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

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Chapter 1

GENERAL INTRODUCTION

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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 endoplasmic 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 then 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 ABCA1 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 esterification 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-lipoxygenase-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. Dyslipidemia 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 derivatives, and HIV protease inhibitors.^{43,44} Dyslipidemia is a major risk factor for coronary heart disease (CHD) (see section 2.2). In addition to dyslipidemia, other lipid-unrelated factors can also increase CHD risk. These lipid unrelated risk factors for CHD include homocysteinemia, hypertension and infection.⁴⁵

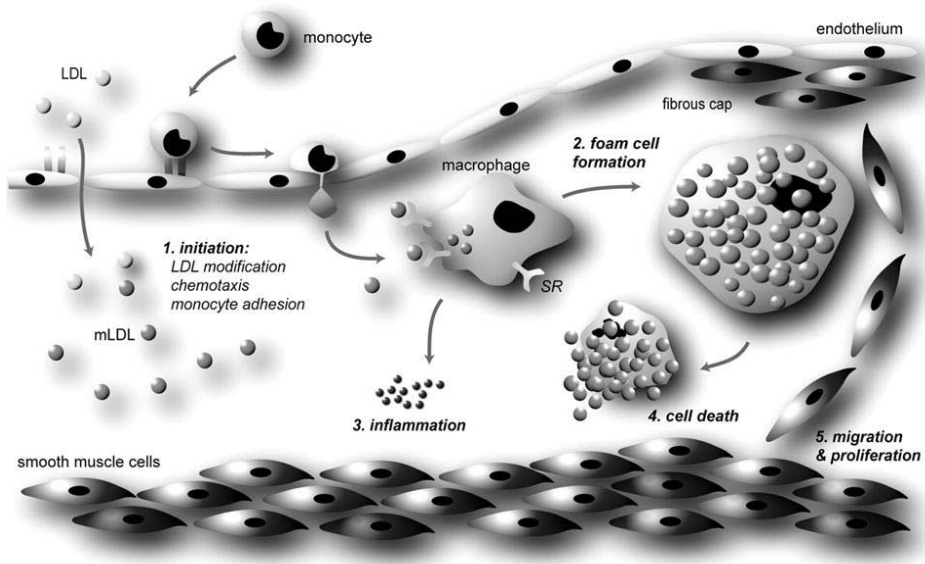


Figure 2. pathogenesis of atherosclerosis development. Adapted from de Winther *et al.*⁴⁶ See text for explanation.

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, β 2 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. Vulnerable 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 quails⁴⁹⁻⁵¹ 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 diet⁶¹⁻⁶⁴ 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 development⁶¹⁻⁶⁴ 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 LPL⁹³ 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 -C629A¹³⁹ 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 atherosclerosis¹⁵⁴ 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 eicosanoids and heterodimerize with RXR to affect gene expression by binding to DR-1 responsive elements. PPAR α 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). PPAR γ is expressed in adipose tissue, macrophages, colon and placenta and is important in regulation of lipid and glucose metabolism and adipocyte differentiation. PPAR γ activation makes tissues more insulin sensitive and agonists are therefore applied in diabetes. PPAR δ is expressed at low levels in a variety of tissues, is involved in lipid and glucose metabolism¹⁵⁹⁻¹⁶² 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 SREBP1c 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, LXR α and LXR β which are both activated by oxysterols (*i.e.* cholesterol derivatives). LXR α is mainly expressed in liver and macrophages while LXR β 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 mass¹⁷⁶⁻¹⁷⁸ 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 PPAR α 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 LPL¹⁸⁶ and downregulation of the LPL inhibitor apoCIII.¹⁸⁷⁻¹⁸⁹ TG may also be reduced by increased hepatic β -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 fibrates¹⁹⁵ 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 progression³⁸ and overall mortality (-11%)²⁰⁸ but is not well tolerated because it causes severe flushing via increasing plasma prostaglandins.

Addition of the prostaglandin D₂ 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-16 α -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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