). This was accompanied by a dose-dependent increase in HDL-cholesterol levels (+118% and +176%) and reductions in total plasma CETP activity (-31% and -61%; *P*<0.01) (not shown).

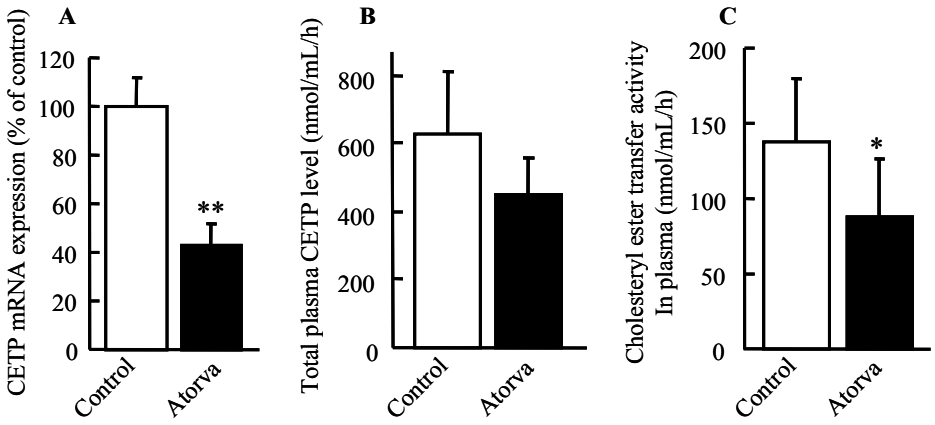

Figure 5. Effect of atorvastatin on hepatic CETP mRNA expression and cholesteryl ester transfer activity in plasma. *E3L.CETP* mice were fed a cholesterol-containing diet without (white bars) or with (black bars) atorvastatin. After 6 weeks, livers were collected to determine CETP mRNA expression (A), and plasma was assayed for total CETP level (B) and CE transfer activity (C). Values are means ± SD (n=4-6 per group). **P*<0.05; ***P*<0.01.

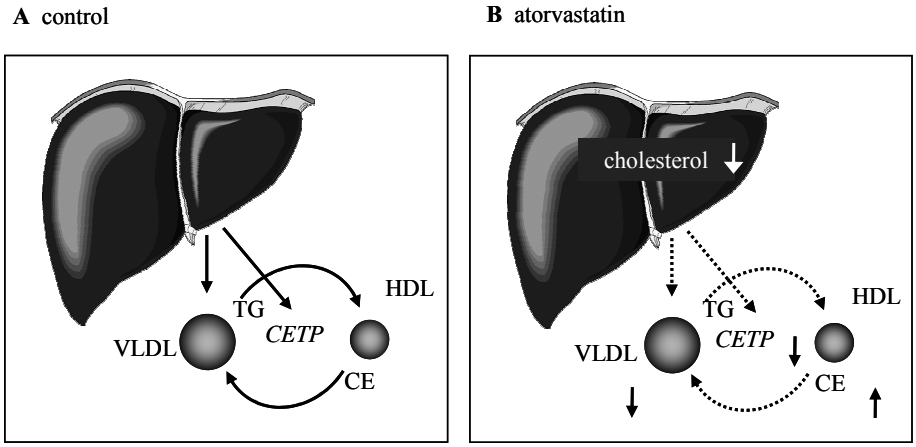

Figure. 6. Proposed mechanism underlying the HDL-raising effect of statins. See text for explanation. CE, cholesteryl ester; TG, triglyceride.

Discussion

E3L mice respond to statin treatment with respect to lowering of apoB-containing lipoproteins and reduced atherosclerosis development similarly as humans,[11,16,17] whereas statins do not affect or even increase plasma cholesterol levels in apoE-deficient mice[18,19] and LDL receptor-deficient mice.[20] However, whereas statins increase HDL in humans, atorvastatin and rosuvastatin did not increase HDL levels in *E3L* mice.[11,16,17]

To investigate whether the statin-induced elevation of HDL-cholesterol in humans depends on CETP expression, we crossbred *E3L* mice with human CETP transgenic mice. We found that atorvastatin decreased (V)LDL in both *E3L* and *E3L.CETP* mice but increased the steady-state HDL-cholesterol level only in *E3L.CETP* mice, which was not observed in *E3L* littermates. We previously showed that atorvastatin reduces plasma cholesterol in *E3L* mice by reducing VLDL production.[11] Since atorvastatin similarly reduces (V)LDL cholesterol in *E3L.CETP* mice as compared to *E3L* mice, and CETP expression *per se* does not affect VLDL production,[21] it is likely that the mechanisms by which atorvastatin reduces (V)LDL-cholesterol are similar in *E3L.CETP* mice and *E3L* mice. In addition, the mild increase in LDLr expression in both *E3L* and *E3L.CETP* mice may contribute to lower plasma cholesterol levels. The increase in HDL was accompanied by decreased hepatic *CETP* mRNA expression levels with a concomitant reduction in plasma CE transfer activity. Apparently, the fact that mice naturally lack CETP expression prevents the atorvastatin-induced increase in HDL-cholesterol in mice.

Since several additional key players in HDL metabolism might have been affected differently by atorvastatin treatment in *E3L.CETP* as compared to *E3L*

mice, and thus participate in the HDL-cholesterol raising effect, we have also evaluated the effect of atorvastatin on the hepatic expression of *Apoa1*, *Abca1*, *Pltp*, and *Sr-b1*. ApoAI is involved in the generation formation nascent HDL particles,[22] which acquire cholesterol via ABCA1. In fact, the HDL-cholesterol level in mice is largely determined by the hepatic expression of ABCA1.[23] PLTP plays an important role in the remodeling of HDL, by facilitating phospholipid transfer to HDL during its maturation from discoidal HDL into spherical HDL.[24] Finally, at least in mice, hepatic SR-BI is crucially involved in the selective uptake of HDL-CE.[25] We found that atorvastatin did not affect the hepatic expression of *Pltp*, *Sr-b1*, and *Apoa1*. Atorvastatin did decrease *Abca1* expression in *E3L* and *E3L.CETP* mice. However, since a decreased *Abca1* expression would be expected to lower HDL levels, it also cannot be a causal factor for the selective elevation of HDL in *E3L.CETP* mice. Previous experiments in mice in which hepatic ABCA1 expression levels were modulated specifically, have shown a causal relationship between hepatic ABCA1 expression and plasma HDL-cholesterol. In our study, atorvastatin primarily decreases CETP expression as related to a reduced hepatic cholesterol content. We speculate that, as a consequence rather than as a cause, the liver attempts to maintain its cholesterol balance by an upregulation of LDL receptors to enhance cholesterol influx and a down-regulation of ABCA1 to decrease cholesterol efflux.

Taken together, the selective raise in HDL-cholesterol in *E3L.CETP* mice cannot be explained by atorvastatin-mediated effects on apoAI, ABCAI, PLTP, or SR-BI, but is primarily caused by the reduction in *CETP* expression. Both a decrease in plasma CETP activity and a reduction in (V)LDL (*i.e.* acceptor of HDL-CE) can account for a reduction in CE transfer activity, which in its turn causes the increase in HDL-cholesterol. In addition to its transfer activity, CETP has also been implicated in the direct[26] and in the SR-BI-mediated[27] HDL-CE uptake by hepatocytes. Inhibition of these uptake pathways by atorvastatin via reducing cellular CETP may thus potentially also contribute to the increase in HDL-cholesterol.

The atorvastatin-induced down-regulation of CETP expression is presumably caused by a reduction in plasma and hepatic cholesterol levels. Cholesterol feeding of *CETP* transgenic mice increases hepatic *CETP* mRNA expression via an LXR responsive element in the CETP promoter.[28] Conversely, atorvastatin may down-regulate CETP expression by reducing LXR signaling, as atorvastatin reduced plasma and hepatic cholesterol levels[16] and consequently probably also hepatic oxysterols, the natural ligands of LXRα. In line with this hypothesis, the expression of other LXR target genes such as ABCG5, ABCG8, LPL and SREBP-1c were also reduced upon atorvastatin treatment. In addition, the CETP promoter activity is affected by several other regulatory transcription factors,[7] which alone or in combination could also be responsible for decreased transcription. The fact that atorvastatin treatment of humans also decreases

plasma CETP[8,9] may well be explained by similar regulation of CETP expression.

Based on our collective data, we thus propose the following mechanism by which statins raise HDL-cholesterol, as summarized in Fig. 6. By inhibiting HMGCoA reductase activity, statins decrease the hepatic lipid content. This results in decreased (V)LDL levels by a lower VLDL production and a higher (V)LDL clearance. In addition, reduction in hepatic cholesterol results in reduced levels of hepatic oxysterols (*i.e.* the natural ligands of LXRα) and, consequently, decreased LXRα-induced hepatic expression of CETP. Therefore, the HDL-cholesterol levels are raised by lower (V)LDL levels and lower CETP expression, resulting in decreased CE transfer activity from HDL to (V)LDL.

Clinical studies have established that statins improve the survival rate of patients with hypercholesterolemia and coronary artery disease by lowering LDL-cholesterol and by their pleiotropic anti-inflammatory effects.[29] However, a high residual cardiovascular risk still remains.[3] Even with aggressive atorvastatin treatment in the PROVE-IT study, the risk remained 60-70% despite greater protection against death or major cardiovascular events.[30] Therefore, concomitant raising of HDL-cholesterol is generally considered to enhance the anti-atherogenic potential of statins. Since our novel *E3L.CETP* mouse model is responsive to modulation of apoB-containing lipoproteins as well as HDL levels, we anticipate that our mouse model will be valuable to study the effect of such HDL-raising therapeutic strategies, alone or in combination with (V)LDL-lowering strategies, on plasma lipid metabolism and atherosclerosis development, and to study the underlying mechanisms.

In conclusion, our results show that atorvastatin increases HDL-cholesterol by reducing the hepatic CETP expression and plasma CE transfer activity in *E3L.CETP* mice. Therefore, we postulate that reduction of CETP expression contributes to the increase in HDL that is found in human subjects treated with statins.

Acknowledgements

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Chapter 4

NIACIN INCREASES HDL BY REDUCING HEPATIC EXPRESSION AND PLASMA LEVELS OF CHOLESTERYL ESTER TRANSFER PROTEIN IN APOE*3LEIDEN.CETP MICE

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Abstract

Objective: Niacin potently decreases plasma triglycerides and LDL-cholesterol. In addition, niacin is also the most potent HDL-cholesterol increasing drug used in the clinic. In the present study, we aimed at elucidation of the mechanism underlying its HDL-raising effect.

Methods and Results: In APOE*3Leiden transgenic mice expressing the human CETP transgene, niacin dose-dependently decreased plasma triglycerides (up to -77%, $P<0.001$) and total cholesterol (up to -66%, $P<0.001$). Concomitantly, niacin dose-dependently increased HDL-cholesterol (up to +87%, $P<0.001$), plasma apoAI (up to +72%, $P<0.001$), as well as the HDL particle size. In contrast, in APOE*3Leiden mice not expressing CETP, niacin also decreased total cholesterol and triglycerides but did not increase HDL-cholesterol. In fact, in APOE*3Leiden.CETP mice, niacin dose-dependently decreased the hepatic expression of CETP (up to -88%; $P<0.01$) as well as plasma CETP mass (up to -45%, $P<0.001$) and CETP activity (up to -52%, $P<0.001$). Additionally, niacin dose-dependently decreased the clearance of apoAI from plasma and reduced the uptake of apoAI by the kidneys (up to -90%, $P<0.01$).

Conclusion: Niacin markedly increases HDL-cholesterol in APOE*3Leiden.CETP mice by reducing the CETP activity, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool, and increases HDL-apoAI by decreasing the clearance of apoAI from plasma.

Introduction

Dyslipidemia is an important risk factor for the development of cardiovascular disease (CVD). Although lowering of LDL-cholesterol (C) by e.g. statins reduces CVD risk by approximately 30%, substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL-C.[1-3] Because of clinical studies, which have shown that HDL-C, independently of LDL-C, is inversely correlated with the risk of CVD,[4,5] attention has shifted toward strategies for targeting HDL composition as adjunctive therapy to prevent and treat CVD. Current strategies to mildly increase HDL-C levels include aggressive overall lifestyle modification (*i.e.* exercise, diet, weight loss, and smoking cessation), and modest increases in HDL-C levels are achieved with statins[6] and fibrates (5-10%).[7]

Niacin (nicotinic acid, vitamin B3) has been described to exhibit lipid-modifying capacities already since the 1950s. Since then various (clinical) studies have shown the beneficial effects of niacin on plasma lipid levels. Treatment with niacin alone was associated with a 27% reduction in non-fatal myocardial infarction and it reduced all cause mortality by 11%.[8,9] In combination with colestipol (FATS trial) or simvastatin (HATS trial), niacin reduced cardiac events by as much as 80-90%.[10,11] These potent atherogenic properties of niacin are thought to be attributable to its marked HDL-elevating effect (+20% to +30%), besides it potent effect on reducing plasma TG (-40% to -50%) and LDL-C (-20%).[7,12] In fact, niacin is currently the most effective therapy for elevating HDL-C.

The mechanism underlying the ability of niacin to reduce the plasma (V)LDL level has been well-studied. By selective binding to GPR109A on adipocytes, niacin suppresses hormone sensitive triglyceride lipase (HSL) activity, resulting in a decreased release of free fatty acids (FFA) from adipose tissue and decreased plasma FFA levels.[13] The resulting reduced supply of FFA towards the liver is believed to bring about a decreased hepatic VLDL-TG production, resulting in reduced VLDL-TG and (V)LDL-C levels.[13,14] In contrast, the mechanism underlying the HDL-C raising effect of niacin has not been elucidated as yet. This is probably related to the lack of suitable animal models that respond in a human-like manner to HDL-raising drug interventions. In wild-type mice and apoE-knockout mice (the classical animal model for hyperlipidemia and atherosclerosis), rats and dogs, niacin only transiently reduced plasma levels of TG but failed to failed to raise HDL-C.[15,16] An HDL-C-elevating effect of niacin has been reported in rabbits, but with 30% ethanol as dosing vehicle and only after 12 weeks of treatment.[17]

Therefore, the aim of this study was to elucidate the mechanism underlying the HDL-C raising effect of niacin. To this end, we used our recently developed APOE*3Leiden (E3L).CETP transgenic mouse model. We have previously demonstrated that E3L mice have a human-like lipoprotein profile in which the

elevated plasma cholesterol and TG levels are mainly confined to the (V)LDL-sized lipoprotein fractions.[18,19] These mice develop atherosclerosis upon dietary cholesterol feeding and respond in a human-like manner to drugs used in the treatment of CVD (e.g. statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II receptor antagonists),[20-23] but they did not yet respond to HDL-modulating interventions. By cross-breeding E3L mice with mice expressing human CETP under control of its natural flanking regions, E3L.CETP were obtained[24] that respond to the HDL-raising effects of fenofibrate,[25] atorvastatin[26] and torcetrapib.[27] We now fed these mice a Western-type diet without or with increasing doses of niacin to reveal the mechanism underlying its HDL-C raising effect.

Methods

Animals

Hemizygous human CETP transgenic (CETP) mice, expressing a human CETP minigene under the control of its natural flanking sequences[28] were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossbred with hemizygous *E3L* mice[18] at our Institutional Animal Facility to obtain E3L and E3L.CETP littermates.[24] In this study, female mice were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 12 weeks, E3L and E3L.CETP mice were fed a semi-synthetic cholesterol-rich diet, containing 15% (w/w) fat and 0.25% (*E3L*) or 0.1% (E3L.CETP) (w/w) cholesterol (Western-type diet; Hope Farms, Woerden, The Netherlands) for three weeks to obtain similar total cholesterol levels in both strains (about 12-14 mmol/L). After matching based on total plasma cholesterol (TC), triglyceride (TG) levels, and age, mice (n=8 per group) received a Western-type diet without or with 0.03% (~36 mg/kg/day), 0.1% (~118 mg/kg/day), 0.3% (~360 mg/kg/day) or 1% (~1180 mg/kg/day) niacin (Sigma, St. Louis, MO, USA) for at least 3 weeks. These doses correspond well to the doses used in humans, if the 10 times faster metabolism of mice as compared to humans is taken into account. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am, unless indicated otherwise. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

Plasma lipid and lipoprotein analysis

Plasma was obtained via tail vein bleeding as described[24] and assayed for TC, TG and phospholipids (PL), using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 'Phospholipids B' (Instruchemie, The Netherlands), respectively. The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) using a Superose 6 column as

described previously.[24] HDL-C was isolated by precipitating the apoB-containing lipoproteins from 20 µL EDTA plasma by adding 10 µL heparin (LEO Pharma, The Netherlands; 500 U/mL) and 10 µL 0.2 M $MnCl_2$. Mixtures were incubated for 20 min at room temperature and centrifuged for 15 min at 13,000 rpm at 4°C. In the supernatant HDL-C was measured using enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA).

Plasma apoAI concentration
Plasma apoAI concentrations were determined using a sandwich ELISA. Hereto, rabbit anti-mouse apoAI polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (at 3 µg/mL) at 4°C and incubated with diluted mouse plasma (dilution 1:400,000) for 90 min at 37°C. Subsequently, goat anti-mouse apoAI antibody (600-101-196; Rockland Immunochemicals, Inc., Gilbertsville, PA; dilution 1:3000) was added and incubated for 90 min at 37°C. Finally, horse radish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (605-4313; Rockland; dilution 1:15000) was added and incubated for 90 min at 37°C. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, ME, USA) was used as a standard.

HDL size by native PAGE
The HDL size was determined essentially as described.[29] Total lipoproteins were isolated from plasma by ultracentrifugation (5 h at 541,000 g) as the d < 1.21 g/mL plasma fraction in a TLA 100.3 rotor (Beckman). Lipoproteins (7.5 µg protein) were loaded onto a 4-20% polyacrylamide Tris.HCl gel (BioRad, Hercules, CA, USA) and electrophoresis was performed according to the manufacturer's protocol. Gels were stained with Coomassie Brilliant Blue (Merck) and HDL size was compared with globular protein standards (HMW native marker kit, GE Healthcare).

Plasma lipolysis
Post-heparin plasma from overnight fasted mice was collected from the tail vein at 20 minutes after intraperitoneal injection of heparin (1.0 U/g body weight). Post-heparin plasma triacylglycerol hydrolase activity was determined in the presence or absence of 1 mol/L NaCl to estimate the hepatic lipase (HL) activity, which was calculated as the portion of total triacylglycerol hydrolase activity not inhibited by 1 mol/L NaCl.[30]

Preparation of ^{125}I-apoAI-labeled autologous HDL
ApoAI was radiolabeled at pH 10 with carrier-free ^{125}I according to the ICl method[31], and separated from unbound ^{125}I by Sephadex G50 gel filtration. ^{125}I-

apoAI (~75 µg) was incubated with 1.4 mL of plasma from E3L.CETP mice (3 h at 37°C), and ^{125}I-apoAI-HDL was isolated after density gradient ultracentrifugation. The specific activity was ~15 cpm /ng HDL protein.

In vivo kinetics of ^{125}I-apoAI-labeled HDL
E3L.CETP mice were injected via the tail vein with ^{125}I-apoAI-HDL (40 µg protein) in a total volume of 200 µL PBS. At the indicated time points after injection, blood was collected from the tail vein to determine the plasma decay of ^{125}I-apoAI. The total plasma volumes of the mice were calculated from the equation V (mL) = 0.04706 x body weight (g), as determined from previous ^{125}I-BSA clearance studies.[32] At 6 h after injection, the mice were sacrificed and organs were taken and counted for ^{125}I-activity. Values were corrected for serum radioactivity present in the liver (84.7 µL/g wet weight), kidneys (135.2 µL/g wet weight), skeletal muscle (13.7 µL/g wet weight) and white adipose tissue (16.1 µL/g wet weight).[33]

Hepatic lipid analysis
Liver tissue samples were homogenized in phosphate-buffered saline (approx. 10% wet w/v), and the protein content was measured according to the method of Lowry *et* al. Lipids were extracted, separated by high-performance thin-layer chromatography on silica gel plates and analyzed with TINA2.09 software (Raytest Isotopen Messgeräte, Straubenhardt, Germany), as described before.[34]

Hepatic mRNA expression
Total RNA extraction from liver tissue samples was performed using RNA-Bee (Amsbio, Oxon, UK) according to the manufacturer's instructions. RNA was converted to single-stranded cDNA by a reverse transcription procedure (Promega) according to the manufacturer's protocol using random primers. cDNA levels were measured by real-time polymerase chain reaction (PCR) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. PCR master mix from Eurogentec was used. Primers and probes were obtained from Biosource (Nivelles, Belgium). The probes were labelled with 3-BHQ1 and 5-FAM or 5-TET. The mRNA levels were normalized to mRNA levels of three housekeeping genes (*i.e.*, cyclophilin, HPRT and GAPDH). Primers and probes used for this study were described previously.[25] The level of mRNA expression for each gene of interest was calculated according to the manufacturer's instructions (Applied Biosystems) as described previously.[35]

CETP mass and activity in plasma
Plasma CETP mass was analyzed by ELISA using kit 'CETP ELISA Daiichi' (Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan). Plasma CETP activity was measured as the transfer of [^{3}H]cholesteryl oleate ([^{3}H]CO) from exogenous

LDL to HDL as described.[36] CETP activity was calculated as μmol CE transfer per mL plasma per hour.

Biliary lipid secretion
The common bile duct of anesthetized mice was ligated, the gall bladder was cannulated, and bile was collected during 90 minutes.[30] Cholesterol, PL and total bile acids in bile were determined using kits '236691' (Roche Molecular Biochemicals, Indianapolis, IN, USA), 'Phospholipids B' (Instruchemie, The Netherlands) and 'Total bile acids assay' (Bio-Stat, UK), respectively.

Fecal excretion of bile acids and neutral sterols
The mice were housed at 3 mice per cage. Feces produced during 2 subsequent periods (48 h each) were separated from the wood shavings by sieving. Aliquots of lyophilized feces were used for determination of neutral and acidic sterol content by gas-liquid-chromatography procedures as described.[30]

Statistical analysis
All data are presented as means ± SD unless indicated otherwise. Data were analyzed parametrically by 1-way ANOVA followed by Dunnett to correct for multiple testing. Probability values less than 0.05 were considered statistically significant. SPSS 14.0 was used for statistical analysis.

Results

Niacin decreases plasma lipids in both E3L and E3L.CETP mice, but increases HDL only in E3L.CETP mice
No adverse clinical signs were observed with increasing dosages of niacin as indicated by absence of differences in weight gain and plasma ALT levels

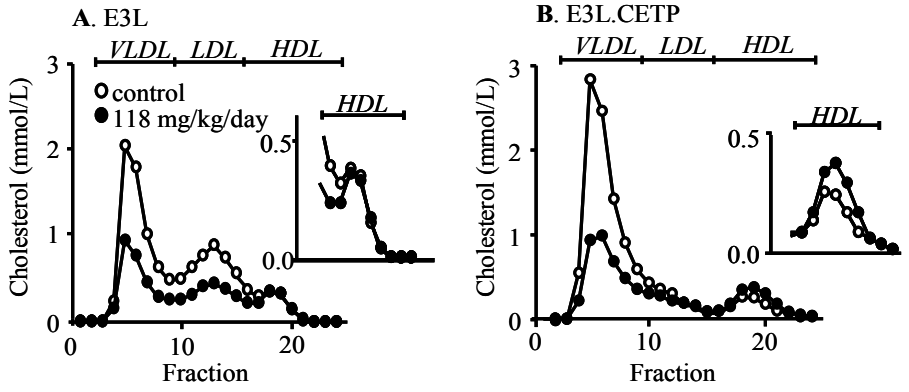

Figure 1. Effect of niacin on lipoprotein profiles. E3L (A) and E3L.CETP (B) mice received a Western-type diet without (open circles) or with (closed circles) niacin (118 mg/kg/day) for 3 weeks. Plasma was pooled per group and the distribution of cholesterol over the individual lipoproteins was determined after separation by FPLC.

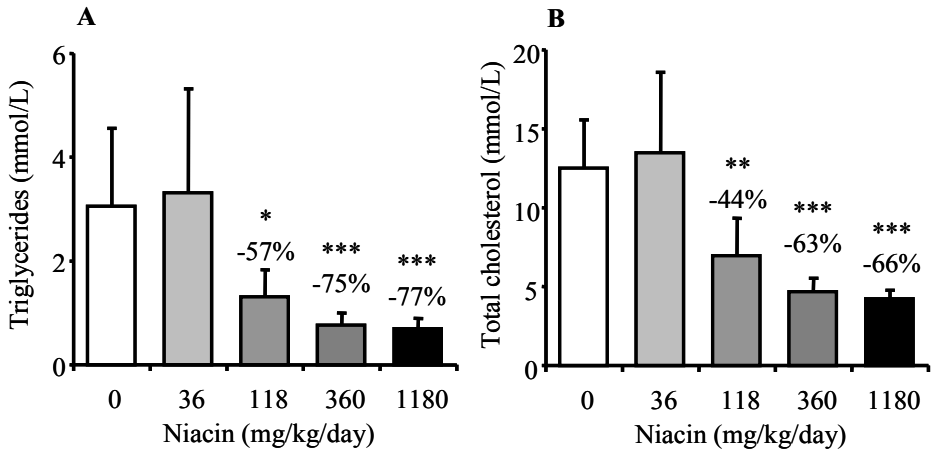

Figure 2. Dose-dependent effect of niacin on plasma triglycerides and total cholesterol. E3L.CETP mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Plasma triglycerides (A) and total cholesterol (B) were determined. Values are means ± SD (n=8 per group). **P*<0.05, ***P*<0.01, ****P*<0.001.

between treatment groups and the control. Treatment of E3L mice with niacin (118 mg/kg/day) caused a sustained reduction in plasma TG by -26% (1.4±0.6 mM vs 1.9±0.6 mM; *P*<0.05) and in plasma TC by -35% (9.2±3.4 mM vs 14.2±4.5 mM; *P*<0.05). Lipoprotein fractionation by FPLC showed that the reduction in cholesterol was confined to the apoB-containing lipoproteins (V)LDL, whereas HDL-C was not affected (Fig. 1A). An equal dose of niacin even more potently reduced plasma TG (-57%, *P*<0.05) and TC (-44%, *P*<0.01) in E3L.CETP mice. As in E3L mice, the TC-decreasing effect of niacin in E3L.CETP mice was caused by a reduction of (V)LDL-C. However, whereas niacin did not affect HDL levels in E3L mice, it increased HDL-C in E3L.CETP mice (Fig. 1B). In E3L.CETP mice, the effects of niacin on plasma TG and TC levels were dose-dependent as shown in figure 2. At the highest dose of 1180 mg/kg/day, niacin reduced TG levels by -77% (*P*<0.001) (Fig. 2A) and TC levels by -66% (*P*<0.001) (Fig. 2B).

The HDL-increasing effect of niacin in E3L.CETP mice is dose-dependent

To investigate whether the HDL-increasing effect of niacin in E3L.CETP mice was also dose-dependent, we determined HDL-C concentrations in whole plasma after precipitation of apoB-containing lipoproteins by heparin/ $MnCl_2$. Indeed, niacin appeared to decrease (V)LDL-C levels up to -79% (*P*<0.001) (Fig. 3A), and to increase HDL-C up to +87% (*P*<0.001) (Fig. 3B), both in a dose-dependent fashion. We next evaluated whether niacin also affects apoAI, the main apolipoprotein constituent of HDL. Indeed, niacin dose-dependently increased apoAI up to +72% (*P*<0.001) (Fig. 3C). Whereas niacin thus increases both HDL-C and apoAI, the effects on HDL-C at the various doses are

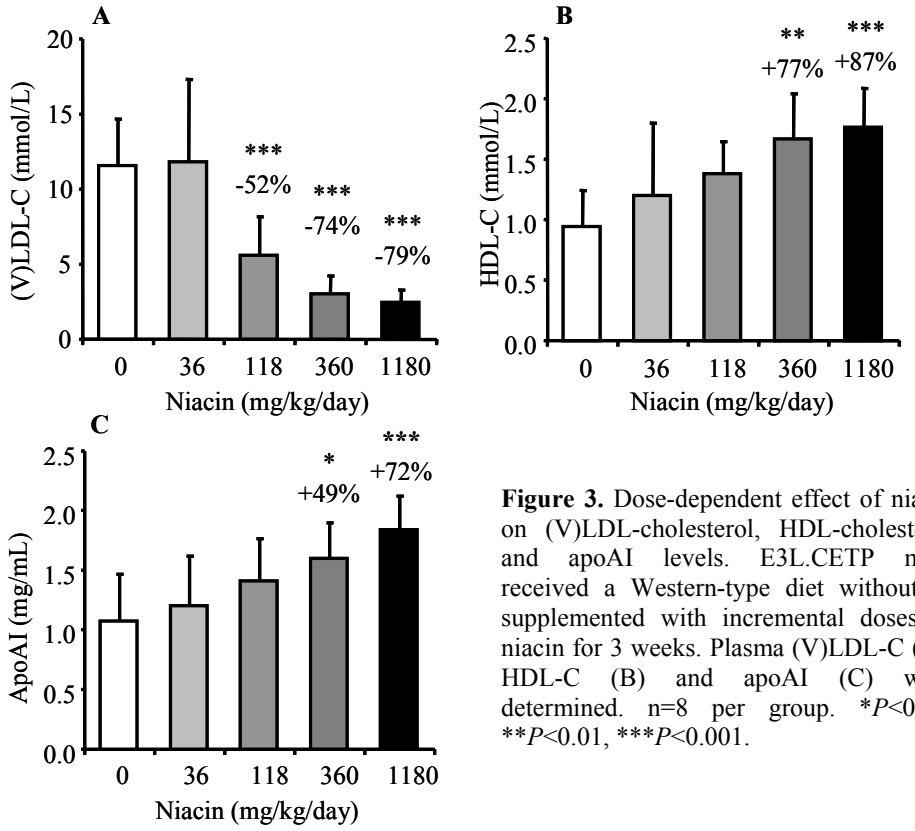

Figure 3. Dose-dependent effect of niacin on (V)LDL-cholesterol, HDL-cholesterol and apoAI levels. E3L.CETP mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Plasma (V)LDL-C (A), HDL-C (B) and apoAI (C) were determined. n=8 per group. **P*<0.05, ***P*<0.01, ****P*<0.001.

somewhat more pronounced than on apoAI, suggesting that niacin increases the lipidation of apoAI. This was reflected by a modest increase of the HDL particle size as determined by native PAGE (Fig. 4). Further analyses of the pooled HDL fractions showed a decrease in triglycerides (-45%) and an increase in cholesteryl ester (+56%) and phospholipids (+66%) (data not shown). Niacin did not seem to affect the hepatic synthesis or clearance of HDL, at least judged from unchanged hepatic mRNA expression of genes involved in HDL synthesis (*apoa1*, *abca1*) or clearance (*sr-b1*; data not shown). Hepatic *pltp* mRNA

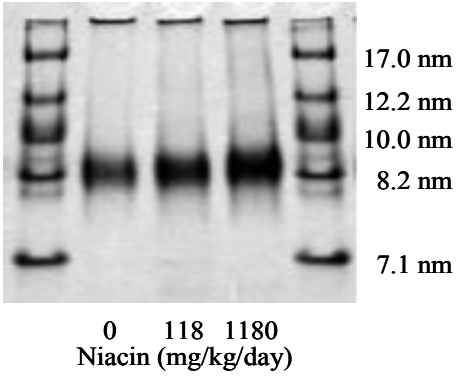

Figure 4. Dose-dependent effect of niacin on the HDL particle size. Total lipoproteins from pooled plasma were subjected to native 4-20% PAGE, and the resulting gel was stained with Coomassie Brilliant Blue.

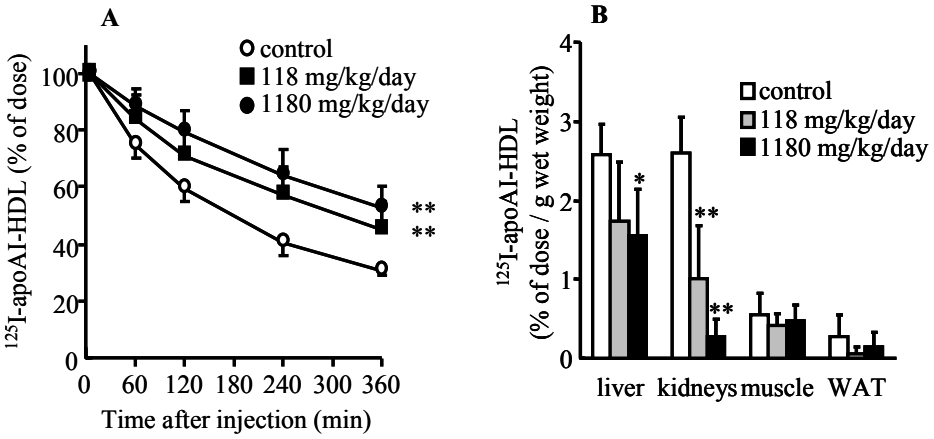

Figure 5. Dose-dependent effect of niacin on plasma apoAI kinetics. Mice were injected with ^{125}I-apoAI-HDL, and plasma ^{125}I activity was determined at the indicated time points (A). Thereafter the mice were euthanized and ^{125}I activity was determined in the liver, kidneys, skeletal (hindlimb) muscle and white adipose tissue (WAT) (B). n=5 per group. *P<0.05, **P<0.01.

expression was slightly increased upon niacin treatment (data not shown). In plasma niacin did decrease the HL activity, albeit that the effect was not dose-dependent (maximal reduction of -47% at 118 mg/kg/day; P<0.05).

Niacin increases the residence time of apoAI in plasma

To evaluate whether the dose- dependently increased plasma apoAI level as induced by niacin-treatment was caused by decreased clearance of apoAI from plasma, we determined the effect of niacin on the plasma kinetics of intravenously injected ^{125}I-apoAI-labeled HDL (Fig. 5). Indeed, niacin dose-dependently increased the residence of ^{125}I-apoAI in plasma (Fig. 5A). From the mono-exponential decay curves it was calculated that the plasma half-life of ^{125}I-apoAI (3.5±0.1 h) was increased by niacin at 118 mg/kg/day (5.5±1.3 h; P<0.01) and 1180 mg/kg/day (6.6±1.3 h; P<0.01). This was accompanied by a dose-dependent reduction in the uptake of ^{125}I-activity by the liver (up to -50%; P<0.05) and the kidneys (up to -90%; P<0.01) (Fig. 5B). For comparison, the uptake of [^{3}H]cholesteryl oleoyl ether-labeled HDL by the liver was much larger (approx. 40% of dose/g wet weight), whereas the uptake by the kidneys was undetectable (data not shown).

Niacin reduces the hepatic lipid content

The effects of niacin on plasma lipid metabolism in E3L.CETP mice are consistent with a niacin-induced reduction in CETP activity. Because CETP expression is regulated by the hepatic cholesterol content,[28] we first examined effects of niacin on liver lipids (Fig. 6A). Niacin decreased the hepatic TG content (-38%, P<0.05). This is consistent with the inhibitory effects of niacin

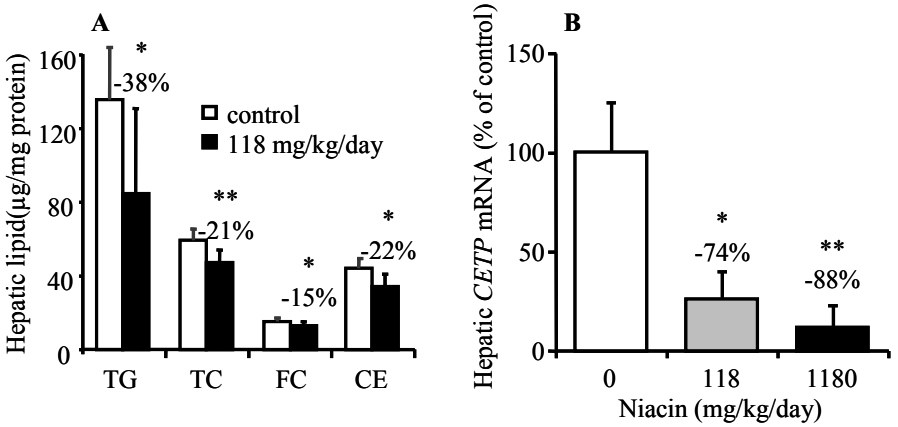

Figure 6. Effect of niacin on hepatic lipid content and *CETP* mRNA expression. E3L.CETP mice received a Western-type diet without (open bars) or with (closed bars) niacin. Hepatic triglycerides (TG), total cholesterol (TC), free cholesterol (FC) and cholesteryl esters (CE) quantified (A) and *CETP* mRNA expression was measured (B). **P*<0.05, ***P*<0.01.

on HSL in adipose tissue,[13] thereby reducing the trafficking of FFA to the liver for TG synthesis. Niacin also decreased the hepatic TC content (-21%, *P*<0.01), which was mainly attributed to a reduction in hepatic cholesteryl esters (-22%, *P*<0.05). This effect was in line with a compensatory increase in hepatic *Hmgcoared* mRNA expression (+232%, *P*<0.05; not shown).

Niacin decreases hepatic CETP mRNA expression and plasma CETP levels

The decrease in hepatic cholesterol was indeed accompanied by a dose-dependent reduction in hepatic *CETP* mRNA up to -88% (*P*<0.01) at 1180 mg/kg/day (Fig. 6B). To evaluate whether the niacin-induced decreased hepatic

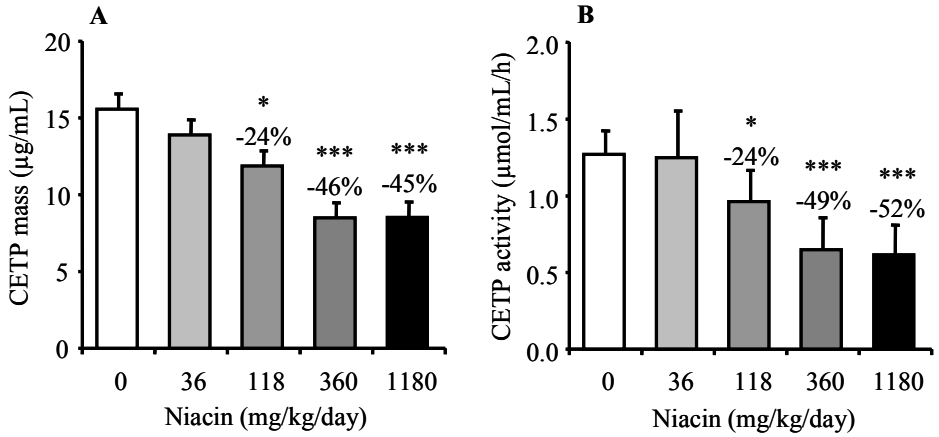

Figure 7. Dose-dependent effect of niacin on plasma CETP mass and activity. E3L.CETP mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Plasma CETP mass (A) and CETP activity (B) were determined. Values are means ± SD (n=8 per group). **P*<0.05, ****P*<0.001.

CETP mRNA expression was reflected by reduced CETP levels in plasma, we determined both CETP mass (Fig. 7A) and activity (Fig. 7B). Indeed, niacin dose-dependently decreased plasma CETP mass and CETP activity to a similar extent (up to -45% and -52%; $P<0.001$).

Table 1. Effect of niacin on biliary and fecal lipid output.

	Control	Niacin 118 mg/kg/d	Niacin 1180 mg/kg/d
Bile			
Bile flow (μL/min/100g bw)	2.0 ± 0.5	2.0 ± 0.4	2.3 ± 0.7
Bile acid output (nmol/min/100g bw)	50 ± 14	67 ± 18	65 ± 25
Cholesterol output (nmol/min/100g bw)	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.2
Phospholipid output (nmol/min/100g bw)	14 ± 3	14 ± 3	16 ± 5
Feces			
Neutral sterols (Fmol/100g bw/d	32.7 ± 2.2	34.8 ± 4.6	36.1 ± 4.5
Bile acids (Fmol/100g bw/d)	8.6 ± 1.5	8.4 ± 1.6	6.7 ± 0.9*
Total sterols (Fmol/100g bw/d)	41.3 ± 3.1	43.2 ± 5.1	42.7 ± 4.7

E3L.CETP mice received a western-type diet without or supplemented with niacin for 3 weeks. The bile bladder was cannulated, and bile flow and composition were measured during 90 minutes (n=6-7). Feces were collected per cage (3 mice per cage) in two subsequent periods of 48 h each (n=8). Fecal composition was measured by gas-liquid-chromatography and fecal sterol output was calculated. Data are presented as mean ± SD, *P<0.05.

Niacin does not affect biliary and fecal cholesterol output

To evaluate the consequences of the niacin-induced alterations in lipid metabolism on lipid excretion into bile and feces, we determined bile flow, biliary lipids and sterols in stool. Niacin did not affect bile flow or the bile composition (cholesterol, phospholipids and bile acids). The highest dose of niacin (1180 mg/kg/day) did affect the composition of the fecal sterols to some extent, as reflected by a slight non-significant increase in neutral sterols and a decrease in bile acids (-22%; $P<0.05$). However, like the dietary input, total fecal sterol output was not affected by niacin (Table 1).

Discussion

In this study, we investigated the mechanism(s) underlying the HDL-raising effect of niacin. We demonstrated that CETP plays a crucial role in the niacin-induced increase in plasma HDL-C and apoAI levels in E3L.CETP mice. Niacin reduced CETP dependent transfer of cholesterol from HDL to (V)LDL as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool.

This resulted in an increased lipidation of apoAI, as reflected by an increased HDL particle size, and a reduced uptake of apoAI by the kidneys.
We previously showed that E3L mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels and that these mice show a human-like response to drug interventions aimed at treatment of CVD (e.g. statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II receptor antagonists)[20-23] with respect to alterations in the lipoprotein profile and/or atherosclerosis development. This is in sheer contrast with wild-type C57Bl/6 mice and conventional hyperlipidemic mice, such as apoE-deficient or LDL receptor-deficient mice, which show either an adverse response or no response to such interventions.[37] In particular, administration of niacin to wild-type mice or apoE-deficient mice did show a transient decrease in plasma TG and FFA levels, but failed to increase plasma HDL-C in these mice.[13,16] Likewise, we now showed that niacin lowered TG and cholesterol

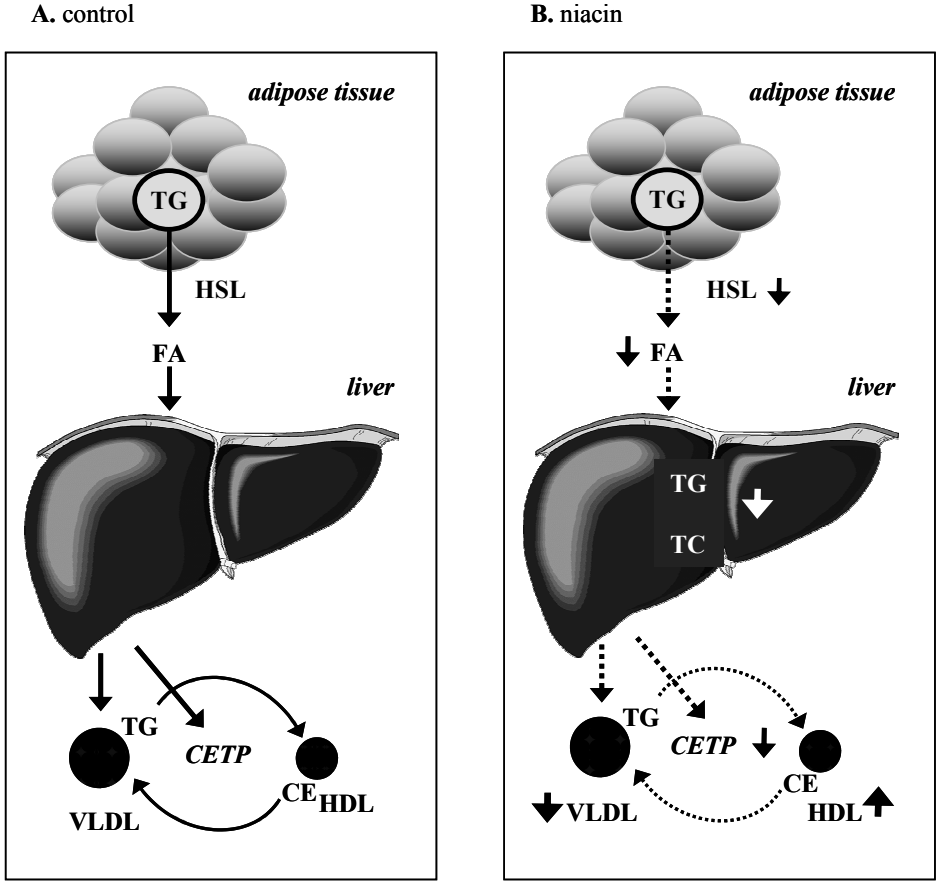

Figure 8. Proposed mechanism underlying the HDL-raising effect of niacin. For explanation see text. CE, cholesteryl ester; FA, fatty acids; HSL, hormone sensitive lipase; TC, total cholesterol; TG, triglycerides.

within apoB-containing lipoproteins in E3L mice, but did not affect HDL-C levels.

Recently, we showed that introduction of the human CETP gene in *E3L* mice results in a mouse model which also shows a human-like response with regard to raising HDL-C after treatment with fenofibrate,[25] atorvastatin[26] and torcetrapib.[27] Since the introduction of CETP permits cross-talk between (V)LDL and HDL metabolism via the exchange of neutral lipids, we reasoned that the E3L.CETP mouse would also be an excellent mouse model to study the effects of niacin on HDL metabolism.

First, we observed that niacin dose-dependently reduced VLDL-TG and (V)LDL-C levels. The primary action of niacin is inhibition of HSL activity in adipose tissue after binding to the GPR109A receptor that is selectively expressed by adipocytes. This results in a decreased liberation of FFA from adipose tissue, and a decreased flux of albumin-bound FA to the liver, which is required for substrate-driven hepatic TG synthesis and VLDL production.[13] As a consequence we thus observed a concentration-dependent drop in VLDL-TG and (V)LDL-C levels. In addition, we observed that niacin reduced the hepatic cholesterol content. This may be caused by reduced input of cholesterol from plasma into the liver, since plasma (V)LDL-C concentrations are reduced and cholesterol-enriched HDL is formed from which cholesteryl esters are presumably not being delivered efficiently to the liver. The decreased hepatic cholesterol content cannot be explained by differences in biliary sterol output, since the excretion of bile acids and cholesterol remained unchanged. Alternatively, niacin may reduce the endogenous hepatic synthesis of cholesterol.

Second, we showed that niacin dose-dependently raised HDL-C levels in E3L.CETP mice, but not in E3L mice, as paralleled by a less pronounced raise in apoAI. The presence of CETP thus plays a crucial role in the HDL-raising effect of niacin, and we reasoned that niacin may dose-dependently inhibit CETP activity. It is well-known that VLDL-TG is a driving force for CETP activity, and the relative proportions of VLDL and HDL have been shown to play a determinant role in CETP activity. It has been demonstrated that the capacity of apoB-containing lipoproteins to accept CE from HDL is closely correlated with the relative TG content of the lipoprotein acceptor particles.[38-41] By decreasing VLDL levels, niacin may thus reduce CETP activity simply by decreasing the availability of VLDL-TG as substrate for CETP.

Our data corroborate recent observations from Hernandez *et al*.[15,42] who showed that niacin increased HDL-C levels in CETP mice and APOB.CETP mice, but not their CETP-deficient wild-type littermates. In fact, they speculated the reduced VLDL levels to be the main mechanism underlying the HDL-raising effect of niacin. However, we observed that niacin not only reduced plasma CETP activity, but also dose-dependently reduced plasma CETP mass to a similar extent, suggesting that niacin reduces the synthesis of CETP leading to

less CETP protein being released in plasma as reflected by similar reductions in CETP mass and activity. Indeed, niacin dose-dependently reduced hepatic *CETP* mRNA expression. It has been reported that hepatic cholesterol determines the hepatic *CETP* mRNA expression in CETP transgenic mice,[28] presumably via an LXR responsive element in the CETP promoter.[43] Therefore, it is likely that niacin decreases the hepatic CETP mRNA expression as a result of the observed decreased cholesterol content of the liver upon niacin treatment.

Besides increasing HDL-C, niacin also dose-dependently increased plasma apoAI levels. Niacin has been shown to inhibit the uptake of HDL-apoAI (but not HDL-CE) by cultured hepatocytes,[44] which we now confirmed *in vivo*. This may partly contribute to the increased apoAI levels. Such a potential effect of niacin should be independent of GPR109A, since expression of this receptor has not been detected in hepatocytes.[13,45,46] Together with our observations that hepatic mRNA expression of genes involved in HDL synthesis (*apoa1*, *acba1*) and clearance (*sr-b1*) were not affected by niacin, and an increase of PLTP would rather lead to a decrease in HDL-C levels,[30,47] it is most likely that the raise in apoAI is explained directly by the niacin-induced decreased CETP activity, which prevents cholesteryl ester transfer from HDL to (V)LDL. This leads to increased lipidation of apoAI, resulting in larger and cholesteryl ester-enriched HDL particles, and thus decreased glomerular filtration and excretion of lipid-poor apoAI via the cubulin/megalin receptor complex.[48] Indeed, we demonstrated a clear dose-dependent reduction in the uptake of ^{125}I-apoAI by the kidney.

Based on our collective data, we thus propose the following mechanism by which niacin reduces TG and (V)LDL-C and concomitantly raises HDL-C, as summarized in figure 8. By inhibiting HSL in adipose tissue upon binding of the niacin receptor GPR109A, niacin decreases TG lipolysis and thereby the supply of FFA to the liver, required for lipid synthesis. The consequently reduced hepatic lipid content results in a lower VLDL production and thus lower (V)LDL levels. In addition, reduction in hepatic cholesterol results in reduced hepatic expression of CETP, as well as diminished release of CETP into the plasma. Additionally, HL activity is reduced which may contribute to reduced remodelling of HDL in plasma, resulting in decreased clearance of HDL. The HDL particles become CE enriched, and less lipid-poor apoAI is cleared by the kidney. Niacin thus increases HDL-C and apoAI levels by 1) reducing levels of (V)LDL, the acceptor of CETP-mediated HDL-CE transfer, 2) decreasing CETP expression, 3) decreasing HL activity, and 4) decreasing the clearance of apoAI.

As concluded from a many clinical trials using statins, lowering LDL-C alone is not longer regarded to be sufficient to treat CVD. Therefore, comprehensive lipid management, in which raising HDL-C is an important target, is becoming a new standard.[4,7] Niacin (at dosages of 2-4 g/day) is unsurpassed in raising HDL-C. We show that niacin (in a clinical relevant range if we take into account the 5-10 times faster metabolism of mice) significantly improves the

plasma lipid levels in E3L.CETP mice, *e.g.* reduces TG and (V)LDL-C and increases HDL-C, albeit that total fecal sterol output is unaffected. Whether this will lead to improved HDL function and HDL-related reductions in CVD in the clinic still remains to be investigated.
Niacin has not been a very successful drug thus far because of its side-effect: severe flushing. Niacin is nowadays produced as an extended release (ER) compound, which enhances the tolerability. Clinical trails AIM-HIGH[49] and ARBITER-6 (HALTS)[50] evaluating the secondary prevention of CVD by ER niacin treatment are currently running. Post-hoc analysis of a subgroup of ARBITER-2, a randomized, placebo-controlled trial, showed increases in HDL-C upon daily intake of ER niacin (+20%), which were related to reduced progression of carotid intima-media thickness in the setting of both normal glycemic status and diabetes mellitus.[51,52] Because the flushing effects of niacin appeared to be prostaglandin D_2 (PGD_2) receptor mediated,[53] a combination therapy is currently being evaluated combining ER niacin and PGD_2 receptor antagonist laropiprant, which is better tolerated than ER niacin alone.[54] Currently one trail evaluating effects of this combination drug on hard clinical endpoints, as myocardial infarction, stroke or revascularisation (HPS2-THRIVE) is underway.
In conclusion, our results show that niacin increases HDL-C by reducing the hepatic *CETP* expression and plasma CETP protein and CE transfer activity in *E3L.CETP* mice. Therefore, we postulate that reduction of *CETP* expression contributes to the increase in HDL that is found in human subjects treated with niacin, which should be subject of further investigation.

Acknowledgements

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Chapter 5

TORCETRAPIB DOES NOT REDUCE ATHEROSCLEROSIS BEYOND ATORVASTATIN AND INDUCES MORE PRO-INFLAMMATORY LESIONS THAN ATORVASTATIN

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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.[2] 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[2] 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%

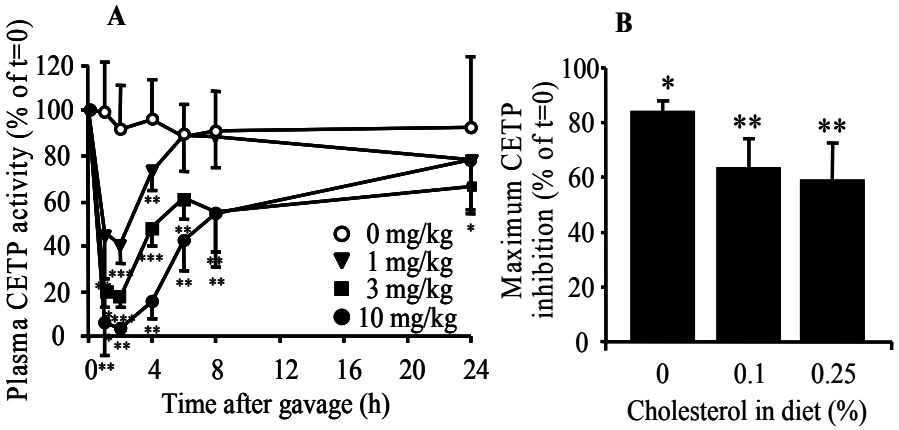


Figure 1. A single dose of torcetrapib inhibits CETP *in vivo*. *E3L.CETP* mice fed a chow diet received the indicated amounts of torcetrapib via intragastric gavage. Blood was drawn at the indicated time points and plasma was assayed for CETP activity (A). *E3L.CETP* mice, fed a chow diet or a diet 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.

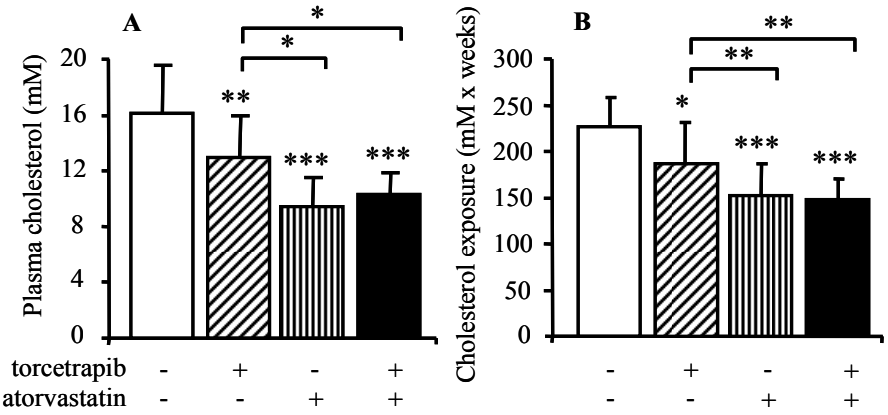


Figure 2. Torcetrapib reduces plasma cholesterol to a lesser extent than atorvastatin. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 9 weeks of drug intervention, blood was drawn and plasma was assayed for cholesterol (A). 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.

(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) 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).

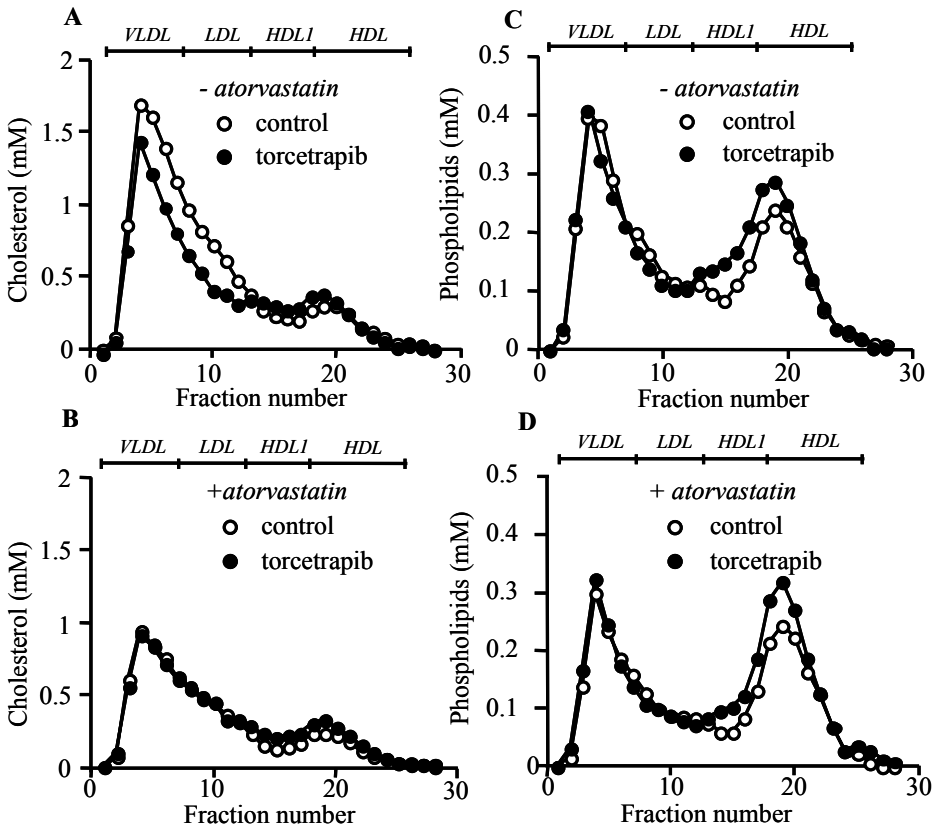


Figure 3. Torcetrapib reduces plasma VLDL and increases HDL levels. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 14 weeks of drug intervention, blood was drawn and plasma was pooled per treatment group (n=14-15). Pooled plasma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

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 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.[19]

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 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% and -46% respectively; $P<0.05$).

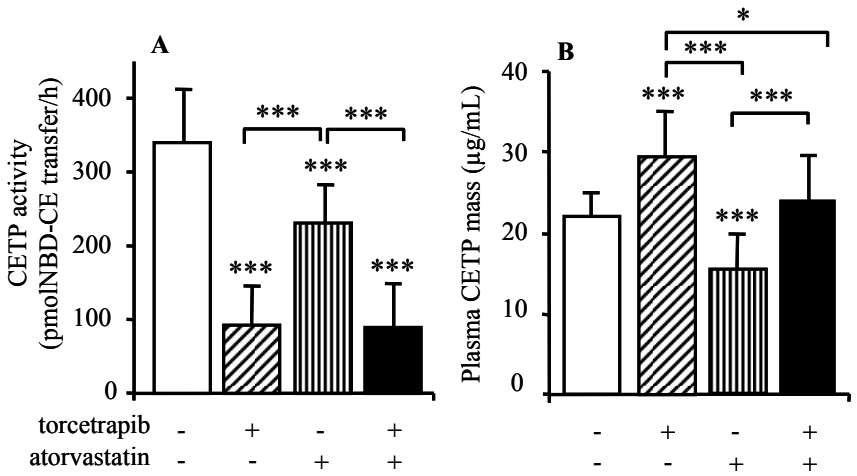


Figure 4. Torcetrapib reduces plasma CETP activity and increases CETP mass. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 9 weeks of drug intervention, blood was drawn and plasma was assayed endogenous CETP activity (A) and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

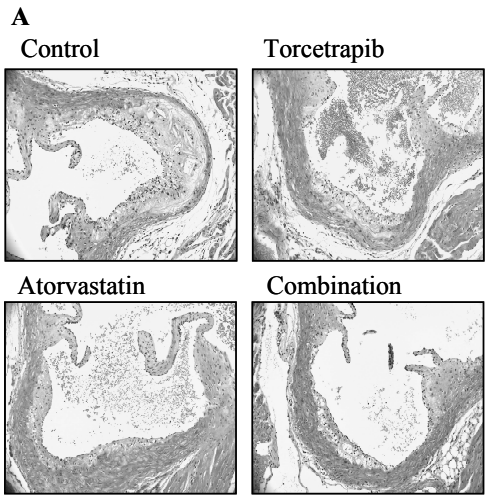
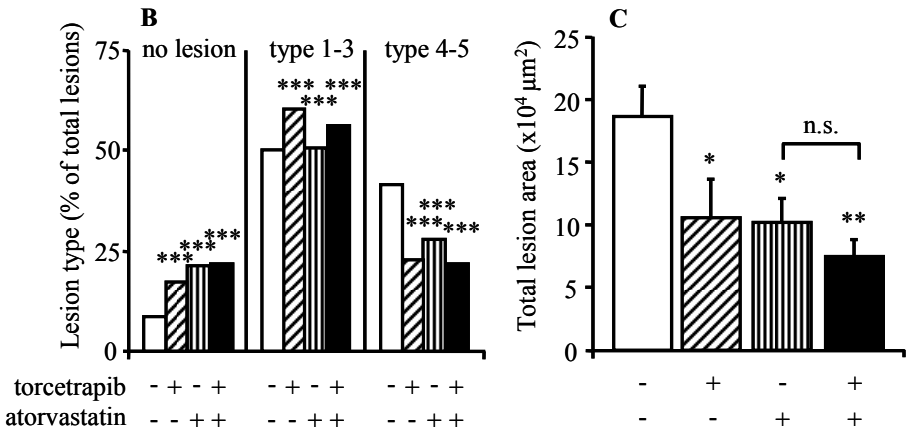


Figure 5. Torcetrapib reduces atherosclerosis development but does not enhance the atherosclerosis reducing effect of atorvastatin. Mice were fed a diet containing 0.25% cholesterol without or with torcetrapib (0.01%), atorvastatin (0.0023%) or both. After 14 weeks of drug intervention, hearts were isolated, 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



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

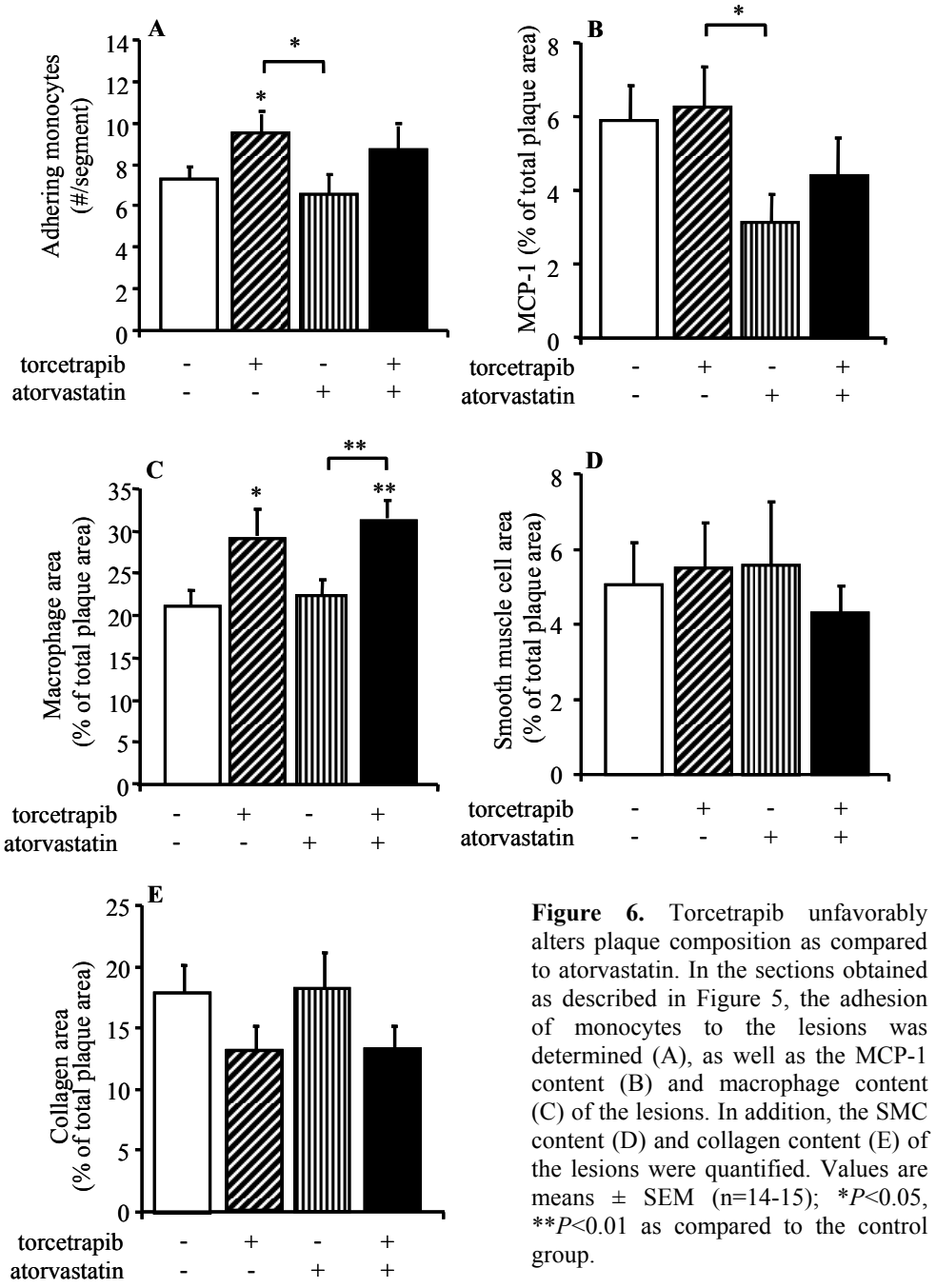


Figure 6. Torcetrapib unfavorably alters plaque composition as compared to atorvastatin. In the sections obtained as described in Figure 5, the adhesion of monocytes to the lesions was determined (A), as well as the MCP-1 content (B) and macrophage content (C) of the lesions. In addition, the SMC content (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.

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.) (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 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 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.

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Chapter 6

PXR AGONISM DECREASES PLASMA HDL LEVELS IN APOE*3-LEIDEN.CETP MICE

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Abstract

Pregnane X receptor (PXR) agonism has been shown to affect multiple steps in both the synthesis and catabolism of HDL, but its integrated effect on HDL metabolism in vivo remains unclear. The aim of this study was to evaluate the net effect of PXR agonism on HDL metabolism in APOE*3-Leiden (E3L) and E3L.CETP mice, well-established models for human-like lipoprotein metabolism. Female mice were fed a diet with increasing amounts of the potent PXR agonist 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN). In E3L and E3L.CETP mice, PCN increased liver lipids as well as plasma cholesterol and triglycerides. However, whereas PCN increased cholesterol contained in large HDL-1 particles in E3L mice, it dose-dependently decreased HDL-cholesterol in E3L.CETP mice, indicating that CETP expression dominates the effect of PCN on HDL metabolism. Analysis of the hepatic expression of genes involved in HDL metabolism showed that PCN decreased expression of genes involved in HDL synthesis (Abca1, Apoa1), maturation (Lcat, Pltp) and clearance (Sr-b1). The HDL-increasing effect of PCN, observed in E3L mice, is likely caused by a marked decrease in hepatic SR-BI protein expression, and completely reversed by CETP expression. We conclude that chronic PXR agonism dose-dependently reduces plasma HDL-cholesterol in the presence of CETP.

Introduction

Since low HDL-cholesterol is a strong and independent risk factor for cardiovascular disease,[1] pharmacological approaches aimed at raising HDL are generally seen as a novel therapeutic strategy to reduce atherosclerosis. However, the recent large phase III trials assessing the effect of the CETP inhibitor torcetrapib in combination with atorvastatin failed, despite achieving a 60% increase in HDL-cholesterol.[2-4] Torcetrapib not only failed to reduce atherosclerosis, as assessed by coronary intima-media thickness (IMT) and intravascular ultrasonography (IVUS) measurements,[2-4] but also increased the risk of cardiovascular events and death rate.[5] Although these data question the therapeutic significance of raising HDL, the adverse effects of torcetrapib may well be compound-specific and related to increased inflammation.[6] Therefore, the search for additional strategies aimed at raising HDL, e.g. via increasing the expression of apoAI, is warranted.

The pregnane X receptor (PXR) may be a novel suitable target to raise HDL. PXR agonism has been shown to increase plasma apoAI and HDL-cholesterol in wild-type mice, but not in PXR-knockout mice, suggesting that PXR agonism may be a new strategy to increase HDL by enhancing apoAI expression.[7] In addition, PXR expression in mice antagonizes the cholic acid-mediated downregulation of plasma HDL-cholesterol and apoAI.[8] PXR activation may also increase HDL formation by the intestine by increasing ABCA1 and ABCG1 expression and protein levels in intestinal cells, which results in an increased cholesterol efflux from intestinal cells to apoAI and HDL in vitro.[9] PXR activation also decreased SR-BI expression in HepG2 cells and primary rat hepatocytes in vitro,[10] which may add to a potential HDL-increasing effect in vivo.

However, some data indicate that PXR agonism may also negatively affect HDL levels. For example, PXR activation decreases the expression of ABCA1 in hepatocytes in vitro,[10] which would reduce HDL formation in an in vivo setting. PXR also increases lipogenesis in the liver leading to an increased hepatic triglyceride (TG) content and increased plasma VLDL-TG levels,[11,12] which may result in reduced HDL-cholesterol levels via CETP-mediated exchange of neutral lipids. An increased hepatic lipid content may increase CETP expression, and increased VLDL-TG will result in a higher rate of cholesteryl ester transfer from HDL to VLDL with a higher reciprocal rate of TG transfer from VLDL to HDL, resulting in a relatively TG-rich HDL that is more rapidly remodeled and cleared via hepatic lipase.[13]

In this study we aimed to examine the integrated effect of PXR agonism by the established PXR agonist PCN[14,15] on HDL metabolism in vivo. Hereto, we used the APOE*3-Leiden (E3L) mouse, a well-established model for human-like lipoprotein metabolism.[16] In addition, we used the E3L.CETP mouse[6,17-19] to

assess the specific contribution of CETP in the PXR-mediated effects on HDL metabolism.

Materials and Methods

Animals and diets
Female APOE*3-Leiden (E3L) and E3L.CETP transgenic mice that express human CETP under control of its natural flanking regions[17] were housed under standard conditions with access to water and food ad libitum. Mice were fed a diet enriched with 15% cacao butter (Diet T; AB Diet Services, Woerden, The Netherlands) for 3 weeks to increase plasma cholesterol levels from 2 mM to ~6 mM. Blood was collected after a 4 h fast from the tail vein into EDTA-containing cups, and both E3L and E3L.CETP mice were randomized according to their plasma total cholesterol, TG and HDL-cholesterol. Subsequently, mice were fed control diet (diet T) or the same diet with 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN; Sigma) at increasing doses of 0.01%, 0.03% and 0.1% (corresponding with 11, 33 and 110 mg/kg/day) for three weeks each. After each treatment period, blood was drawn after 4 h of fasting into EDTA-containing cups via tail bleeding. After the last treatment period with the highest dosage, mice were sacrificed and livers were isolated. All experiments were approved by the Institutional Committee on Animal Care and Experimentation.

Plasma lipids and lipoprotein profiles
Plasma total cholesterol and triglycerides (TG) were measured using commercially available enzymatic kits (236691 and 1488872, respectively, Roche Molecular Biochemicals, Indianapolis IN, USA) according to the manufacturer's instructions. Phospholipids were determined using an enzymatic Phospholipids kit (Spinreact, Sant Esteve de Bas, Spain). To determine the lipid distribution over plasma lipoproteins, lipoproteins were separated using 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 as described above. In E3L.CETP mice, plasma HDL-cholesterol was measured after precipitation of the apoB-containing lipoproteins from 20 µL EDTA plasma by adding 10 µL heparin (LEO Pharma, The Netherlands; 500 U/mL) and 10 µL 0.2 M $MnCl_2$. Mixtures were incubated during 20 min at room temperature and centrifuged for 15 min at 13,000 rpm at 4°C. In the supernatant HDL-C was measured.

Hepatic lipid levels
Liver samples (~50 mg) were vigorously shaken (20 sec at 4800 rpm) in ice-cold methanol (10 µL/mg tissue) using a Mini Bead Beater (BioSpec Products,

Bartlesville, USA). Tissue homogenates (45 µL~4.5 mg tissue) were diluted with ice-cold methanol (450 µL) and ice-cold chloroform (1350 µL), and further shaken (20 sec at 4800 rpm) to extract lipids from the tissue samples. Mixtures were centrifuged (15 min at 14,000 rpm; 4°C) and supernatant was transferred into a new tube, dried under nitrogen gas. Lipids were dissolved in 100 µL 2% Triton-X100. Total cholesterol, TG and phospholipid levels were assayed as described above.

Plasma CETP activity

Total (lipoprotein-independent) CETP activity was measured as the transfer of [^{3}H]cholesteryl oleate (CO) from LDL to HD,[20] exactly as described.[6] Endogenous (lipoprotein-dependent) CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (RB-CETP, Roar Biomedical, New York),[6] as described.[21]

Table 1. Primers used for rtPCR

Gene	Forward primer	Reverse primer
Abca1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Apoa1	GGAGCTGCAAGGGAGACTGT	TGCGCAGAGAGTCTACGTGTGT
CETP	CAGATCAGCCACTTGTCCAT	CAGCTGTGTGTTGATCTGGA
Cyp3A11	CTTTCCTTCACCCTGCATTCC	CTCATCCTGCAGTTTTTTCTGGAT
Cyp7a1	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Hl	CAGCCTGGGAGCGCAC	CAATCTTGTTCTTCCCGTCCA
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Lcat	GGCAAGACCGAATCTGTTGAG	ACCAGATTCTGCACCAGTGTGT
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Pltp	TCAGTCTGCGCTGGAGTCTCT	AAGGCATCACTCCGATTTGC
Sr-b1	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA

Abca1, ATP-binding cassette transporter A1; Apoa1, apolipoprotein AI; CETP, human cholesteryl ester transfer protein; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hl, hepatic lipase; Hprt, hypoxanthine-guanine phosphoribosyl transferase; Lcat, lecithin: cholesterol acyltransferase; Cyclo, cyclophilin; Pltp, phospholipid transfer protein; Sr-b1, scavenger receptor class B type I.

Hepatic mRNA expression

Total mRNA extraction from liver tissue samples was performed using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. mRNA quality was confirmed with lab-on-a-chip (Bio-Rad Laboratories, Hercules, CA, USA), and mRNA was converted to single-stranded cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). RT-PCR was performed using the IQ5 multicolor real-time PCR detection system using the SYBR Green RT-PCR mix (Bio-Rad Laboratories, Hercules,

CA, USA). mRNA levels were normalized to mRNA levels of hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers are listed in Table 1.

HDL apolipoprotein composition
Plasma was pooled per group and lipoproteins were separated using FPLC. HDL fractions (7.5 μL) were run on a 4-20% SDS-PAGE gel (Bio-Rad Laboratories, Hercules CA, USA). Gels were stained with Coomassie brilliant blue.

Hepatic ABCA1 and SR-BI protein
Immunoblot analysis of hepatic ABCA1 and SR-BI was performed as described.[22] In short, liver samples were lysed, cell debris was removed, and protein concentration was determined. Equal amounts of protein (20 μg) were separated on 7.5% SDS-PAGE gels and transferred to nitrocellulose membrane. Loading of equal amounts of cell protein was confirmed with Ponceau S staining of the resulting blots. Immunolabeling was performed using murine monoclonal αABCA1 (AC-10) or rabbit polyclonal αSRBI (anti-BI[495]) as primary antibody and goat-anti-mouse IgG and goat-anti-rabbit IgG, respectively, as secondary antibodies. Immunolabeling was detected by enhanced chemiluminescence.

Statistical analysis
Data are presented as means ± SD. Statistical differences were assessed using the Student T Test (hepatic mRNA expression) or the Mann Whitney U test (all other analyses). SPSS 14.0 was used for statistical analysis and $p<0.05$ was regarded as statistically significant.

Results

PXR agonism affects plasma lipid levels
E3L and E3L.CETP mice were fed a control diet or a diet with increasing doses of the PXR agonist PCN (0, 0.01, 0.03 and 0.1%), and plasma TG and cholesterol were determined (Fig. 1). PCN dose-dependently increased plasma TG in both E3L mice (up to + 218%; $p<0.01$) (Fig. 1A) and E3L.CETP mice (up to + 185%; $p<0.05$) (Fig. 1B), indicating that the effect of PCN on plasma TG is independent of CETP expression. However, whereas PCN significantly increased plasma cholesterol in E3L mice (up to +19%; $p<0.01$) (Fig. 1C), PCN only tended to increase plasma cholesterol in E3L.CETP mice (Fig. 1D).

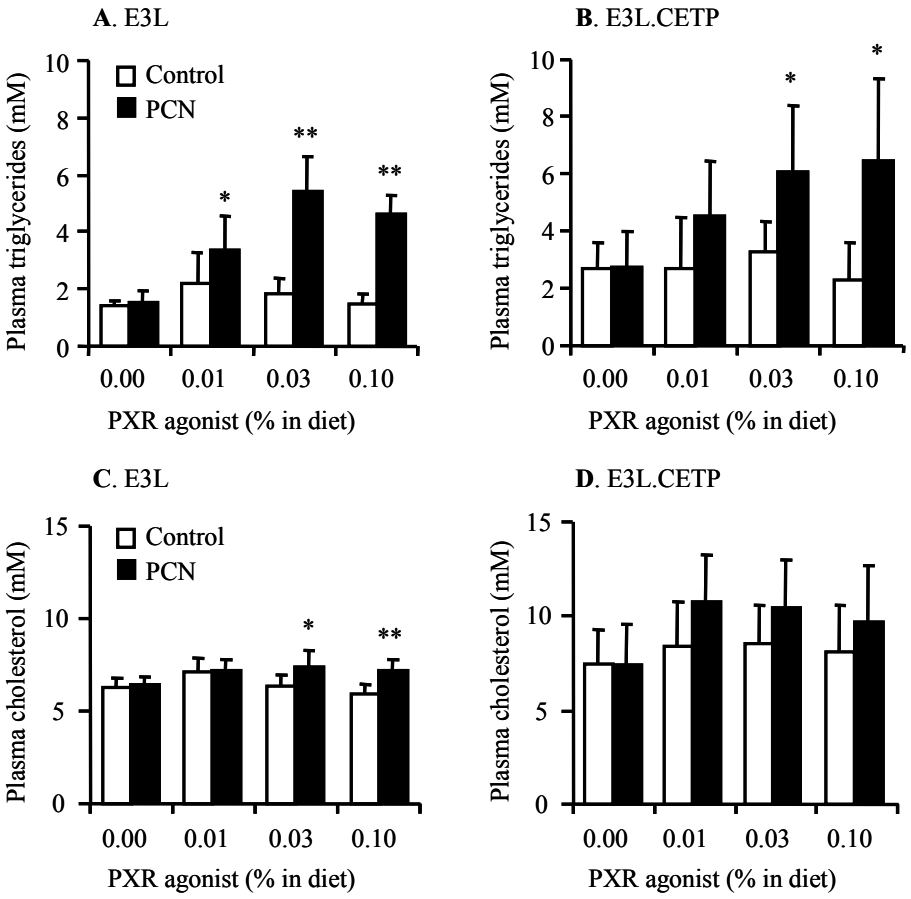

Figure 1. PXR agonism dose-dependently increases plasma cholesterol and triglycerides. APOE*3-Leiden (E3L) mice (A, C) and E3L.CETP mice (B, D) were fed a control diet (time-matched control group) or a diet with increasing doses of 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) (0, 0.01, 0.03, and 0.10%) for three weeks each. Before treatment and at the end of the 3 week periods, blood was drawn from both PCN-treated and time-matched control mice and plasma was assayed for triglycerides (A, B) and cholesterol (C, D). Values are means ± SD (n= 6-7 per group); * $p<0.05$, ** $p<0.01$ versus control group.

PXR agonism increases hepatic lipid levels

Since the effects of the PXR agonist on plasma lipids may be caused by an altered hepatic lipid homeostasis, the effect of PCN on hepatic lipid composition was determined (Fig. 2). In E3L mice, PCN increased the levels of TG (+342%; $p<0.01$), total cholesterol (+159%; $p<0.01$) and phospholipids (+100%; $p<0.01$) (Fig. 2A). Similar effects of PCN were observed in E3L.CETP mice (Fig. 2B), indicating that the effect of PCN on hepatic lipid levels is also independent from CETP expression.

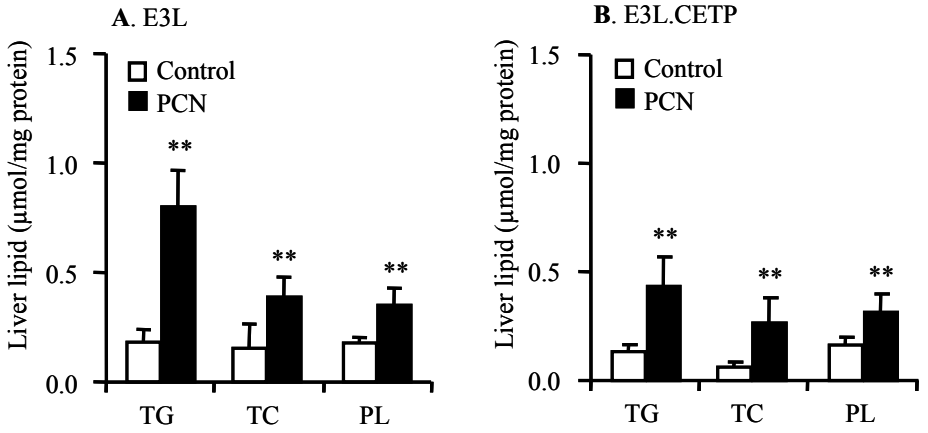

Figure 2. PXR agonism increases hepatic lipid levels. E3L mice (A) and E3L.CETP mice (B) were fed a control diet (time-matched control group) or a diet with increasing doses of PCN for three weeks each. After the last treatment period (0.10% PCN and time-matched control), mice were sacrificed and livers were isolated. Liver were homogenized, lipids were extracted, and triglycerides (TG), total cholesterol (TC) and phospholipids (PL) were quantified. Values are means ± SD (n= 6-7 per group); ** $p<0.01$ versus control group.

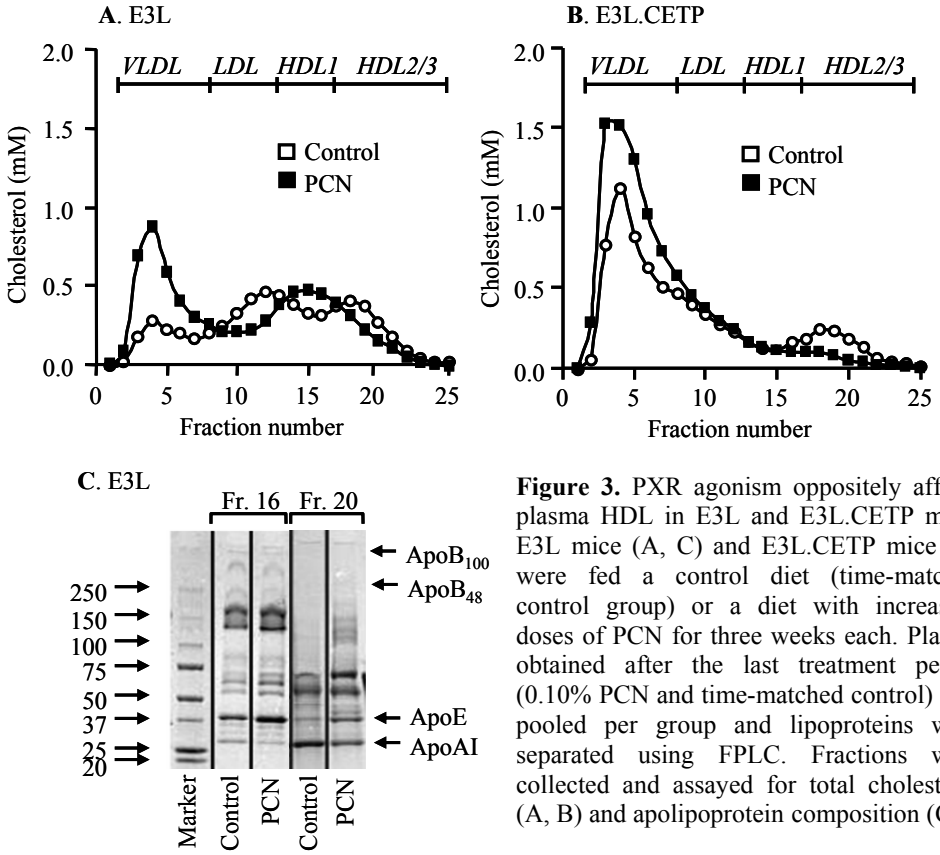

Figure 3. PXR agonism oppositely affects plasma HDL in E3L and E3L.CETP mice. E3L mice (A, C) and E3L.CETP mice (B) were fed a control diet (time-matched control group) or a diet with increasing doses of PCN for three weeks each. Plasma obtained after the last treatment period (0.10% PCN and time-matched control) was pooled per group and lipoproteins were separated using FPLC. Fractions were collected and assayed for total cholesterol (A, B) and apolipoprotein composition (C).

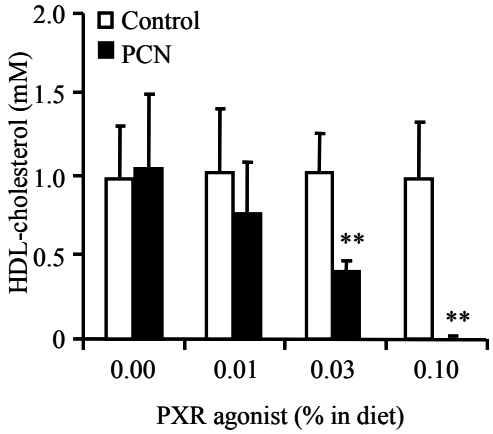

Figure 4. PXR agonism dose-dependently decreases plasma HDL in E3L.CETP mice. E3L.CETP mice were fed a control diet (time-matched control group) or a diet with increasing doses of PCN for 3 weeks each. After the last treatment period (0.10% PCN and time-matched control), blood was drawn and plasma was assayed for HDL-cholesterol after precipitation of apoB-containing lipoproteins. Values are means ± SD (n= 6-7 per group); ** $p<0.01$ versus control group.

PXR agonism decreases plasma HDL levels in presence of CETP

We next investigated the effect of PCN on the cholesterol distribution over lipoproteins after separation by FPLC (Fig. 3). In both E3L and E3L.CETP mice, PCN increased the amount of cholesterol in VLDL. HDL-cholesterol contained in large HDL particles increased in E3L mice (Fig. 3A). On the other hand, HDL-cholesterol of all sizes was markedly decreased in E3L.CETP mice upon PCN treatment (Fig. 3B). Analysis of the apolipoprotein composition of FPLC fractions 16 and 20 of plasma from E3L mice, showed that PCN induces the appearance of large apoE-rich HDL-1 as apparent from a high ratio of apoE to apoAI (fraction 16), and that PCN treatment reduced the amount of apoAI in HDL of regular size (fraction 20) (Fig. 3C). Analysis of HDL-cholesterol in plasma after precipitation of apoB-containing lipoproteins showed that the HDL-decreasing effect of PCN in E3L.CETP mice was dose-dependent (Fig. 4).

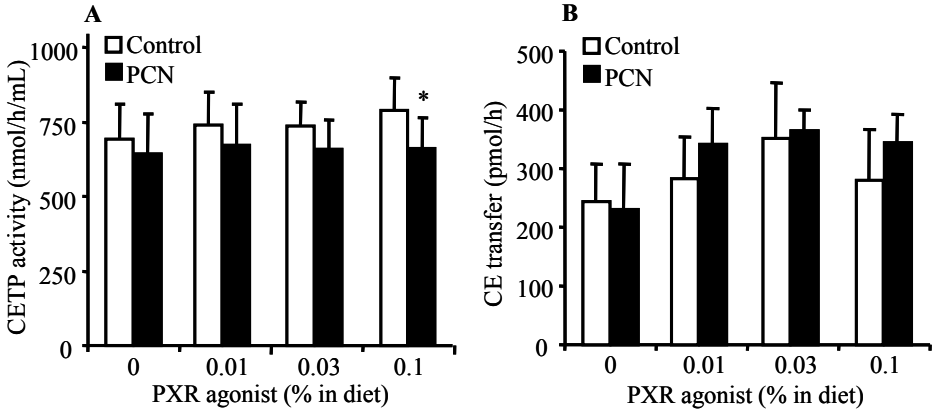

Figure 5. PXR agonism does not affect plasma CETP activity in E3L.CETP mice. E3L.CETP mice were fed a control diet (time-matched control group) or a diet with increasing doses of PCN for three weeks each. After the last treatment period (0.10% PCN and time-matched control), blood was drawn and plasma was assayed for total CETP activity (A) and endogenous CETP activity (B). Values are means ± SD (n= 6-7 per group); * $p<0.05$, ** $p<0.01$ versus control group.

PXR agonism does not affect plasma CETP activity

We have previously observed that a decrease in the hepatic cholesterol content of E3L.CETP mice e.g. by treatment with fenofibrate [18] or atorvastatin [19] decreases both the hepatic expression of CETP and the activity of CETP in plasma. Since PXR agonism strongly increases hepatic cholesterol content, we questioned whether the reduction in plasma HDL in E3L.CETP mice may be related to increased plasma CETP activity. Therefore, the effect of PCN was determined on hepatic CETP expression as well as on total and endogenous CETP activity in plasma of E3L.CETP mice (Fig. 5). Albeit that a small effect was observed on total plasma CETP activity at the highest dose, PCN in general did not affect either the total CETP activity (Fig. 5A) or endogenous (Fig. 5B) CETP activity, which is in line with an unaltered hepatic gene expression (Table 1). The reduction of HDL in E3L.CETP mice can thus not be explained by increased CETP activity.

Table 2. PXR agonism affects hepatic gene expression.

A. PXR targets

	E3L control	PCN	E3L.CETP control	PCN
Cyp3a11	1.00 ± 0.35	12.74 ± 5.05***	1.00 ± 0.38	13.37 ± 2.76***
Cyp7a1	1.00 ± 0.53	0.32 ± 0.15**	1.00 ± 0.41	0.59 ± 0.34

B. HDL metabolism

	E3L Control	PCN	E3L.CETP control	PCN
Abca1	1.00 ± 0.24	0.76 ± 0.23	1.00 ± 0.16	0.81 ± 0.31
Apoa1	1.00 ± 0.33	0.60 ± 0.20*	1.00 ± 0.38	0.50 ± 0.10*
Hl	1.00 ± 0.17	0.67 ± 0.16**	1.00 ± 0.28	0.73 ± 0.17
Lcat	1.00 ± 0.48	0.80 ± 0.18	1.00 ± 0.23	0.68 ± 0.13*
Pltp	1.00 ± 0.22	0.45 ± 0.12***	1.00 ± 0.29	0.54 ± 0.18**
Sr-b1	1.00 ± 0.16	0.45 ± 0.09***	1.00 ± 0.28	0.61 ± 0.16*
CETP	n.d.	n.d.	1.00 ± 0.53	1.77 ± 0.80

E3L and E3L.CETP mice were fed a control diet or a diet with increasing doses of PCN. After the last treatment period (0.10% PCN or time-matched control), mice were sacrificed and livers were isolated. mRNA was isolated and mRNA expression of the indicated genes was quantified by RT-PCR. Genes are grouped as established PXR targets (A) and genes involved in HDL metabolism (B). Data are calculated as fold difference as compared to the control group. Values are means ± SD (n= 6-7 per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control group. N.d., not detected.

PXR agonism affects hepatic expression of genes involved in HDL metabolism

To get further insight into the mechanism(s) underlying the effects of PCN on HDL metabolism, we evaluated the hepatic expression of genes involved in HDL metabolism (Table 2).

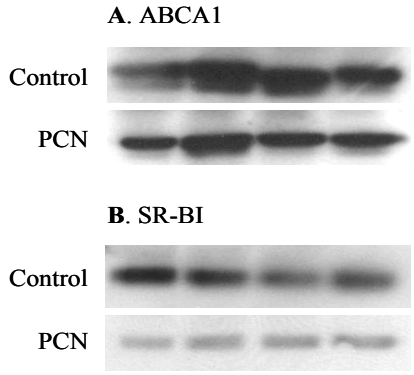

Figure 6. PXR agonism reduces hepatic SR-BI protein. E3L.CETP mice were fed a control diet (time-matched control group) or a diet with increasing doses of PCN for three weeks each. After the last treatment period (0.10% PCN and time-matched control), mice were sacrificed and livers were isolated. Livers were homogenized and equal amounts of hepatic proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. ABCA1 (A) and SR-BI (B) were visualized by immunolabeling. Results of 4 individual mice per group are shown.

As a control for PXR agonism, we determined the effects of PCN on the expression of Cyp3a11, which is an important target gene of PXR,[23,24] and Cyp7a1, which is negatively regulated by PXR.[25] PCN strongly upregulated Cyp3a11 (13-14-fold) and down-regulated Cyp7a1 (-60 -70%) in E3L mice and E3L.CETP mice, which confirms that PCN is a potent PXR agonist in these mouse models.
PCN had similar effects on the hepatic expression of the various genes involved in HDL metabolism in E3L and E3L.CETP mice. PCN decreased proteins involved in HDL assembly including Abca1 (~20%, n.s.) and Apoa1 (~40-50%; $p<0.05$). In addition, PCN decreased the expression of genes involved in HDL maturation such as Lcat (~20-30%), Hl (~30%) and Pltp (~50%), as well as the gene involved in hepatic clearance of HDL-cholesterol, Sr-b1 (~40-50%).

PXR agonism decreases hepatic SR-BI protein levels
Since a decrease in SR-BI can explain the increase in HDL-cholesterol contained in large HDL-1 particles in E3L mice, we determined whether the relatively large effect of PCN on the hepatic expression of Sr-b1 was reflected by reduced hepatic protein levels. Western blot analysis of hepatic homogenates of E3L.CETP mice indicated that PCN did not substantially reduce hepatic ABCA1 protein (-21%, n.s.), which is in line with Abca1 expression analysis, but substantially reduced SR-BI protein (-77%, $p<0.05$) (Fig. 6). Similar results were obtained for E3L mice (data not shown).

Discussion

Studies on the effect of PXR activation on HDL metabolism have generated conflicting data with respect to their net effect on HDL levels. However, these data have been derived either from in vitro studies of from in vivo studies in wild-type mice that naturally have very low (V)LDL and high HDL levels, and do not express CETP. Therefore, we have evaluated the effect of PXR agonism

on HDL metabolism in E3L mice, which have a more favorable ratio of (V)LDL to HDL and is a well-established model for human-like lipoprotein metabolism, as well as E3L.CETP mice.

We have demonstrated that E3L and E3L.CETP mice respond well to PXR agonism. PCN not only considerably increased hepatic Cyp3a11 expression and reduced Cyp7a1 expression, but also induced fatty livers as judged from an increased liver weight, increased levels of hepatic TG, total cholesterol and phospholipid, as well as formation of lipid droplets (not shown). Hepatic steatosis appears to be a common effect of PXR agonism, since 1) expression of activated PXR in the livers of transgenic mice increases hepatic TG levels,[12] 2) PXR agonism in mice expressing the human PXR gene increases hepatic TG levels,[12] and 3) PCN increases hepatic TG levels in wild-type mice, but not in PXR knockout mice.[11]

We showed that PCN markedly increased plasma TG accompanied by a modest increase in plasma cholesterol, as reflected by increased (V)LDL levels. This is most probably a consequence of the increased hepatic lipid levels, which may result in an increased substrate-driven hepatic VLDL production.

The effect of PXR agonism on plasma HDL levels appeared more complex. In E3L mice, PCN increased cholesterol contained in large HDL-1. Accumulation of apoE-rich large HDL-1 is a common characteristic of SR-BI deficient mice, since SR-BI appears solely responsible for the selective clearance of HDL-cholesteryl esters in mice.[26] Indeed, we observed that PCN largely decreased the hepatic expression of Sr-b1 as well as hepatic SR-BI protein in both E3L and E3L.CETP mice. This strongly suggests that the decrease in hepatic SR-BI may be a causal factor for the increase in large HDL. Based on these data we speculate that a decrease in hepatic SR-BI may also contribute to the increase in HDL-cholesterol and apoAI in wild-type mice as previously observed by Bachman et al.[7] In contrast, PXR agonism by PCN failed to increase cholesterol within large HDL-1 in E3L.CETP mice. This can be explained by the fact that large HDL-1 is a preferred substrate for CETP, since CETP expression in SR-BI-deficient mice normalized both the particle size and plasma levels of HDL.[27]

PXR agonism not only failed to increase the HDL particle size in E3L.CETP mice as compared to E3L mice, but even dose-dependently decreased the HDL-cholesterol level. We have previously shown that fenofibrate[18] and atorvastatin[19] increase HDL-cholesterol levels by decreasing hepatic CETP expression related to lower liver lipid levels, suggesting that the PXR-induced increased liver lipid levels may conversely reduce HDL levels by increased CETP expression. However, PCN did not affect hepatic CETP expression or total CETP activity in plasma in E3L.CETP mice. One could argue that the PCN-induced increase in VLDL-TG levels may result in a substrate-driven increase in CETP activity, resulting in a relatively TG-rich HDL that would be more rapidly remodeled and cleared via hepatic lipase.[13] However, such a mechanism is less plausible

since we were also unable to detect an increase in the endogenous (lipoprotein-dependent) CETP activity.
Albeit that CETP expression per se seems to be the main contributor to the PXR-induced decrease in HDL-cholesterol in E3L.CETP mice, other players involved in HDL metabolism may contribute as well. Previous studies showed that PXR agonism decreased ABCA1 expression in hepatocytes,[10] but we only observed a tendency towards reduced hepatic Abca1 mRNA (~20%) and protein (~20%). However, PCN markedly reduced hepatic Apoa1 mRNA (~40-50%). Given the fact that both apoAI and ABCA1 are important for the generation of discoidal HDL precursors (i.e. apoAI) and their subsequent lipidation (i.e. ABCA1), genetic deficiency for either apoAI[28] or ABCA1[29] dramatically decreases HDL levels. Therefore, a potential modest reduction of ABCA1 accompanied by the large reduction in apoAI may well have synergistically contributed to the dose-dependent marked decrease in HDL in our in vivo study in mice, but only in the mice that express CETP. In addition, PCN decreased the expression of genes involved in HDL maturation such as Hl (~30%), Lcat (~20-30%) and Pltp (~50%). Although HL-deficiency mildly increases HDL,[30] LCAT-deficiency[31] and PLTP-deficiency[32] both reduce plasma HDL levels. Therefore, the effects of PXR agonism on the hepatic expression of Lcat and Pltp, but not Hl, may also have contributed to some extent to the observed reduction in HDL.
Together, our data show that PXR agonism increases cholesterol contained in large HDL-1 particles in E3L mice, as related to decreased hepatic SR-BI levels, and decreases HDL-cholesterol in E3L.CETP mice primarily resulting from CETP expression per se. Since the E3L.CETP mouse has proven a valuable model to predict drug-induced responses in humans with respect to HDL metabolism[6,18,19] we anticipate that PXR agonism is not a valid strategy to raise HDL.

Acknowledgements

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Chapter 7

APOLIPOPROTEIN CI INHIBITS SCAVENGER RECEPTOR BI AND INCREASES PLASMA HDL LEVELS *IN VIVO*

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Abstract

Apolipoprotein CI (apoCI) has been suggested to influence HDL metabolism by activation of LCAT and inhibition of HL and CETP. However, the effect of apoCI on scavenger receptor BI (SR-BI)-mediated uptake of HDL-cholesteryl esters (CE), as well as the net effect of apoCI on HDL metabolism *in vivo* is unknown. Therefore, we evaluated the effect of apoCI on the SR-BI-mediated uptake of HDL-CE *in vitro* and determined the net effect of apoCI on HDL metabolism in mice. Enrichment of HDL with apoCI dose-dependently decreased the SR-BI-dependent association of [^{3}H]CE-labeled HDL with primary murine hepatocytes, similar to the established SR-BI-inhibitors apoCIII and oxLDL. ApoCI-deficiency in mice gene dose-dependently decreased HDL-cholesterol levels. Adenovirus-mediated expression of human apoCI in mice increased HDL levels at a low dose and increased the HDL particle size at higher doses. We conclude that apoCI is a novel inhibitor of SR-BI *in vitro* and increases HDL levels *in vivo*.

Introduction

Apolipoprotein CI (ApoCI) is a 6.6 kDa protein that is mainly synthesized by the liver, and also by other tissues such as lung, spleen, intestine, brain and adipose tissue. After secretion by the liver, apoCI associates with chylomicrons, VLDL and HDL and is exchangeable between these lipoproteins.[1] ApoCI is highly positively charged, and is present in a relatively high plasma concentration of about 10 mg/dL.[1]

Several functions of apoCI in lipoprotein metabolism have been described. ApoCI affects the metabolism of apoB-containing lipoproteins. Using apoCI-deficient and apoCI-overexpressing mice, it has been shown that apoCI attenuates the clearance of VLDL by inhibition of the lipolytic conversion of VLDL by lipoprotein lipase (LPL), either directly[2] or indirectly via product inhibition due to its fatty acid-binding properties.[3] In addition, apoCI decreases the clearance of VLDL by inhibition of the binding and uptake of VLDL by the classical apoE-recognizing receptors, including the LDL receptor (LDLr)[4] and LDLr-related protein (LRP)[5] on the liver, as well as the VLDL receptor (VLDLr)[6] that is mainly present on peripheral tissues. ApoCI has also been shown to increase the production of VLDL.[7] By these combined actions, apoCI thus increases the plasma levels of VLDL-associated triglyceride (TG) and cholesterol (C) in mice.[1,2]

ApoCI has also been suggested to be involved in HDL metabolism, although such a role has only been derived from *in vitro* observations. ApoCI is involved in HDL remodeling by activation of lecithin: cholesterol acyltransferase (LCAT) that esterifies cholesterol in HDL and, therefore, increases HDL-C levels and HDL particle size,[8,9] and by inhibition of hepatic lipase (HL) that lipolyzes TG and phospholipids (PL) in HDL.[10,11] However, the *in vivo* relevance of these actions of apoCI is unknown, but it is conceivable that apoCI increases the plasma levels and/or the particle size of HDL. ApoCI has also been identified as an inhibitor of the activity of cholesteryl ester (CE) transfer protein (CETP),[12] which may add to a potential HDL-raising effect of apoCI, at least in CETP-expressing species.

ApoCI may also affect the uptake of HDL-CE via SR-BI, as two homologues of apoCI (*i.e.* apoCII and apoCIII) have recently been demonstrated to inhibit SR-BI.[13] Although such an effect of apoCI has not been reported before, SR-BI inhibition would add to a potential HDL-raising effect of apoCI *in vivo*, since SR-BI is solely responsible for the selective hepatic uptake of HDL-CE in mice.[14]

Therefore, in the present study we evaluated whether apoCI would represent a novel modulator of SR-BI by evaluating the effect of apoCI on the uptake of HDL-CE by freshly isolated mouse hepatocytes. In addition, we examined the *in vivo* relevance of the combined effects of apoCI on HDL metabolism using

apoCI-deficient mice and mice that overexpress apoCI using adenoviral expression.

Methods

Mice
Heterozygous apoCI knockout (apoc1$^{-/-}$) mice[15] were crossbred to obtain wild-type (WT), apoc1$^{+/-}$ and apoc1$^{-/-}$ littermates (C57Bl/6 background). Overexpression of apoCI was achieved via injection of a recombinant adenovirus that expresses human apoCI as described.[16] MX1Cre:LRP$^{lox/lox}$.LDLr$^{-/-}$.VLDLr$^{-/-}$ mice (C57Bl/6 background), that are deficient for the LDLr, VLDLr and hepatic LRP after three intraperitoneal injections of polyinosinic: polycytidylic ribonucleic acid (pI:pC), have been generated as described previously.[17] Mice had access to regular chow and water *ad libitum*. When indicated, blood was drawn via the tail vein into paraoxon-coated capillaries after 4 h fasting at 13.00 h and plasma was collected after centrifugation.

Radiolabeling of HDL
HDL was isolated from human plasma by density gradient ultracentrifugation and labeled with [^{3}H]cholesteryl oleoyl ether (COEth) as described previously.[18]

In vitro hepatocyte studies
Hepatocytes were isolated from anesthetized WT and LRP^{-}.LDLr$^{-/-}$.VLDLr$^{-/-}$ mice by perfusion of the liver with collagenase.[19] Freshly isolated cells (1x10^6/mL) were incubated (3h at 37°C) in DMEM + 2% BSA with [^{3}H]COEth-labeled HDL (20 μg protein/mL) in the absence or presence of apoCI (Protein Chemistry Technology Center, Dallas TX, USA), apoCIII (Biodesign International, Saco, ME, USA), or oxidized (oxLDL) (100 μg protein/mL) under gentle shaking. After incubation, cells were pelleted by centrifugation and unbound label was removed by repeated washing with Tris-buffered saline. The pellet was lysed in 0.1 M NaOH and cell-associated radioactivity and protein content were measured. [^{3}H]COEth association was calculated as dpm/mg cell protein.

Biochemical analysis
Plasma cholesterol levels were measured with a commercially available enzymatic kit (236691, Roche Molecular Biochemicals, Indianapolis IN, USA). Plasma apoCI was measured by ELISA[2] and apoAI was measured by western blotting.[20] Lipoproteins were fractionated using fast performance liquid chromatography (FPLC). Hereto, 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 as described above.

Statistical analysis
Data were analyzed using the Mann-Whitney nonparametric test. Analyses were performed with SPSS 14.0 (SPSS inc, Chicago, USA).

Results

ApoCI inhibits the association of [^{3}H]COEth-labeled HDL with hepatocytes
To examine whether apoCI affects the SR-BI-mediated uptake of HDL-CE, we evaluated the effect of apoCI on the association [^{3}H]COEth-labeled HDL with primary hepatocytes from WT mice. Enrichment of HDL (20 µg protein/mL)

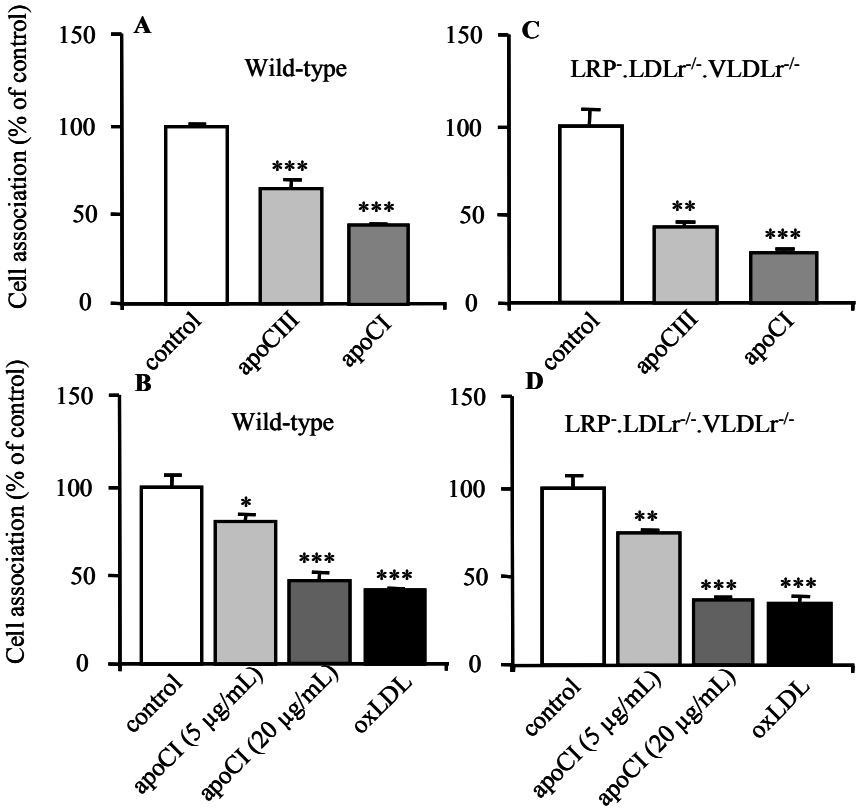

Figure 1. ApoCI dose dependently inhibits SR-BI *in vitro.* Primary hepatocytes were isolated from WT mice (A, B) and LRP$^-$.LDLr$^{-/-}$.VLDLr$^{-/-}$ mice (C, D) and incubated (3 h at 37°C) with [^{3}H]COEth-HDL (20 µg/mL) enriched with apoCIII (20 µg/mL) or apoCI (20 µg/mL) (A, C). In a separate experiment, cells were incubated with apoCI (5 and 20 µg/mL) or oxLDL (100 µg/mL) (B, D). Cell-associated radioactivity was expressed as dpm/mg cell protein. Values represent means ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001.

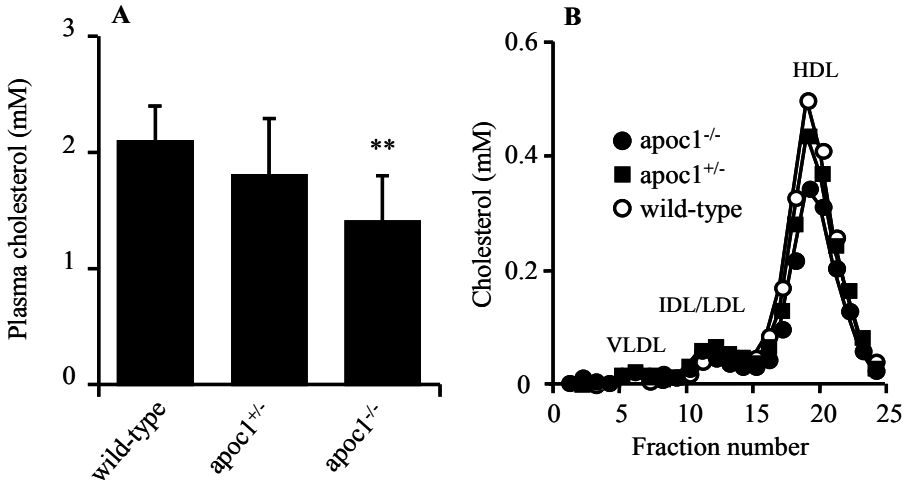

Figure 2. ApoCI deficiency in mice decreases HDL *in vivo.* Blood was drawn from WT, apoc1$^{+/-}$ and apoc1$^{-/-}$ littermate mice after 4 h fasting, and plasma was assayed for total cholesterol (A). Values represent means ± SD (n=12). **P<0.01. Plasma was pooled per group (n=12), lipoproteins were fractionated by FPLC and cholesterol in fractions was measured (B).

with the established SR-BI inhibitor apoCIII (20 µg/mL) inhibited HDL-CE association with WT hepatocytes (-37%; P<0.001), which is in line with the findings of Huard *et al.*[13] ApoCI, at the same concentration, inhibited HDL-CE association even more effic iently than apoCIII (-57%; P<0.001) (Fig. 1A). In a second experiment, we showed that the inhibition of HDL-CE association by apoCI is dose-dependent (-19% at 5 µg/mL; P<0.05 and -53% at 20 µg/mL; P<0.001), and that the highest apoCI concentration was equally effective as the established SR-BI inhibitor oxLDL (-57% at 100 µg/mL; P<0.001) (Fig. 1B). As apoCI is also an inhibitor of the classical apoE recognizing receptors (*i.e.* LRP, LDLr and VLDLr), we repeated this experiment with hepatocytes from LRP, LDLr and VLDLr triple-knockout mice. ApoCI affected the association of HDL-CE with LRP^{-}.LDLr$^{-/-}$.VLDLr$^{-/-}$ hepatocytes (Fig. 1C and D) similarly as with WT cells (Fig. 1A and B). This shows that the inhibitory effect of apoCI on HDL-CE association with hepatocytes is independent of the apoE-recognizing receptors, and confirms that HDL-CE association with hepatocytes is strictly dependent on SR-BI.[14]

ApoCI deficiency decreases plasma HDL levels in mice

Previous *in vitro* studies have demonstrated that apoCI activates LCAT[8,9] and inhibits HL [10,11] and we thus now show that apoCI additionally inhibits SR-BI. To evaluate the consequences of these combined effects for HDL-C metabolism *in vivo*, we first examined the effect of genetic apoCI-deficiency in mice on HDL-C levels. Hereto, blood was drawn from 4 hours fasted WT, apoc1$^{+/-}$ and apoc1$^{-/-}$ littermates. ApoCI-deficiency resulted in a gene dose-dependent

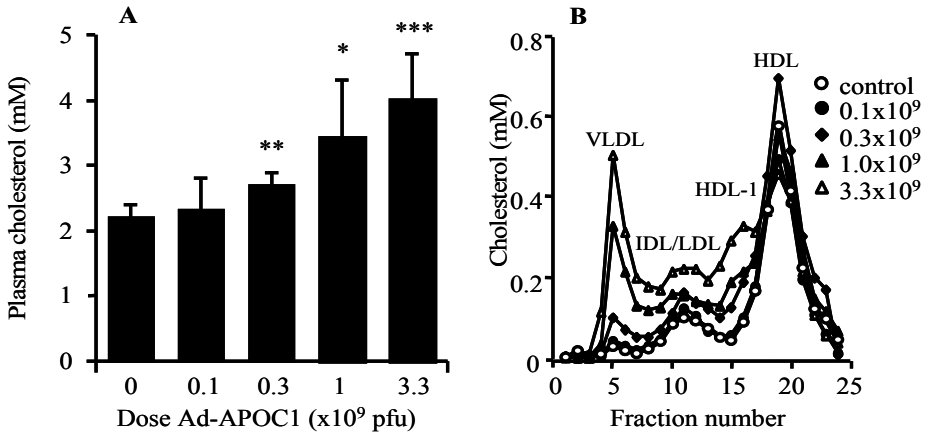

Figure 3. ApoCI overexpression in mice increases HDL *in vivo*. WT mice were injected with a recombinant adenovirus expressing human apoCI to dose-dependently increase apoCI plasma levels. After 5 days, blood was drawn after a 4 h fast, and plasma total cholesterol was measured (A). Values represent means ± SD (n=5). *P<0.05, **P<0.01, ***P<0.001. Plasma was pooled per group (n=5), lipoproteins were fractionated by FPLC and cholesterol in fractions was measured (B).

decrease in plasma cholesterol up to -32% (P<0.01) upon homozygous apoCI deficiency (Fig. 2A). Separation of the various lipoprotein fractions from plasma by FPLC showed that the decrease in plasma cholesterol was mainly confined to the HDL fraction. A gene dose-dependent decrease in HDL-C was observed up to -29% in apoc1$^{-/-}$ mice (Fig. 2B). In contrast, apoCI deficiency did not affect plasma apoAI levels (not shown).

ApoCI overexpression increases plasma HDL levels and enlarges HDL

To examine the effect of apoCI overexpression on HDL-C levels and HDL size, WT mice were injected with a recombinant adenovirus expressing human apoCI (0.1, 0.3, 1, and 3.3 x10^9 pfu/mouse).[16] This resulted in a virus dose-dependent increase in plasma levels of apoCI (0, 12, 23 and 33 mg/dL) and cholesterol (up to +48%; P<0.001) (Fig. 3A). Fractionation of lipoproteins by FPLC indicated that overexpression of apoCI led to a dose-dependent increase in VLDL, which is explained by the well-known attenuation of the catabolism of apoB-containing lipoproteins. Interestingly, overexpression of a low dose of apoCI led to an increase of the normal sized HDL (+15%), whereas higher doses rather led to an increase of HDL particle size as shown by the appearance of HDL-1 (Fig. 3B).

Discussion

The aim of our study was to determine the effect of apoCI on HDL metabolism, by addressing the effect of apoCI on the hepatic HDL receptor SR-BI *in vitro*

and by evaluating the net effect of apoCI on plasma HDL *in vivo*. We showed that apoCI inhibits the SR-BI-mediated association of HDL-CE with primary murine hepatocytes. Furthermore, we showed in mice that apoCI-deficiency reduces HDL levels and, conversely, that modest and high apoCI overexpression increases and enlarges HDL, respectively.

Previous *in vitro* studies have suggested that apoCI may be involved in HDL metabolism by stimulation of LCAT,[8,9] inhibition of HL[10,11] and inhibition of CETP,[12] which are all involved in the remodeling of HDL in the circulation. Based on the structural homology of apoCI with apoCII and apoCIII, which have been shown to inhibit the of SR-BI-mediated selective uptake of HDL-CE by HepG2 cells,[13] we postulated that apoCI may also inhibit SR-BI. Indeed, apoCI appeared even more effective in inhibiting the SR-BI-mediated uptake of HDL-CE by primary mouse hepatocytes than apoCIII.

It is interesting to speculate how apoCI affects SR-BI function. As compared to apoCII and apoCIII, apoCI is unusually rich in positively-charged lysine residues, which is important for both its lipopolysaccharide-binding[21] and CETP-inhibiting[22] properties. The fact that apoCI, apoCII and apoCIII all inhibit SR-BI suggests that the high positive charge of apoCI is not essential for this effect. The mechanism by which apoCII and apoCIII inhibit SR-BI, has not yet been resolved.[13] SR-BI binds HDL via multiple binding sites[23] and subsequently mediates selective CE uptake from this particle.[24] Therefore, apoCI, apoCII and apoCIII may interfere with this process by 1) modifying HDL particles in a way that binding of SR-BI to HDL is reduced, 2) stabilizing HDL particles in a way that CE can not easily be removed, and/or 3) interacting directly with SR-BI, thereby preventing binding to HDL.

Interestingly, the effects of apoCI on the various HDL-modulating proteins, including activation of LCAT, inhibition of HL, and inhibition of SR-BI, should theoretically all lead to an increase in HDL levels and/or particle size. First, overexpression of LCAT, an HDL-associated plasma enzyme that is responsible for cholesterol esterification in HDL,[25-27] increases both HDL-C and HDL size.[28] Second, homozygous deficiency for HL, a plasma enzyme that degrades TG and PL within HDL,[29] does not have a large impact on HDL-C, but does result in accumulation of large HDL-1 particles.[30] Third, heterozygous SR-BI deficiency primarily results in an increase in HDL-C, whereas homozygous SR-BI deficiency leads to accumulation of large HDL-1.[31] Therefore, we postulated that apoCI expression would positively correlate with HDL-C levels and HDL size. Indeed, we showed that apoCI deficiency reduced HDL-C. Conversely, overexpression of increasing amounts of apoCI increased HDL-C at moderate expression and gradually increased the formation of large HDL-1 at higher apoCI expression levels. These effects are thus all consistent with the expected effects of apoCI on LCAT, HL and SR-BI, albeit that it is not feasible to quantify the relative contribution of the individual pathways.

Regarding the current interest in raising HDL as a novel strategy to reduce cardiovascular risk, apoCI may be an exciting new lead. It is interesting to note that, in addition to its effect on LCAT, HL and SR-BI, apoCI is also the main endogenous protein inhibitor of CETP.[12] CETP is absent in mice but present in plasma of humans and may therefore also add to the HDL-raising effect of apoCI in humans. Although a causal effect of apoCI on determining HDL levels in humans would be difficult to study, more than 90% of plasma apoCI appears to be associated with HDL in normolipidemic subjects[32] and we recently demonstrated a positive correlation between plasma apoCI and HDL.[33] Of course, since levels of HDL do not always reflect the atheroprotective properties of HDL (*i.e.* its role in reverse cholesterol transport, its anti-inflammatory and antioxidative properties), studies on the effect of apoCI on HDL functionality are still warranted.

A drawback of apoCI as a lead in HDL-raising therapy would be that apoCI not only increases HDL levels, but also increases VLDL levels mainly by inhibition of LPL activity.[2] This appeared the predominant reason why apoCI overexpression on a hyperlipidemic apoE-deficient background aggravated atherosclerosis,[11] whereas apoCI-deficiency attenuated atherosclerosis.[34] Furthermore, SR-BI has recently been described to facilitate the hepatic uptake of VLDL and chylomicrons.[35,36] These effects may thus add to some extent to the VLDL-raising effect of apoCI. Therefore, it would be interesting to perform *in vitro* structure-and-function studies to determine the minimal domain of apoCI that targets subsets of HDL-modulating proteins, including preferentially LCAT, HL and CETP, without adversely affecting VLDL metabolism via LPL and potentially SR-BI.

In conclusion, we have demonstrated that apoCI is a novel inhibitor of the SR-BI-mediated uptake of HDL-CE by hepatocytes, and that apoCI is a determinant for the plasma levels and size of HDL *in vivo*.

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Chapter 8

IDENTIFICATION OF A C-TERMINAL DOMAIN IN APOLIPOPROTEIN CI THAT INHIBITS CETP ACTIVITY BUT NOT LPL ACTIVITY, BY STRUCTURE-FUNCTION ANALYSIS

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Abstract

Apolipoprotein CI (apoCI) is a highly positively charged plasma apolipoprotein of 57 amino acids and has a dual role in plasma lipoprotein metabolism and atherosclerosis. On one hand, apoCI inhibits the cholesteryl ester transfer protein (CETP), which increases the plasma level of anti-atherogenic high density lipoproteins (HDL). On the other hand, apoCI inhibits lipoprotein lipase (LPL), which increases proatherogenic (very) low density lipoproteins ((V)LDL). In this study, we show that the CETP-inhibitory function of apoCI resides in the C-terminal domain, whereas the C terminus of apoCI is not sufficient for the LPL-inhibitory function of apoCI. The C-terminal peptide $apoCI_{32-57}$ potently inhibited CETP activity, mainly caused by positively charged amino acids, with a negligible effect on LPL activity. Therefore, $apoCI_{32-57}$ may be an interesting lead in the search for novel CETP inhibitors as a new strategy to increase HDL thereby reducing cardiovascular risk.

Introduction

Apolipoprotein CI (apoCI) is the smallest known apolipoprotein. ApoCI consists of only 57 amino acids, arranged in two amphipathic helices connected by a flexible hinge region, and is unusually rich in positively charged lysine (K) and arginine (R) residues (21 mol%). ApoCI is mainly produced by hepatocytes and secreted into plasma, where it associates with predominantly anti-atherogenic high density lipoproteins (HDL), but also with atherogenic very low density lipoproteins (VLDL).[1]

ApoCI has various functions in lipoprotein metabolism. ApoCI has been identified as the main endogenous HDL-associated inhibitor of cholesteryl ester transfer protein (CETP) activity,[2] probably because of its large amount of positively charged amino acids,[3] which is regarded as an anti-atherogenic property. CETP is responsible for the transfer of cholesteryl esters (CE) from HDL to low density lipoproteins (LDL) and VLDL in plasma. In this way, CETP lowers HDL-cholesterol (C) and at the same time increases (V)LDL-C, thereby unfavourably modifying two risk factors for atherosclerosis. CETP inhibition is generally regarded as an effective new therapeutic strategy to increase HDL-C levels and reduce cardiovascular disease (CVD) risk.[4,5] In fact, apoCI-deficiency in human CETP transgenic mice increases CETP activity and lowers HDL-C levels.[6] Conversely, human apoCI overexpressing (APOC1) transgenic mice have reduced specific CETP activity.[7] ApoCI could therefore be an interesting lead for a new generation of CETP inhibitors.

However, apoCI is also a major inhibitor of lipoprotein lipase (LPL),[8] which is a pro-atherogenic property. LPL hydrolyses triglycerides (TG) within TG-rich lipoproteins such as VLDL, resulting in the liberation of fatty acids (FA) that can be stored in adipose tissue or used as energy supply for skeletal muscle and heart. Therefore, apoCI-deficient mice on a hyperlipidemic apoE-deficient background have decreased VLDL levels[9] and decreased atherosclerosis.[10] Conversely, APOC1 transgenic mice have severely increased plasma VLDL levels,[8] and APOC1 mice on a hyperlipidemic apoE-deficient background have increased atherosclerosis.[11,12] Because of apoCI-induced LPL inhibition, human apoCI overexpression in CETP transgenic mice not only reduces specific CETP activity, but also largely increases VLDL levels. The increase in VLDL levels consequently increases hepatic CETP gene expression, which precludes an increase in HDL-C resulting from CETP inhibition only.[7]

To investigate whether apoCI may represent a suitable lead for novel CETP inhibitors, we aimed in this study to identify by structure-function analysis the minimal CETP-inhibitory domain of apoCI without LPL-inhibitory activity. Studies in baboons with high HDL have identified the N-terminal domain $apoCI_{1-38}$ as the CETP inhibitor,[13,14] whereas studies on the interaction of human apoCI with human CETP indicated that the C-terminal domain $apoCI_{34-54,}$ rather than the N-terminal domain $apoCI_{4-25}$, inhibited CETP.[3] Therefore, we

generated full-length apoCI as well as an array of N-terminal and C-terminal apoCI-derived peptides by solid-phase synthesis, and determined their effect on the activity of CETP as well as of LPL. To get more insight into the mechanism by which apoCI inhibits CETP activity, we also evaluated the contribution of the positively charged amino acids by replacing K and R residues by electroneutral alanine (A) residues.

Materials and Methods

ApoCI and apoCI peptides.
Full-length human apoCI ($apoCI_{1-57}$; purity >95%) was synthesized by the Protein Chemistry Technology Center (UT Southwestern Medical Center, Dallas, TX). ApoCI-derived peptides, without or with replacement of positively charged K and R by electroneutral A, were synthesized by the Peptide Synthesis Facility of the Department of Immunohematology and Blood Transfusion at the Leiden University Medical Center (Leiden, The Netherlands) by solid phase peptide synthesis on a TentagelS-AC (Rap, Tübingen, Germany) using 9-fluorenylmethoxycarbonyl/t-Bu chemistry, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate/*N*-methylmorpholine for activation and 20% piperidine in *N*-methylpyrrolidone for fluorenylmethoxycarbonyl removal.[15] The peptides were cleaved from the resin, deprotected with trifluoroacetic acid/water, and purified on Vydac C18. The purified peptides were analyzed by reverse-phase HPLC and their molecular masses were confirmed by MALDI-TOF mass spectrometry (purity >95%). The primary sequences of apoCI and apoCI-derived peptides are shown in Table 1.

Table 1. Primary sequences and isoelectric points (pI) of full-length human apoCI ($apoCI_{1-57}$) and apoCI-derived peptides.

ApoCI peptides	pI	Primary sequence
1-57	7.93	TPDVSSALD**K**L**K**EFGNTLED**K**A**R**ELIS**R**I**K**QSELSA**K**M**R**EWFSETFQ**K**V**K**E**K**L**K**IDS
1-20	4.27	TPDVSSALD**K**L**K**EFGNTLED
1-23	4.86	TPDVSSALD**K**L**K**EFGNTLED**K**A**R**
1-30	5.98	TPDVSSALD**K**L**K**EFGNTLED**K**A**R**ELIS**R**I**K**
1-38	6.02	TPDVSSALD**K**L**K**EFGNTLED**K**A**R**ELIS**R**I**K**QSELSA**K**M
46-57	9.53	FQ**K**V**K**E**K**L**K**IDS
35-57	9.40	SA**K**M**R**EWFSETFQ**K**V**K**E**K**L**K**IDS
32-57	8.11	SELSA**K**M**R**EWFSETFQ**K**V**K**E**K**L**K**IDS
32-57 A	5.00	SELSAAMAEWFSETFQ**K**V**K**E**K**L**K**IDS
32-57 B	4.25	SELSA**K**M**R**EWFSETFQAVAEALAIDS
32-57 C	3.45	SELSAAMAEWFSETFQAVAEALAIDS

The positively charged basic amino acids Lys (K) and Arg (R) are represented in boldface.

CETP activity.
The effect of apoCI and apoCI-derived peptides on the CETP-mediated transfer of [^{3}H]cholesteryl oleate (CO) from LDL to HDL was determined essentially as described.[16] HDL, LDL and lipoprotein deficient serum (LPDS) were isolated from human plasma by ultracentrifugation, and LDL was radiolabelled by incubation with [^{3}H]CO (Amersham Biosciences, Buckinghamshire, UK)-containing phosphatidylcholine vesicles in the presence of LPDS exactly as described.[16] ApoCI and apoCI-derived peptides were incubated (6 h at 37°C) with [^{3}H]CO-LDL (31.25 nmol total cholesterol) and HDL (12.5 nmol total cholesterol) in a total volume of 175 µL 0.1 M phosphate buffer, pH 7.4, in the presence of the 10 µL LPDS as CETP source and 8 mM of the lecithin-cholesterol acyltransferase (LCAT) inhibitor 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma). After incubation, LDL was precipitated with 75 µL 0.1 M phosphate buffer and 25 µL 0.167 mM manganese chloride, and the supernatant was counted for HDL-associated [^{3}H]CO in Ultima Gold (Perkin Elmer, Boston, MA, USA). CETP activity was calculated as nmol CE transfer/ mL/ min and expressed as % of control.

LPL activity assay.
The effect of apoCI and apoCI peptides on the triacylglycerol hydrolase activity of LPL was assessed by determining their effect on the LPL-mediated hydrolysis of TG within VLDL-like emulsion particles,[8] using either soluble LPL as described[17] or heparan sulfate proteoglycan (HSPG)-bound LPL. Hereto, 0.25 µg/well HSPG (Sigma) was bound to 96-wells plates by overnight incubation at 4°C. Wells were blocked (1 h at 37°C) with 1% BSA in 0.1 M Tris.HCl pH 8.5, Bovine LPL (0.2 U/well; Sigma) was attached to HSPG by incubation (2 h at 37°C) in 12% BSA in 0.1 M Tris.HCl, pH 8.5. After washing, [^{3}H]TO-labelled VLDL-like emulsion particles (0.5 mg TG/mL), pre-incubated with apoCI (30 min at 37°C), were added in the presence of essentially fatty acid-free BSA (60 mg/mL, Sigma) and the LPL-cofactor apoCII (2.5 µg/mL) in 0.1 M Tris.HCl, pH 8.5. After incubation (20 min at 37°C) the reaction was stopped by placing plates on ice and 20 µL sample was added to 1.5 mL of methanol: chloroform: hexane: oleic acid (1,410: 1,250: 1,000: 1, v/v/v/v) and 0.5 mL of 0.1 N NaOH was added. To quantify the [^{3}H]oleate generated, 0.5 mL of the aqueous phase obtained after vigorous mixing (15 sec) and centrifugation (15 min at 3,600 rpm) was counted in Ultima Gold.

Statistical analysis. Data were analyzed using the Mann-Whitney nonparametric test. Analyses were performed with SPSS 14.0 (SPSS Inc, Chicago, USA).

Results

ApoCI dose-dependently inhibits CETP activity.
To confirm the previously reported CETP-inhibitory effect of full-length human apoCI, we determined the dose-dependent effect of $apoCI_{1-57}$ on the transfer of [^{3}H]CO from human LDL to human HDL in the presence of LPDS as source of CETP (Fig. 1). Indeed, apoCI dose-dependently inhibited CETP activity, with an IC_{50} of approx. 4 μM, and nearly completely inhibited CETP activity at 16 μM (-91%; $P<0.05$).

The CETP-inhibitory property of apoCI is confined to its C-terminal domain.
Since the CETP-inhibitory effect of apoCI has been attributed to either its N-terminus[13,14] or C-terminus,[3] we generated an array of N-terminal and C-terminal apoCI-derived peptides by solid-phase synthesis (shown in Table 1), and determined their effect on CETP activity (Fig. 2). The N-terminal peptides $apoCI_{1-20}$ to $apoCI_{1-38}$ did not affect CETP activity at 16 μM, and only marginally decreased CETP activity at 64 μM in a length-dependent way (-19% for $apoCI_{1-38}$; $P<0.05$) (Fig. 2A). In contrast, the C-terminal peptides $apoCI_{35-57}$ and $apoCI_{32-57}$ already affected CETP at 16 μM (-37% for $apoCI_{32-57}$; $P<0.05$), and largely decreased CETP activity at 64 μM, again in a length-dependent way (-100% for $apoCI_{32-57}$; $P<0.05$) (Fig. 2B).

The CETP-inhibitory property of $apoCI_{32-57}$ depends on positively charged amino acids.
It has been shown that the ability of apoCI to inhibit CETP activity could be explained by reduction of the electronegative surface charge of HDL.[3] Since apoCI is unusually rich in electropositive K and R residues, we thus determined the contribution of these residues to the CETP-inhibitory effect of $apoCI_{32-57}$ by site-specific replacement into A residues that are electroneutral and do not affect the overall peptide structure (Fig. 3). At a concentration of 16 μM,

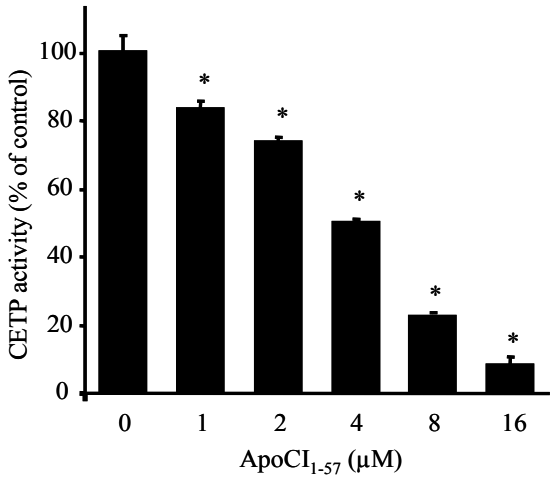

Figure 1. ApoCI dose-dependently inhibits CETP activity. Full-length apoCI (apoCI1-57; 0-16 μM) was incubated (6 h at 37°C) with [3H]CO-LDL and HDL in PBS in the presence of LPDS as source of CETP. The amount of [^{3}H]CO transferred from LDL to HDL was determined by counting [^{3}H]CO-HDL after precipitation of LDL. CETP activity was calculated relatively to control incubations without apoCI. Values are means ±SD, n=3, * $P<0.05$, as compared to control without peptides added.

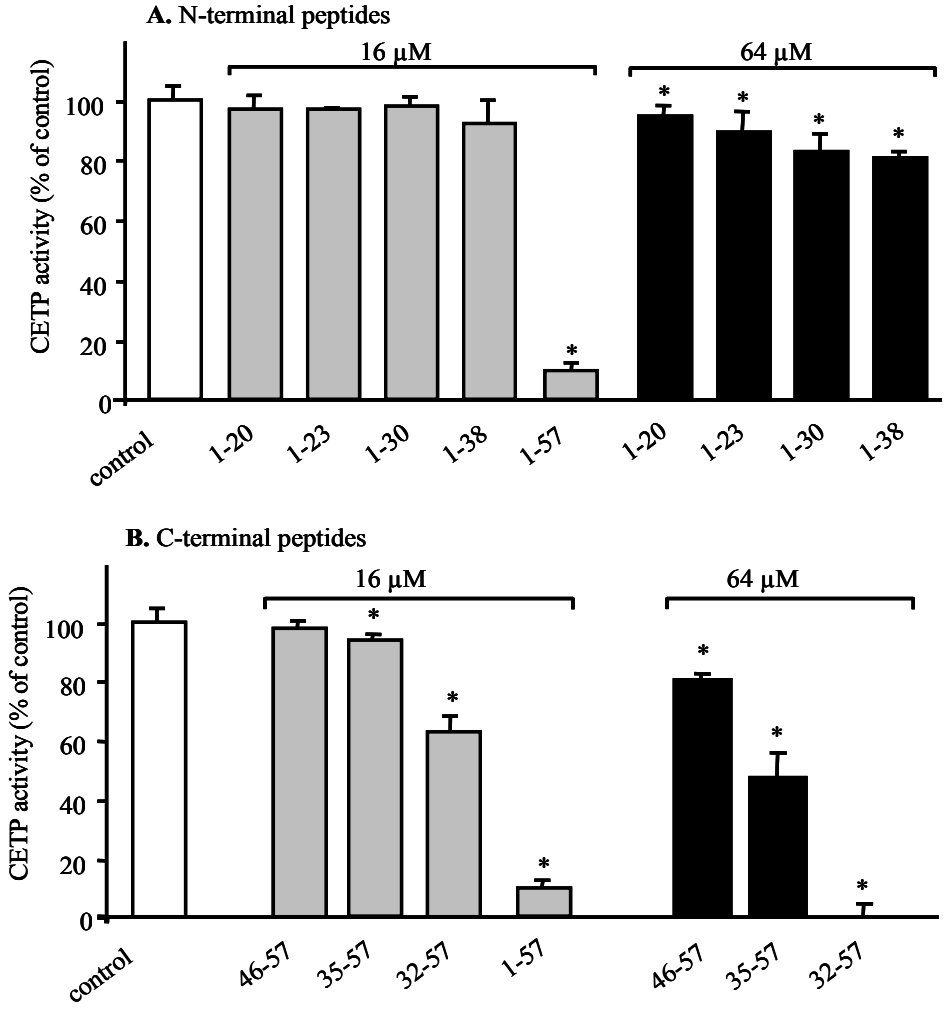

Figure 2. The CETP-inhibitory property of apoCI is confined to its C-terminal domain. ApoCI peptides derived from the N-terminal domain (A) or C-terminal domain (B) were incubated (6 h at 37°C) at concentrations of 16 µM and 64 µM with [^{3}H]CO-LDL and HDL in PBS in the presence of LPDS as source of CETP. CETP activity was calculated relatively to control incubations without apoCI. Values are means ±SD, n=3, * P<0.05, as compared to control without peptides added.

wild-type apoCI$_{32\text{-}57}$ decreased CETP activity appreciably (-37%; P<0.05). Neutralization of positively charged residues, within either the KMR cluster at the N-terminal end or within the KVKEKLK cluster at the C-terminal end, reduced CETP inhibition partially (-22% and -21%; P<0.05), whereas neutralization of all positively charged amino acids completely abolished the CETP-inhibitory property of apoCI$_{32\text{-}57}$. A similar pattern was observed at 64 µM, albeit that the peptide in which all K and R residues have been replaced by A still inhibit CETP activity to some extent (-31%; P<0.05).

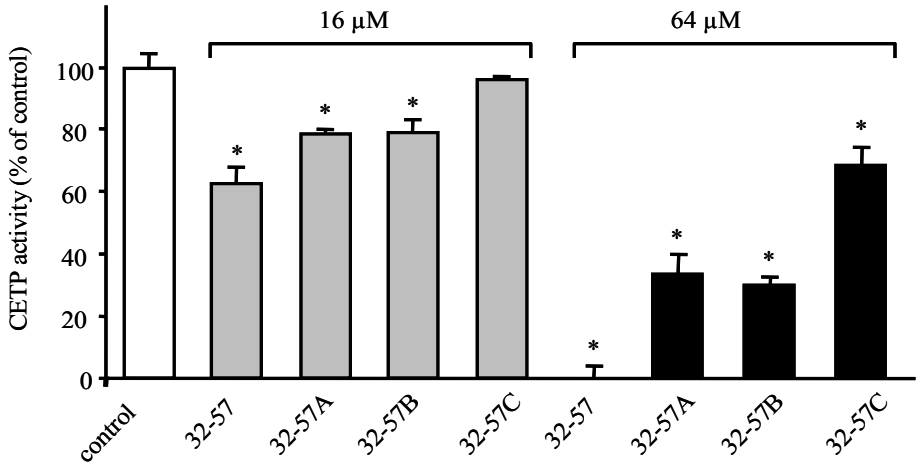

Figure 3. The CETP-inhibitory property of apoCI$_{32-57}$ depends on positively charged amino acids. ApoCI$_{32-57}$ or apoCI$_{32-57}$ in which positively charged K and R are replaced at the N-terminal end (A), C-terminal end (B), or both ends (C) were incubated (6 h at 37°C) at concentrations of 16 µM and 64 µM with [^{3}H]CO-LDL and HDL in PBS in the presence of LPDS as source of CETP. CETP activity was calculated relatively to control incubations without apoCI. Values are means ±SD, n=3, * P<0.05, as compared to control without peptides added.

ApoCI$_{32-57}$ does not inhibit LPL activity as compared to full-length apoCI$_{1-57}$. ApoCI$_{32-57}$, which is the minimal apoCI peptide that results in maximum CETP inhibition, may have therapeutic value provided that it selectively inhibits the activity of CETP as compared to LPL. Therefore, we next determined the effect of full-length apoCI$_{1-57}$ and of apoCI$_{32-57}$ on LPL activity by incubation with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and HSPG-bound LPL (Fig. 4). Indeed, whereas apoCI$_{1-57}$ dose-dependently decreased LPL activity (-90% at 16 µM; P<0.05), LPL activity was not significantly affected by apoCI$_{32-57}$.

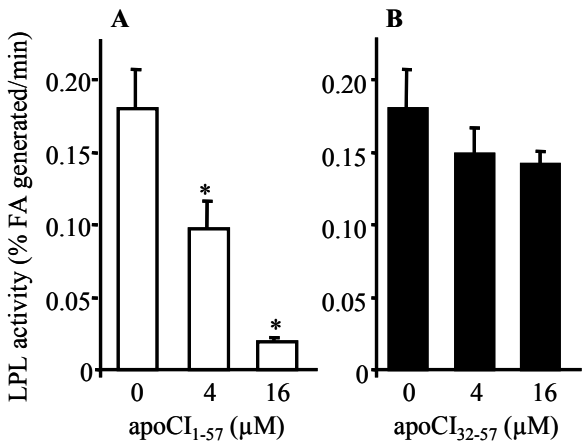

Figure 4. Full-length apoCI, but not apoCI$_{32-57}$, inhibits LPL activity. ApoCI1-57 (A) and apoCI$_{32-57}$ (B) were incubated (20 min at 37°C) at concentrations of 4 µM and 16 µM with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles and HSPG-bound LPL. Generated [^{3}H]oleate was separated from glycerol tri[3H]oleate by extraction and quantified. LPL activity was determined as the percentage of fatty acids (FA) generated per minute. Values are means ±SD, n=3, * P<0.05, as compared to control without peptides added.

Discussion

Using an array of apoCI-derived peptides, we demonstrated that the CETP-inhibitory property of apoCI is restricted to the C-terminal domain of apoCI. We identified $apoCI_{32-57}$ as a minimal CETP inhibitory sequence that does not inhibit LPL activity, and showed that the positively charged amino acids K and R are largely responsible for the CETP-inhibitory effect.

First, we thus demonstrated that the CETP-inhibitory property of apoCI resides in its C-terminal domain. This observation is consistent with a previous study comparing the CETP-inhibitory effect of $apoCI_{34-54}$ and $apoCI_{4-25}$.[3] By direct comparison, we showed that $apoCI_{32-57}$ is more effective in CETP inhibition than $apoCI_{34-54}$ (not shown). However, our data contradict earlier findings in a strain of baboons with high HDL in which $apoCI_{1-38}$ was identified as the naturally occurring CETP inhibitor in plasma.[13] Notably, human $apoCI_{1-38}$ also inhibits baboon CETP.[14] It is therefore likely that the discrepancy between the various studies is explained by species differences on the level of CETP: the N-terminus of apoCI affects baboon CETP whereas the C-terminus of apoCI affects human CETP. It should be noted that we did show a modest effect of $apoCI_{1-38}$ on CETP activity (Fig. 2A), and the studies on baboon CETP did not compare $apoCI_{1-38}$ with a C-terminal peptide.[14] Therefore, it is still possible that the baboon CETP activity assay is more sensitive than the human CETP activity assay, and thus detects a larger CETP-inhibitory effect of $apoCI_{1-38}$, without ruling out that $apoCI_{32-57}$ is even more effective.

It has been shown that the ability of apoCI to inhibit CETP activity could be explained by reduction of the electronegative surface charge of HDL,[3] thereby inhibiting the physical association of CETP with HDL. Therefore, we evaluated the contribution of the positively charged amino acids within $apoCI_{32-57}$ to its CETP-inhibitory effect by replacement of K and R residues in the KMR cluster at the N-terminal end or within the KVKEKLK cluster at the C-terminal end by electroneutral A. This is a small amino acid that does not affect the overall peptide structure as predicted from secondary structure analysis using Peptide Companion software (not shown). We demonstrated that replacement of positively charged amino acids in either the KMR or KVKEKLK motif both reduced the CETP inhibitory effect of $apoCI_{32-57}$. In addition, despite the presence of positively charged amino acids in both the N-terminus and C-terminus of apoCI, the C-terminal peptide $apoCI_{32-57}$ had a higher isoelectric point (pI = 8.11) than $apoCI_{1-38}$ (pI = 6.02). These observations thus confirm the hypothesis that the CETP-inhibitory property of apoCI is dependent on its overall positive charge rather than on specific electropositive residues.

In addition to electropositive charge, other structural properties of apoCI likely contribute to inhibition of CETP activity. First, $apoCI_{32-57}$ appeared to be a stronger CETP inhibitor than $apoCI_{35-57}$ whereas there is no difference in the amount of positively charged residues. Second, $apoCI_{46-57}$ has a higher isoelectric point (pI = 9.53) as compared to $apoCI_{32-57}$ (pI = 8.11), but is a less

efficient CETP inhibitor. Third, replacement of all K and R residues in apoCI$_{32-57}$ did not completely abrogate its CETP-inhibitory effect. It is thus conceivable that, in addition to the importance of the peptide charge, the peptide length positively influences the α-helical conformation of the peptides and the binding affinity of the peptide for HDL.

Whereas full-length apoCI is an effective inhibitor of LPL activity, apoCI$_{32-57}$ did not affect LPL activity. In fact, none of the applied apoCI-derived peptides were able to inhibit LPL activity (not shown). The mechanism by which apoCI inhibits LPL activity has not been fully elucidated. ApoCI may directly interact with LPL and/or interfere with other apolipoproteins that modulate LPL activity such as the LPL co-activator apoCII or apoCIII or apoAV.[8] Alternatively, apoCI may affect LPL activity by product inhibition, since apoCI effectively binds free fatty acids.[18] The lack of an LPL-inhibiting effect of the truncated apoCI peptides could thus result from reduced interference with any of these three processes. The fact that C-terminal peptides inhibit the activity of CETP, but not that of LPL, suggests that these peptides may still bind avidly with HDL (thereby interfering with CETP activity), but weakly with VLDL (thereby precluding interference with LPL activity). Since the anti-apoCI antibody that we used in our ELISA does not react with apoCI-peptides even after minimal truncation of apoCI, such a hypothesis is difficult to test by routine techniques.

In our study, we focused on the most prominent features of apoCI in lipid metabolism, *i.e.* CETP inhibition and LPL inhibition. ApoCI has also been described to stimulate LCAT,[19,20] inhibit HL[11,12] and inhibit the HDL receptor scavenger receptor class B type I (SR-BI),[21] which may contribute to HDL increase. In addition, apoCI inhibits (V)LDL clearance via apoE-recognizing receptors,[22,23] which may contribute to hyperlipidemia. Therefore, *in vivo* studies using CETP transgenic mice and wild-type mice are needed to evaluate whether apoCI$_{32-57}$ is capable to increase HDL without inducing hyperlipidemia and whether such an effect can be ascribed to CETP inhibition only.

The fact that apoCI$_{32-57}$ inhibits CETP activity without affecting LPL activity suggests that apoCI$_{32-57}$ is a valuable lead for a new anti-atherogenic therapy, as raising HDL by inhibiting CETP is generally seen as a protective lipoprotein in atherosclerosis development. This is especially relevant considering the recent clinical failure of the CETP inhibitor torcetrapib.[24] Despite evoking a large increase in HDL-cholesterol levels, torcetrapib did not potentiate the anti-atherogenic potency of atorvastatin as judged from coronary intima-media thickness (IMT) and intravascular ultrasonography (IVUS) measurements.[25-27] In fact, torcetrapib increased overall mortality and the amount of non fatal cardiovascular events.[24] These disappointing results are probably explained by compound-specific off target toxic effects of torcetrapib, including hypertension and hyperaldosteronism.[28] Being derived from an endogenous protein, apoCI$_{32-57}$ is not expected to induce such side effects. Also, whereas torcetrapib forms an inactive complex between HDL and CETP, thereby resulting in an accumulation of CETP protein in plasma,[29] apoCI is not expected to result in

accumulated CETP in plasma as its proposed working mechanism (*i.e.* reduction of the interaction between HDL and CETP) is different from that of torcetrapib.
In conclusion, we identified $apoCI_{32-57}$ as the minimal domain in apoCI that inhibits CETP activity without affecting LPL activity. Therefore, future studies are warranted to evaluate whether $apoCI_{32-57}$ will raise HDL without inducing hyperlipidemia and may be a valuable lead in the search for new CETP inhibitors that aim at raising HDL and reducing atherosclerosis.

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Chapter 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the western world, and is mainly caused by atherosclerosis. In the Netherlands, about one third of all deaths are due to CVD. Dyslipidemia (i.e. high plasma (V)LDL-cholesterol (C), high triglycerides and low HDL-C) is a major risk factor for atherosclerosis development and cardiovascular disease. Patients with dyslipidemia are usually treated with cholesterol lowering drugs including statins[1] and fibrates.[2] These drugs lower plasma cholesterol very efficiently (up to 40%),[1,2] however, they prevent only a fraction (about 30%) of cardiovascular events. Therefore new therapeutic strategies to reduce CVD risk more efficiently are necessary. Since HDL is clearly inversely correlated with CVD risk, and has been attributed multiple protective effects in atherosclerosis by its role in reverse cholesterol transport (RCT) and its anti-inflammatory, anti-oxidative and anti-thrombotic properties, HDL-raising therapy is currently considered as a promising strategy to further reduce CVD risk.[3,4]
The research described in this thesis was performed to elucidate the mechanisms underlying the HDL-C raising effects of classic lipid-lowering drugs (fenofibrate, atorvastatin, niacin) as well as an experimental HDL-raising compound (torcetrapib), and to evaluate the HDL-raising potential of novel strategies (PXR agonism, apoCI). The major conclusions and implications of our findings and future perspectives will be discussed here.

HDL modulation by classical lipid lowering drugs

The classical lipid-lowering drugs statins, fibrates and niacin have been shown to modestly raise HDL-C levels in humans up to 10, 15, and 30%, respectively.[5] Interestingly, these drugs failed to show human-like HDL-increasing effects in either normolipidemic or classical hyperlipidemic mice (i.e. LDL receptor-knockout and apoE-knockout mice). Although fenofibrate has been shown to increase HDL in wild-type mice, the raised HDL had an increased particle size, as opposed to the raised HDL in humans. Likewise, although these drugs did evoke human-like lipid-lowering effects in ApoE*3Leiden transgenic (E3L) mice[6] with respect to dose-dependent decreases of (V)LDL-C and triglycerides, they generally failed to raise HDL-C in E3L mice.[7]
As mice naturally lack the cholesteryl ester transfer protein (CETP), which is an important determinant of HDL metabolism in humans, we reasoned that the HDL-raising effect of the classical lipid-lowering drugs may relate to the presence of CETP. Therefore, we crossbred E3L mice with CETP mice to generate the novel E3L.CETP mouse model.[8] In this thesis we show that E3L.CETP mice indeed respond with an increase in HDL besides a decrease in (V)LDL to both a fibrate (fenofibrate; chapter 2), a statin (atorvastatin; chapter 3) and niacin (chapter 4). This indicates not only that the E3L.CETP mouse is a valuable model to study the effect of HDL modulating drugs on lipid

metabolism, but also that CETP plays an essential role in the HDL increase observed with these drugs.

It is interesting to note that these various classes of drugs have a similar HDL-increasing effect, as dependent on CETP expression, whereas they evoke their lipid-lowering effects through different mechanisms. Statins, fibrates and niacin reduce plasma lipids by primarily by inhibition of the *de novo* hepatic cholesterol synthesis,[9,10] stimulation of triglyceride hydrolysis in plasma,[11-13] and inhibition of lipolysis in adipocytes,[14] respectively. Despite these different primary actions on lipid metabolism, our studies demonstrated that they all reduce the activity and mass of CETP in plasma. In fact, we showed that atorvastatin, fenofibrate and niacin all reduced the hepatic mRNA expression of CETP, which is likely the main causal factor for the reduction in plasma CETP. In line with this hypothesis, increasing the hepatic cholesterol content of CETP transgenic mice by cholesterol feeding increases hepatic CETP expression via LXR-dependent mechanisms as well as plasma CETP mass.[15,16] The three classes of lipid-lowering drugs not only decrease CETP expression, but also decrease (V)LDL levels. Since (V)LDL is an acceptor of HDL-derived cholesteryl esters and, therefore, also a driving force for CETP activity, the decrease in (V)LDL adds to the drug-induced reduction in CETP activity.

Based on our observations, we speculate that lipid-lowering drugs in general will thus all increase HDL-C to a certain extent by reducing plasma CETP activity, as a result of a reduction in both hepatic CETP expression and plasma (V)LDL. In fact, observations in human subjects indeed showed that statins and fibrates both reduce CETP mass and activity.[17-21] The effect of niacin on plasma CETP in humans has not been reported as yet, but niacin will thus probably also reduce CETP mass and activity. Interestingly, hyperlipidemic patients carrying the CETP TaqIB1 polymorphism, who have therefore higher plasma CETP levels than people with the TaqIB2 variant, benefit more from statin treatment with respect to the development of coronary atherosclerosis, [22] suggesting that the reduction of CETP activity is indeed a relevant contributor to the protective effects of lipid-lowering drugs in atherosclerosis.

The apparent robust causal relation between hepatic lipid content and hepatic CETP expression raises the question whether the pathological condition of hepatic steatosis would be a causal factor for reducing HDL-levels by increasing CETP expression. Interestingly, a recent study in obese subjects showed that liver fat indeed inversely correlated with HDL levels.[23] However, since (V)LDL levels are also increased in patients with a fatty liver,[23] more research is needed to confirm the hypothesis that hepatic steatosis results in increased plasma CETP levels thereby decreasing HDL.

HDL modulation by CETP inhibition

CETP inhibition has been regarded as a novel promising HDL-C raising strategy to reduce atherosclerosis and cardiovascular disease. Large clinical studies with the CETP inhibitor torcetrapib have been performed to evaluate whether CETP inhibition is able to increase HDL levels and reduce atherosclerosis. Torcetrapib indeed increased HDL-C to a marked extent (+60% at 60 mg/day), but did not reduce atherosclerosis as measured by Intima Media Thickness (IMT) and Intravascular Ultrasound (IVUS)[24-26] and had unwanted effects including increased overall mortality as well as fatal and non fatal cardiovascular events.[27]

There are several possible explanations for the disappointing results of the torcetrapib studies, related to 1) inclusion criteria of the patients, 2) combination therapy with atorvastatin, 3) properties of the newly formed HDL, or 4) compound specific effects.

First, in the torcetrapib studies, patients were included who had undergone cardiac catheterization[26] or have (familial) dyslipidemia.[24,25] These broad inclusion criteria led to the inclusion of a very heterogeneous patient group that may not all benefit from CETP inhibition, as the metabolic context, including baseline CETP activity, HDL-C levels and TG levels, is likely to be an important determinant of the effect of CETP inhibition on atherosclerosis development and CVD risk. The view that the metabolic context is important in the effect of CETP inhibition on atherosclerosis is supported by mouse studies, which in general show that CETP expression is atheroprotective in mice with increased HDL levels while CETP expression is atherogenic in mice with elevated (V)LDL levels. CETP expression in mice with high HDL because of LCAT overexpression or SR-BI deficiency reduces atherosclerosis.[28,29] In line with these mouse data, it has been shown that subjects with highly elevated HDL-C levels which were mainly associated with CETP mutations, have higher prevalence of ischemic ECG changes.[30] CETP inhibition in subjects who have already high HDL may, therefore, not lead to protection against atherosclerosis and CVD. These findings may also suggest that HDL elevation by CETP inhibition is only protective when this normalizes or elevates HDL mildly compared to normolipidemic subjects and CETP inhibitors should not be used to further increase HDL in subjects who have cardiovascular risk factors but normal or elevated HDL levels. In contrast, CETP is a clear atherogenic factor in hyperlipidemic mouse models with impaired (V)LDL clearance, including apoE knockout,[31] LDL receptor knockout[31] and E3L mice.[8] When translated to humans, dyslipidemic subjects with high plasma TG may therefore benefit from CETP inhibition since CETP transfers HDL-CE to (V)LDL that is inefficiently cleared from plasma as indicated by high TG. This suggests that CETP inhibition could be particularly promising in those dyslipidemic patients, who besides elevated (V)LDL also have low HDL and/or high CETP activity.

Further subgroup analyses should be performed in the recent torcetrapib studies to study whether patients with low HDL, high CETP activity and/or high TG at baseline benefit from torcetrapib with respect to atherosclerosis development.
Second, the clinical studies with torcetrapib were all performed in combination with atorvastatin,[24-26] which by itself reduces CETP activity in plasma.[17-20,32,33] By investigating the effect of torcetrapib on atherosclerosis development in E3L.CETP mice, either in combination with atorvastatin or alone (chapter 5), we showed that torcetrapib *per se* did in fact reduce atherosclerosis while torcetrapib did not reduce atherosclerosis in mice that were also treated with atorvastatin, indicating that combination treatment with atorvastatin and other lipid lowering drugs may attenuate or mask the effect of torcetrapib on atherosclerosis.
Third, it is not clear whether the increased HDL as induced by CETP inhibition by torcetrapib is atheroprotective or not. HDL is thought to be protective in atherosclerosis via mediating RCT and by its proposed anti-inflammatory, anti-oxidative and anti-thrombotic properties. Torcetrapib alters HDL by increasing its size, by the formation of a torcetrapib/CETP complex that associates with HDL, and torcetrapib may alter the protein composition of HDL which may alter HDL functionality. In addition, torcetrapib may reduce HDL-CE clearance as it has been described that in humans, the majority of HDL-CE reaches the liver via (V)LDL after CETP-mediated transfer of HDL-CE from HDL to (V)LDL, and this pathway is blocked by torcetrapib.[34,35]
Fourth, torcetrapib may have had compound-specific adverse side effects with respect to increased mortality and increased cardiovascular events, and compound-specific side effects may explain why torcetrapib did not add to the atherosclerosis-reducing effect of atorvastatin. Torcetrapib has been found to increase blood pressure by approx. 5 mm Hg,[24-26,36] which is not observed with novel CETP inhibitors including anacetrapib and JTT-705. This mild increase in blood pressure is unlikely to completely have counteracted potential protective effects of the HDL increase and is unlikely to have caused increased mortality, but it may be indicative for other underlying adverse effects. Analysis of plasma samples from patients treated with torcetrapib showed an increase in sodium, bicarbonate and aldosterone levels.[27] Torcetrapib also increased plasma plasma aldosterone in E3L.CETP mice (chapter 5). The raise in aldosterone may not only explain the increase in blood pressure, but animal studies have also shown that aldosterone causally increases atherosclerosis, inflammation and oxidative stress,[37-40] indicating that the increase in aldosterone may have counteracted potentially atheroprotective effects of the torcetrapib-induced increase in HDL.
We have demonstrated that torcetrapib increases the macrophage to collagen ratio within atherosclerotic plaques in E3L.CETP mice (chapter 5). Whereas plaques of mice do not easily rupture spontaneously, such a phenotype is considered more prone to spontaneous rupture in humans. Extrapolation of our data to humans may thus suggest that the increase in cardiovascular events and

death may have been caused, at least partly, by increased incidence of plaque rupture. As aldosterone is associated with more inflammation and increased activity of matrix metalloproteinases (MMP),[39,41] which cause breakdown of collagen, it can be reasoned that the increased aldosterone levels may have contributed to the plaque phenotype. However, this hypothesis should be underscored by experimental studies. The effects of torcetrapib on aldosterone and blood pressure are compound-specific and independent of CETP, as torcetrapib induced these effects in both humans, CETP transgenic mice and non-transgenic mice.[42] In contrast, anacetrapib does not increase blood pressure and aldosterone levels.[42,43] The effect of novel CETP inhibitors on plaque composition and cardiovascular events is thus eagerly awaited.

In addition to chemical CETP inhibitors, endogenous CETP inhibitors have also been described. Whereas the lipid transfer inhibitor protein (LTIP) as present on LDL inhibits CETP activity,[44] apoCI is the major endogenous CETP inhibitor present on HDL.[45] being an endogenous protein, apoCI may be a lead for novel safe CETP inhibitors. However, apoCI is also an inhibitor of LPL.[46] Because of apoCI-induced LPL inhibition, human apoCI overexpression in CETP transgenic mice not only reduces specific CETP activity, but also largely increases VLDL levels. The increase in VLDL levels consequently increases hepatic CETP gene expression, which precludes an increase in HDL-C resulting from CETP inhibition only.[47]
To find an apoCI derived CETP inhibitor without LPL inhibitory properties, we performed structure-function analysis using an array of apoCI derived peptides. $ApoCI_{32-57}$ was the most efficient CETP inhibitory peptide tested, and this peptide had only minimal effects on LPL. Therefore we expect that this peptide should be able to increase HDL without inducing hyperlipidemia. Pilot experiments showed that intravenous injections with apoCI and $apoCI_{32-57}$ were unable to modulate lipid levels *in vivo* in CETP transgenic mice (unpublished data), which may relate to dosing and/or adverse pharmacokinetics of the peptides. Short-term elevation of plasma levels of apoCI and $apoCI_{32-57}$, e.g. by using recombinant adenoviruses that induce a relatively high hepatic protein expression of apoCI and $apoCI_{32-57}$ in CETP expressing mice could therefore be useful as a tool to show the potential of $apoCI_{32-57}$ to increase HDL levels without affecting plasma TG. If so, $apoCI_{32-57}$ mimicking agents could be developed that can be used orally.
In addition to CETP, apoCI also affects other HDL modifying enzymes, including LCAT[48,49] HL[50,51] and SR-BI (chapter 7). These combined actions increase HDL in naturally CETP-deficient wild-type mice (chapter 7), but functionality of the increased HDL is unknown. It is also unknown how $apoCI_{32-57}$ would act on these various proteins involved in HDL metabolism. Therefore, additional *in vitro* and *in vivo* experiments in wild-type mice should be performed to show whether potential HDL-increasing effects of $apoCI_{32-57}$

are CETP dependent. In addition, studies in E3L.CETP mice will be useful to reveal whether apoCI$_{32-57}$ will reduce atherosclerosis. It would be interesting to study the effect of apoCI$_{32-57}$ on the plaque phenotype as well as to evaluate whether the effect of torcetrapib on plaque composition was indeed compound specific, especially because apoCI has a different mechanism to inhibit CETP as compared to torcetrapib, i.e. reduction of affinity of CETP with HDL[52] versus formation of an inactive HDL/CETP complex.[53]

Novel strategies to reduce cardiovascular disease

Cholesterol lowering is a proven effective strategy to reduce cardiovascular disease. Therefore a large number of new drugs to reduce plasma lipid levels is under development, including microsomal triglyceride transfer protein (MTP) inhibitors, squalene synthase inhibitors, and apoB expression inhibitors that are all aimed at reducing lipid production by the liver.[54-58] However, as statins already efficiently reduce plasma (V)LDL-C levels without severe side effects, and additional safe (V)LDL-C lowering agents to treat patients that do not respond well to statins are available (e.g. cholesterol binding resins) it will be difficult to develop novel lipid lowering drugs that lead to more clinical benefit with respect to protection against cardiovascular disease.
After the disappointing results from the torcetrapib studies, it is difficult to predict whether novel HDL raising agents will prevent CVD in the future. Despite that there is no direct evidence for the protective effect of HDL in atherosclerosis, HDL is thought to play a role in RCT, and is thought to be anti-inflammatory, anti-oxidative and anti-thrombotic. HDL raising my be achieved by 1) reducing HDL clearance, 2) increasing HDL maturation and modification of HDL metabolism or 3) enhancing HDL production.
In mice, clearance of HDL-C is almost exclusively mediated via SR-BI. In humans, Cla-1 (i.e. the human orthologue of SR-BI) is thought to play a less important role, since HDL-CE is mainly cleared from plasma after CETP-mediated transfer to (V)LDL.[34] Albeit that raising HDL by reducing its clearance may increase the anti-inflammatory and anti-oxidative properties of HDL, the role of HDL in RCT (i.e. transfer of cholesterol to the liver, followed by excretion via the feces) is possibly the most important protective function of HDL. Together with the fact that SR-BI-deficiency in mice aggravates atherosclerosis, reducing HDL clearance may probably not be the most valid HDL-raising strategy.
LCAT plays an important role in the maturation of HDL as this enzyme esterifies HDL-associated cholesterol into CE. Mutations in LCAT which lower LCAT activity reduce HDL-C levels and mildly enhance atherosclerosis development (as measured by IMT).[59] However, since overexpression of LCAT in mice increases atherosclerosis, LCAT-targeted interventions to raise HDL should be pursued with care. As niacin increases HDL via CETP reduction

(chapter 4) niacin can also be considered as a compound that increases HDL via HDL modulation. Niacin is however not well tolerated because it induces severe flushing. A recent study shows that addition of the prostaglandin D_2 receptor 1 blocker laropiprant reduces niacin mediated flushing which may increase niacin use.[60] In addition novel compounds targeting the niacin receptor GRP109A are under development.[61] However, it is uncertain whether the protective effects of niacin on IMT progression and mortality are mediated via its HDL raising or via its (V)LDL reducing properties [62,63]

HDL production is presumably mainly initiated by the synthesis and secretion of apoAI by the liver and intestine, and lipidation of apoAI in plasma via ABCA1. Indeed, apoAI-deficiency and ABCA1-deficiency in mice largely decrease HDL-C. Interestingly, humans carrying mutations in ABCAI and apoAI[59] have a more severe increase in atherosclerosis (as measured by IMT) as compared to carriers of CETP or LCAT mutations, suggesting that enhancing HDL production would be the most promising strategy in the prevention of atherosclerosis and CVD. Upregulation of ABCA1 in macrophages can be achieved with compounds like LXR agonists.[64] However, these compounds will also induce the expression of lipogenic genes in the liver which counteract potential protective effects of ABCAI upregulation in atherosclerosis. In addition, LXR activation will result in upregulation of multiple proteins involved in cholesterol efflux, including apoE, which hampers evaluation of the effect of upregulation of ABCA1 only. Therefore, compounds that specifically upregulate ABCA1 expression in macrophages, preferably in the atherosclerotic vessel wall, should be developed. Increase of apoAI can be achieved via administration of apoAI or apoAI mimicking compounds, or via upregulation of apoAI expression. ApoAI, infused either as a lipid-free protein or contained in recombinant HDL, increases HDL levels. Clinical studies show that short-term apoAI infusion lead to a quick reduction of atheroma volumes in patients with acute coronary syndrome.[65] However, a drawback of apoAI infusion is that treatment is invasive and very expensive, and that long term effects are still unknown.[66] Alternatively, apoAI mimicking agents have been developed which can be given orally.[67] Agents that increase apoAI production would also be promising to increase endogenous HDL production. It has been suggested from studies in wild-type mice that PXR agonism would increase apoAI production and raise HDL levels.[68] However, we observed in our more human-like E3L.CETP mice that PXR agonists reduced HDL of all sizes concomitant with a reduction (rather than an increase) in apoAI expression (chapter 6). This study thus indicates that PXR agonism is likely also unable to raise apoAI and HDL in humans. Recently, rvx-208 has been developed to induce apoAI expression and raise HDL levels, as shown in mice and non-human primates. Provided that this compound is specific for apoAI, it would be promising new lead in the ongoing search for HDL-raising strategies to prevent CVD.[69]

Concluding remarks

Besides a large number of new lipid-lowering agents, drugs that are aimed to specifically raise HDL are currently under development. The therapeutic value of such HDL-raising therapies, however, is still unclear, especially since the first HDL-raising strategy by torcetrapib failed in large human trials. The question whether raising HDL will add to the atheroprotective effect of lipid-lowering therapy is thus still unanswered. Albeit that high HDL-C is clearly associated with reduced CVD risk, high HDL-C is also associated with low (V)LDL-C and TG, a balance that is probably dictated by bidirectional transfer of neutral lipids by CETP. Therefore, it is difficult to predict the importance of HDL independent of other risk factors, and virtually no direct evidence is currently available to show that HDL *per se* is protective in atherosclerosis development. Therefore, other therapeutic strategies such as inhibition of inflammation, which besides dyslipidemia is also a driving force for atherosclerosis, should not be overlooked.
The torcetrapib trials taught us that care should be taken in selecting appropriate subjects in human studies that are expected to benefit most from a novel experimental approach to reduce CVD. Future studies will show which of these new strategies will eventually be used in the future therapy of those patients that are prone to develop CVD. Moreover, our studies indicate the importance of testing effects of experimental drugs on lipid metabolism, atherosclerosis and plaque composition in appropriate animal models. We expect that our newly developed E3L.CETP mouse model with a human-like lipoprotein metabolism will largely contribute to the development of such compounds by reliably predicting human responses to experimental drugs and revealing underlying mechanisms.

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Chapter 10

SUMMARY
SAMENVATTING
ABBREVIATIONS
LIST OF PUBLICATIONS
CURRICULUM VITAE

Summary

Cardiovascular disease (CVD) is a major cause of mortality and morbidity in the Western world. CVD is mainly caused by atherosclerosis, for which dyslipidemia, characterized by high a plasma level of (very) low density lipoprotein ((V)LDL) and a low plasma level of high density lipoprotein (HDL), is a major risk factor. To reduce the risk to develop CVD, drugs aimed at correcting dyslipidemia by lowering (V)LDL are currently the first choice of treatment. Albeit that these drugs lower (V)LDL-C very efficiently (up to ~40%), and generally result in a slight increase in HDL-C, they only prevent a fraction of all cardiovascular events (~30%). Therefore new therapeutic strategies to reduce cardiovascular events more efficiently are necessary. Since HDL is has been attributed multiple protective effects in atherosclerosis by its role in reverse cholesterol transport and its anti-inflammatory and anti-oxidative properties, HDL-raising therapy is currently considered as a promising strategy to further reduce CVD risk. In this thesis, the mechanisms underlying the HDL-raising effects of the classical lipid-lowering drugs fenofibrate, atorvastatin and niacin were elucidated. Furthermore, the effects of potential novel HDL-raising strategies, including torcetrapib, PXR agonism and apoCI, on HDL metabolism were addressed. For these studies, we used the APOE*3-Leiden.CETP (E3L.CETP) transgenic mouse, a valuable model for human-like lipoprotein metabolism.

The lipid-lowering drugs fibrates and statins efficiently reduce plasma cholesterol and triglycerides (TG) in dyslipidemic subjects. In addition, they also increase HDL-C to some extent (~5-15%), but the mechanism underlying the increase in HDL-C was unclear. Since both fibrates and statins 1) reduce the concentration of cholesteryl ester transfer protein (CETP) in human plasma, and 2) do not increase HDL-C in naturally CETP-deficient mice, we reasoned that CETP plays a dominant role in the HDL-increasing effect of both fibrates and statins. To evaluate the role of CETP in the HDL-C increase as seen in humans, we used E3L mice that have been shown to respond in a human-like manner with respect to the lipid-lowering effects of fibrates and statins, and E3L.CETP littermates. In **chapter 2**, E3L and E3L.CETP mice were fed a Western-type diet with or without fenofibrate. Fenofibrate decreased (V)LDL-TG and (V)LDL-C in both E3L and E3L.CETP mice, but fenofibrate increased HDL-C only in E3L.CETP mice. Fenofibrate did not affect the turnover of HDL-CE, indicating that fenofibrate causes a higher steady-state HDL-C level without altering the HDL-C flux through plasma. Analysis of the hepatic gene expression profile showed that fenofibrate did not differentially affect the main players in HDL metabolism, including phospholipid transfer protein (Pltp), ATP-binding cassette transporter A1 (Abca1), scavenger receptor class B type I (Sr-b1), and apolipoprotein AI (Apoa1), in E3L.CETP mice as compared to E3L

mice. However, in E3L.CETP mice, fenofibrate reduced hepatic CETP mRNA as well as the CE transfer activity in plasma. In **chapter 3**, a similar approach was used to evaluate whether atorvastatin increases HDL-C via CETP modulation. Atorvastatin reduced plasma cholesterol in both E3L and E3L.CETP mice, which was specific for (V)LDL, but atorvastatin increased HDL-C only in E3L.CETP mice. Hepatic mRNA expression levels of genes involved in HDL metabolism, including Pltp, Abca1, Sr-b1 and Apoa1, were not differently affected by atorvastatin in E3L.CETP mice as compared to E3L mice. However, in E3L.CETP mice, atorvastatin down-regulated the hepatic CETP mRNA expression as well as the total CETP level and cholesteryl ester (CE) transfer activity in plasma. Therefore, we concluded that both fenofibrate and atorvastatin increase HDL-C by reducing the CETP-dependent transfer of cholesterol from HDL to (V)LDL, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool.
The most potent HDL-raising drug currently available is niacin, but the mechanism underlying the effects of niacin on HDL metabolism is still unknown. In **chapter 4**, E3L and E3L.CETP mice on a western-type diet were treated with niacin. In E3L.CETP mice, niacin dose-dependently decreased plasma cholesterol and TG and dose-dependently increased HDL-C, plasma apoAI as well as the HDL particle size. In E3L mice, niacin also reduced plasma cholesterol and TG, but had no effect on HDL. In E3L.CETP mice, niacin dose-dependently decreased the hepatic expression of CETP as well as plasma CETP mass and CETP activity. Additionally, niacin dose-dependently decreased the clearance of apoAI from plasma and reduced the uptake of apoAI by the kidneys. Therefore, we concluded that niacin markedly increases HDL-C in E3L.CETP mice by reducing CETP activity, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool, and increases HDL-apoAI by decreasing the clearance of apoAI from plasma.

Torcetrapib is the first compound designed specifically to increase HDL-C levels via inhibition of CETP activity. In large clinical trials, torcetrapib increases HDL-C by about 60%, but did not potentiate the effect of atorvastatin on atherosclerosis, as determined by measurement of intima-media thickness (IMT) and intravascular ultrasound (IVUS). The effect of torcetrapib alone is unknown. Moreover, torcetrapib treatment led to adverse effects including an increase in cardiovascular events and increased death rate. In **chapter 5** we aimed to study the effects of torcetrapib with and without atorvastatin on the development of atherosclerosis and to study the adverse effects of torcetrapib. E3L.CETP mice on a western-type diet were treated with torcetrapib, atorvastatin or both, and atherosclerosis development was determined in the aortic root. Torcetrapib decreased plasma cholesterol, albeit to a lesser extent than atorvastatin or the combination of torcetrapib and atorvastatin. Torcetrapib increased HDL-C in the absence and presence of atorvastatin. Torcetrapib and

atorvastatin alone both reduced atherosclerotic lesion size to a similar extent, 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 (MCP-1) and resulted in lesions of a more inflammatory phenotype, as reflected by an increased macrophage content and reduced collagen content. We thus concluded that CETP inhibition by torcetrapib per se reduces atherosclerotic lesion size but does not enhance the anti-atherogenic potential of atorvastatin. In addition, as compared to atorvastatin, torcetrapib introduces lesions of a less stable phenotype.

Pregnane X receptor (PXR) agonism has been suggested to increase HDL levels in wild-type mice, but its effect on integrated HDL metabolism in a model with human-like lipoprotein metabolism was still unknown. In **chapter 6**, we treated E3L and E3L.CETP mice with the PXR agonist pregnenolone-16α-carbonitrile (PCN). In E3L and E3L.CETP mice, PCN increased liver lipids as well as plasma cholesterol and TG. Whereas PCN increased HDL-C, especially within large HDL-1 particles in E3L mice, it dose-dependently decreased HDL-C in E3L.CETP mice. PCN decreased expression of genes involved in HDL synthesis (Abca1, Apoa1), maturation (lecithin: cholesterol acyltransferase (Lcat), Pltp) and clearance (Sr-b1). The HDL-increasing effect of PCN, observed in E3L mice, is likely caused by a marked decrease in hepatic SR-BI protein expression, and is completely reversed by CETP expression. We concluded, therefore, that chronic PXR agonism dose-dependently reduces plasma HDL-C in the presence of CETP and is thus not a relevant target for the development of HDL raising therapy.

Apolipoprotein CI (apoCI) has been suggested to influence HDL metabolism by activation of LCAT and inhibition of HL and CETP. However, the effect of apoCI on SR-BI, as well as the net effect of apoCI on HDL metabolism *in vivo* is unknown. Therefore, we evaluated in **chapter 7** the effect of apoCI on the SR-BI-mediated uptake of HDL-CE in vitro and determined the net effect of apoCI on HDL metabolism in mice. We demonstrated that apoCI dose-dependently decreased the SR-BI-dependent association of HDL-CE with primary murine hepatocytes *in vitro*. Subsequent *in vivo* studies showed that apoCI-deficiency in mice gene dose-dependently decreased HDL-C levels, and adenovirus-mediated expression of human apoCI in mice increased HDL levels at a low dose and increased the HDL particle size at higher doses. Therefore, we concluded that apoCI is a novel inhibitor of SR-BI *in vitro* and increases HDL levels *in vivo*.
Since apoCI is the main endogenous HDL-associated CETP inhibitor, it can be a lead for the development of a new generation CETP inhibitors aimed at increasing HDL levels and reducing CVD risk. However, apoCI also inhibits

LPL activity which leads to hypertriglyceridemia, a risk factor for CVD. Therefore, in **chapter 8** we aimed to identify the minimal CETP-inhibitory domain of apoCI without LPL-inhibitory activity. We show that the CETP-inhibitory function of apoCI resides in the C-terminal helix, whereas the C terminus is not sufficient for the LPL-inhibitory function of apoCI. The C-terminal peptide $apoCI_{32-57}$ potently inhibited CETP activity, mainly caused by positively charged amino acids, with a negligible effect on LPL activity. Therefore, we concluded that $apoCI_{32-57}$ may be an interesting lead in the search for novel CETP inhibitors as a new strategy to increase HDL thereby reducing cardiovascular risk.

Taken together, the studies described in this thesis contribute to the understanding of the mechanisms underlying HDL-modulating strategies. We demonstrated that the HDL-raising effect of classical lipid-lowering drugs depends on the presence of CETP. We also showed that CETP inhibition may still be a potential atherogenic strategy, provided that CETP inhibitors do not adversely affect lesion composition. In addition, we identified the minimal CETP-inhibitory domain within apoCI that may provide a lead towards a new generation of safe CETP inhibitors.

Samenvatting

Hart- en vaatziekten vormen een belangrijke oorzaak van mortaliteit en morbiditeit in de westerse wereld, en worden voornamelijk veroorzaakt door atherosclerose. Een belangrijke risicofactor voor atherosclerose is dyslipidemie, die gekarakteriseerd wordt door een hoog plasmaniveau van het lage dichtheids lipoproteïne ((V)LDL) en een laag plasmaniveau van het hoge dichtheid lipoproteïne (HDL). Dyslipidemie wordt voornamelijk behandeld met lipidenverlagende medicijnen. Deze medicijnen verlagen de plasmaniveaus van (V)LDL-C zeer efficiënt (~40%) en geven tevens een milde verhoging van HDL, maar desondanks voorkomen zij slechts een fractie van alle hart- en vaatziekten (~30%). Daarom zijn nieuwe therapeutische strategieën om hart- en vaatziekten verder terug te dingen noodzakelijk. Omdat HDL op verschillende manieren kan beschermen tegen atherosclerose, namelijk door zijn rol in het reverse cholesterol transport en/of door middel zijn van anti-inflammatoire en anti-oxidatieve eigenschappen, wordt HDL verhoging gezien als een veelbelovende nieuwe manier om hart- en vaatziekten te voorkomen. In dit proefschrift werden de mechanismen onderzocht die aan de HDL-verhogende werking van de klassieke lipidenverlagende middelen atorvastatine, fenofibraat en niacine ten grondslag liggen. Daarnaast werden de effecten van potentiële nieuwe HDL verhogende strategieën, inclusief het experimentele geneesmiddel torcetrapib, en de lichaamseigen eiwitten pregnaan X receptor (PXR) en apolipoproteïne CI (apoCI), op het HDL metabolisme onderzocht. Voor deze studies maakten we gebruik van APOE*3-Leiden.CETP (E3L.CETP) transgene muizen, een waardevol nieuw muismodel voor het menselijke lipoproteïnen metabolisme.

Fibraten en statines verlagen plasmaniveaus van cholesterol en triglyceriden efficiënt in dyslipidemische patiënten. Daarnaast laten deze middelen een milde verhoging van het cholesterol in HDL (5-15%) zien terwijl het mechanisme dat hieraan ten grondslag ligt onduidelijk is. Omdat fibraten en statines de concentratie van het cholesteryl ester transfer proteïne (CETP) in het plasma verlagen, en omdat fibraten en statines HDL niet verhogen in muizen die van nature geen CETP tot expressie brengen, onderzochten wij de hypothese dat CETP een essentiële rol speelt bij het HDL-verhogende effect van zowel fibraten als statines. Hiervoor werd gebruik gemaakt van E3L en E3L.CETP transgene muizen die hetzelfde als de mens reageren op klassieke lipidenverlagende medicijnen zoals statines en fibrates met betrekking tot verlaging van lipiden in het plasma. In **hoofdstuk 2** werden E3L en E3L.CETP muizen gevoed met een vetrijk dieet in aan- en afwezigheid van fenofibraat. Fenofibraat verlaagde plasma (V)LDL-cholesterol (C) en (V)LDL-triglyceriden (TG) in zowel E3L als E3L.CETP muizen, maar verhoogde HDL-C alleen in E3L.CETP muizen. Fenofibraat beïnvloedde de klaring van HDL-cholesteryl

esters (CE) uit het plasma niet, wat erop wijst dat fenofibraat een hoger plasmaniveau van HDL-C veroorzaakt zonder de flux van het HDL-C te veranderen. Analyse van de expressie van genen in de lever die betrokken zijn bij het HDL metabolisme toonde aan dat fenofibraat de belangrijkste spelers in het HDL metabolisme, waaronder het fosfolipiden transport proteïne (Pltp), de ATP-bindende cassette transporter A1 (Abca1), de scavenger receptor B type I (SR-B1), en het apolipoproteïne AI (Apoa1)) niet verschillend beïnvloedde in E3L.CETP en E3L muizen. In E3L.CETP muizen verminderde fenofibraat wel de genexpressie van CETP evenals de overdracht van CE tussen lipoproteïnen in plasma. In **hoofdstuk 3** gebruikten we een vergelijkbare aanpak om te evalueren of atorvastatine HDL-C ook zou verhogen via CETP modulatie. Atorvastatine verlaagde plasma (V)LDL-C niveaus in zowel E3L als E3L.CETP muizen, maar verhoogde HDL-C uitsluitend in E3L.CETP muizen. Expressie van genen in de lever die betrokken zijn bij het HDL metabolisme (Pltp, Abca1, Sr-b1 en Apoa1) werden niet verschillend beïnvloed door atorvastatine in E3L.CETP en E3L muizen. In E3L.CETP muizen verlaagde atorvastatine echter wel de genexpressie van het CETP evenals het niveau van CETP en de activiteit van CETP in plasma. Daarom concludeerden wij dat zowel fenofibraat als atorvastatine HDL-C verhogen door de CETP-afhankelijke overdracht van CE van HDL naar (V)LDL te verlagen. Dit wordt veroorzaakt door een lagere hepatische CETP expressie en een lagere hoeveelheid (V)LDL in het plasma.
Het meest krachtige medicijn wat momenteel beschikbaar is om HDL te verhogen is niacine. Het mechanisme dat aan de verhoging van HDL ten grondslag ligt is echter nog onbekend. In **hoofdstuk 4** voerden wij E3L en E3L.CETP muizen een vet rijk dieet met of zonder niacine. In E3L.CETP muizen verlaagde niacine het plasma cholesterol en TG op een dosisafhankelijke wijze. Niacine verhoogde het HDL-C niveau ook op een dosisafhankelijke manier, net als het plasmaniveau van apoAI en de deeltjesgrootte van het HDL. In E3L muizen verlaagde niacine het plasmaniveau van cholesterol en TG terwijl de verhoging van HDL-C niet werd waargenomen. In E3L.CETP muizen verminderde niacine dosisafhankelijk de hepatische expressie van CETP evenals de massa en activiteit van CETP in plasma. Bovendien reduceerde niacine op een dosisafhankelijke manier de opname van apoAI vanuit het plasma door de nieren. Daarom concludeerden wij dat niacine het HDL-C in E3L.CETP muizen verhoogt door de CETP activiteit te verlagen, als gevolg van zowel een verlaagde CETP expressie als een verminderde hoeveelheid (V)LDL in het plasma. Daarnaast verhoogt niacine apoAI door de klaring van apoAI uit plasma te verminderen.

Torcetrapib is het eerste medicijn dat specifiek is ontworpen om HDL te verhogen, namelijk door de activiteit van CETP in het plasma te remmen. In grote klinische studies bleek torcetrapib inderdaad in staat het HDL met ongeveer 60% verhogen. Ondanks deze forse HDL verhoging werd in patiënten

die met atorvastatine en torcetrapib werden behandeld echter geen vermindering van atherosclerose gevonden in vergelijking met patiënten die alleen met atorvastatine werden behandeld. Het effect van torcetrapib zonder atorvastatine was vooralsnog onbekend. Daarnaast leidde de behandeling met torcetrapib tot ongunstige bijwerkingen zoals een verhoging van cardiovasculaire gebeurtenissen en een verhoogd sterftecijfer. In **hoofdstuk 5** werd het effect van torcetrapib met en zonder atorvastatine in muizen bestudeerd om meer inzicht te krijgen in de potentiële anti-atherogene werking en de bijwerkingen van torcetrapib. E3L.CETP muizen werden gevoerd met een vetrijk dieet waaraan torcetrapib, atorvastatine of beide werden toegevoegd, en de ontwikkeling van atherosclerose werd vervolgens na 14 weken beoordeeld in het kleppengebied van het hart. Torcetrapib verminderde het plasmaniveau van cholesterol, hoewel in mindere mate dan atorvastatine en combinatie van torcetrapib en atorvastatine. Daarnaast verhoogde torcetrapib het HDL-C. Zowel torcetrapib als atorvastatine verminderden atherosclerose, en wel in gelijke mate, maar de combinatie van beide middelen had geen additionele anti-atherogene werking. Vergeleken met atorvastatine veroorzaakte torcetrapib een verhoogde binding van monocyten aan het vaatendotheel, wat gepaard ging met een verhoogde hoeveelheid van het monocyt chemoattractant proteïne-1 (MCP-1) in de atherosclerotische plaques. Dit resulteerde in laesies met meer macrofagen en minder collageen, die in de mens minder stabiel zijn en kunnen scheuren. Wij concludeerden daarom dat remming van CETP door torcetrapib op zich atherosclerose kan verminderen. Echter, net zoals in de mens is torcetrapib niet in staat het anti-atherogene effect van atorvastatine te versterken. Bovendien introduceert torcetrapib laesies van een minder stabiel fenotype vergeleken met atorvastatine.

Er is in de literatuur gesuggereerd dat agonisten van de pregnaan X receptor (PXR) het HDL kunnen verhogen. Deze suggestie hebben wij onderzocht in ons muismodel met een menselijk lipoproteïnen metabolisme. In **hoofdstuk 6** behandelden wij E3L en E3L.CETP muizen met de PXR agonist pregnenolon-16α-carbonitril (PCN). In E3L en E3L.CETP muizen verhoogde PCN lipiden in de lever evenals cholesterol en TG in plasma, terwijl PCN in E3L muizen het cholesterol specifiek in HDL verhoogde, vooral in grote HDL-1 deeltjes. In E3L.CETP muizen gaf PCN een dosisafhankelijke verlaging van het HDL. PCN verminderde de expressie van genen in de lever betrokken bij de synthese (Abca1, Apoa1), maturatie (lecithin: cholesterol acyltransferase (Lcat), Pltp) en klaring (SR-B1) van het HDL. Het HDL-verhogende effect van PCN, dat in E3L muizen werd waargenomen, wordt waarschijnlijk veroorzaakt door een daling van de HDL receptor SR-BI in de lever. Deze HDL verhoging wordt echter door CETP expressie volledig tenietgedaan. Wij concludeerden daarom dat chronisch PXR agonisme in de aanwezigheid van CETP het HDL-C

dosisafhankelijk verlaagt en daarom geen relevante strategie is voor de ontwikkeling van HDL-verhogende medicijnen.

Er is aangetoond dat het plasma eiwit apoCI het HDL metabolisme zou kunnen beïnvloeden door activatie van LCAT, en remming van HL en CETP. Het effect van apoCI op SR-BI en het netto effect van apoCI op HDL niveaus was echter nog onbekend. In **hoofdstuk 7** werd daarom het effect van apoCI op de SR-BI-gemedieerde opname van HDL-CE *in vitro* geëvalueerd en het netto effect van apoCI op het HDL metabolisme in muizen bepaald. Wij toonden aan dat apoCI de SR-BI-afhankelijke associatie van HDL-CE met muizenhepatocyten vermindert. Daarnaast verminderde apoCI-deficiëntie in muizen op een dosisafhankelijk wijze het HDL-C niveau. Een matige overexpressie van apoCI in muizen leidde tot een verhoogd HDL-C niveau, en een hogere overexpressie van apoCI leidde tot een vergroting van de HDL deeltjes. Daarom concludeerden wij dat apoCI een nieuwe remmer is van SR-BI *in vitro* en dat apoCI het HDL niveau verhoogt *in vivo*.
Omdat apoCI de belangrijkste fysiologische HDL-geassocieerde CETP remmer is kan apoCI een basis vormen voor de ontwikkeling van nieuwe CETP remmers om HDL te verhogen en het risico op hart- en vaatziekten te verminderen. ApoCI remt echter ook de activiteit van LPL wat leidt tot hypertriglyceridemia, en dus een atherogene eigenschap is. Daarom was het doel in **hoofdstuk 8** om het minimale CETP remmende domein van apoCI zonder LPL remmende eigenschappen te identificeren. Wij toonden aan dat de CETP remmende functie van apoCI zich vooral bevindt in het C-terminale domein van apoCI, en dat de C terminus niet voldoende is voor de LPL remmende functie van apoCI. $ApoCI_{32\text{-}57}$ remde de activiteit van CETP krachtig, hoofdzakelijk gemedieerd door positief geladen aminozuren, terwijl $apoCI_{32\text{-}57}$ LPL niet remde. Daarom concludeerden wij dat $apoCI_{32\text{-}57}$ een interessante basis kan zijn in de zoektocht naar nieuwe CETP remmers om HDL te verhogen en het risico op hart- en vaatziekten te verlagen.

Samengenomen dragen de studies die in dit proefschrift worden beschreven bij tot het begrip van de mechanismen die aan HDL modulerende strategieën ten grondslag liggen. Wij toonden aan dat het HDL verhogende effect van klassieke lipidenverlagende middelen afhankelijk is van de aanwezigheid van CETP. Daarnaast toonden wij aan dat de remming van CETP nog steeds een potentiële anti-atherogene strategie kan zijn, met als voorwaarde dat nieuwe CETP remmers de laesie samenstelling niet ongunstig beïnvloeden. Bovendien identificeerden wij het minimale CETP remmende domein binnen apoCI dat een basis voor een nieuwe generatie van veilige CETP remmers kan vormen.

List of abbreviations

aa	amino acid
ABCA1/G1/G4	adenosine triphosphate-binding cassette transporter A1/G1/G4
AcLDL	acetylated LDL
AdAPOC1	adenovirus expressing human APOC1
AdLacZ	adenovirus expressing ß-galactosidase
APOC1	human apoCI expressing mice
Apo	apolipoprotein
Bp	base pair
BSA	bovine serum albumin
C	cholesterol
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CM	chylomicron
CO	cholesteryl oleate
COEth	cholesteryl oleoyl ether
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E3L	APOE*3-Leiden
FC	free cholesterol
FCR	fractional catabolic rate
FFA	free fatty acids
FPLC	fast-performance liquid chromatography
HDL	high density lipoprotein
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPRT	hypoxanthine phosphoribosyltransferase
HRP	horse radish peroxidase
HSL	hormone sensitive triglyceride lipase
HSPG	heparin sulfate proteoglycans
IDL	intermediate density lipoprotein
IMT	intima-media thickness
IVUS	intravascular ultrasonography
LCAT	lecithin:cholesterol acyl transferase
LDL	low density cholesterol
LDLr	LDL receptor
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LRP	LDL receptor related protein

LXR	liver X receptor
MCP1	monocyte chemotactic protein-1
MTP	microsomal triglyceride transfer protein
mRNA	messenger RNA
PBS	phosphate buffered phosphate
PCN	pregnenolone-16α-carbonitrile
PCR	polymerase chain reaction
Pfu	plaque forming unit
PL	phospholipid
PLTP	phospholipid transfer protein
PPARα	peroxisome proliferator-activated receptor α
PPRE	peroxisome proliferator response element
PXR	pregnane X receptor
RCT	reverse cholesterol transport
RNA	ribonucleic acid
RXR	retinoid X receptor
SD	standard deviation
SEM	standard error of the mean
SRA	scavenger receptor class A
SR-BI	scavenger receptor class B type I
TC	total cholesterol
TG	triglyceride
VLDL	very low density lipoprotein
VLDLr	VLDL receptor
WAT	white adipose tissue
WT	wild type

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Curriculum Vitae

Willemke de Haan werd geboren op 12 september 1980 te Drachten. Na het behalen van haar VWO diploma in 1999 aan de Christelijke Scholengemeenschap Liudger te Drachten studeerde zij Biomedische Wetenschappen aan de Universiteit van Leiden. Ze behaalde in 2000 haar propedeutisch diploma en in 2002 haar Bachelor diploma. Tijdens de Master fase heeft zij een drietal stages doorlopen. Tijdens haar eerste stage bij de afdeling Immunohematologie en Bloedtransfusie van het Leids Universitair Medisch Centrum (LUMC) deed zij onderzoek naar de gevoeligheid van gluten voor enzymatische afbraak in het maag-darmkanaal onder begeleiding van Dr F Koning. Tijdens haar tweede stage heeft zij op de afdeling Humane en Klinische Genetica, begeleid door Dr CHM van Moorsel en Dr CL Harteveld, meegeholpen met het opzetten van een microarray om β-thalassemie mutaties te detecteren. Haar derde stage voerde ze uit onder begeleiding van Dr M Westerterp en Dr PCN Rensen bij TNO-Kwaliteit van Leven waarbij zij onderzoek verrichtte naar het mechanisme achter de lipidenverhogende effecten van het bloedeiwit apoCI. In 2004 behaalde zij haar Master diploma (*cum laude*) en startte ze als assistent in opleiding met haar promotieonderzoek op de afdeling Endocrinologie en Stofwisselingsziekten van het LUMC onder begeleiding van haar promotores Dr LM Havekes en Dr JW Jukema en haar copromotor Dr PCN Rensen. Zij werkte nauw samen met de groep van Dr HMG Princen, werkzaam bij TNO-Kwaliteit van Leven. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Zij won een Poster Award tijdens de Gordon Research Conference on Lipoprotein Metabolism in zowel 2006 als 2008, de Poster Award van de 29th Annual Meeting of the European Lipoprotein Club in 2006 en de 11^{e} Vasculaire Biologie Werkgroep Jaarprijs 2008 (derde prijs) voor haar publicatie in Circulation. Vanaf maart 2009 hoopt zij als postdoc onderzoek te verrichten naar de relatie tussen het lipidenmetabolisme en diabetes in de groep van Prof Dr MR Hayden te Vancouver, Canada. Hiervoor heeft zij een Rubicon subsidie van NWO toegekend gekregen.