Novel mechanistic insight in cholesteryl ester transfer protein production and pharmacological inhibition

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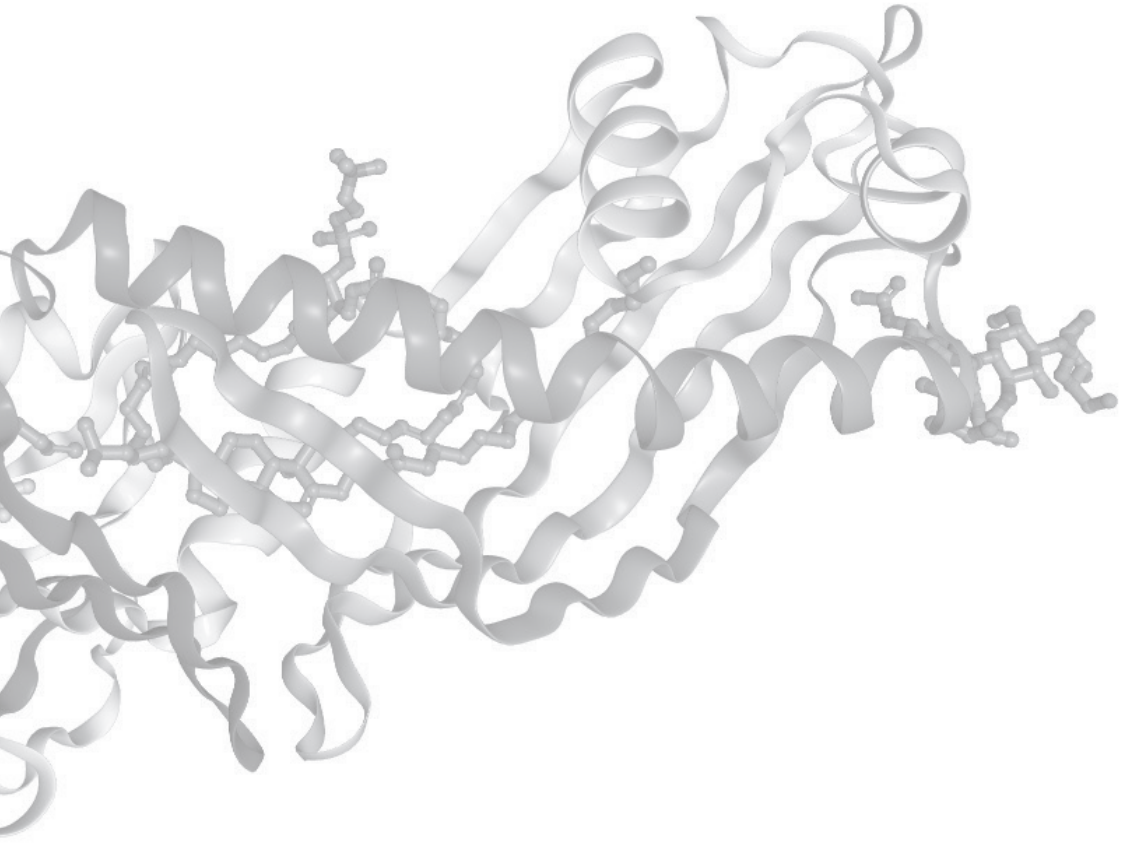
Chapter 1

General introduction



One of the largest health problems in the Western world is cardiovascular disease (CVD),[1, 2] with atherosclerosis being the main pathophysiological cause, resulting in cardiovascular morbidity and mortality. Atherosclerosis is a disease affecting the vessel wall associated with the local accumulation of lipids, immune cells, smooth muscle cells and connective tissue. Accumulation of these constituents leads to the progressive narrowing of the vessel wall and subsequently to a decrease in blood flow to the organs. Progression of local narrowing and disease are associated with pain (angina pectoris) and may ultimately lead to plaque rupture and subsequent heart attack, stroke or even death.

Epidemiological studies have identified several risk factors associated with the development of atherosclerosis e.g. genetic predisposition, smoking, hypertension, age, gender, obesity, inflammation and dyslipidaemia.[3, 4] Dyslipidaemia is characterized by increased levels of triglycerides (TG) and (very) low density lipoprotein [(V)LDL]-cholesterol (C), and decreased levels of high density lipoprotein (HDL)-C. Inflammation is characterized by e.g. increase levels of the acute phase marker C-reactive protein (CRP) and cytokines such as tumor necrosis factor α (TNFα). Currently, the standard treatment for the reduction of CVD risk is statin therapy aimed at reducing plasma (V)LDL-C, with lowering of inflammation as a pleiotropic effect. However, a substantial residual risk remains, which has triggered the search for additional treatment strategies.[5, 6] The observation of an inverse association between HDL-C level and CVD risk,[7, 8] and the fact that cholesteryl ester transfer protein (CETP) decreases HDL-C, has made CETP an important therapeutic target for lowering CVD risk. This has led to the development of several CETP-inhibitors, which are in different stages of clinical trials.

LIPIDS AND LIPOPROTEIN METABOLISM

The main lipid components of our diet are TG and cholesterol. TG are an important source of energy in the body and cholesterol is an essential component of cell membranes as well as the precursor for bile acids, hormones and vitamin D. Since lipids are hydrophobic and thus insoluble in blood they are transported in hydrophilic lipoproteins. Lipoproteins consist of a hydrophobic core containing TG and cholesteryl esters (CE), and a surface containing phospholipids, unesterified cholesterol and apolipoproteins. Based on their density, lipoproteins can be divided into 5 main groups (from lowest to highest density): chylomicrons, VLDL, intermediate density lipoprotein, LDL and HDL. In the subsequent paragraphs, specific aspects of lipoprotein metabolism and especially CETP that are relevant to the subsequent chapters are explained, and their potential roles in atherogenesis are discussed shortly.

Chylomicrons and (V)LDL

Figure 1 represents a schematic overview of lipoprotein metabolism. After a meal, dietary TG and cholesteryl esters are broken down in the intestinal lumen, absorbed by enterocytes in the intestine and resynthesized, assembled in chylomicrons and secreted via the lymph into the circulation. In periods between meals, the liver produces TG-rich VLDL. The principal function of chylomicrons/VLDL is the transport TG from the intestine/liver to peripheral organs. TG used for VLDL assembly are synthesised *de novo*, or are derived from TG-derived fatty acids (FA), after hepatic uptake of chylomicron remnants or VLDL remnants. However, VLDL also contains both free and esterified cholesterol. Cholesterol is obtained from lipoprotein remnants or by *de novo* cholesterol synthesis. Cholesterol is synthesized predominantly by liver cells from relatively simple molecules via a complex 37-step process. The reduction of 3-hydroxy-3-methylglutaryl CoA to mevalonate by the enzyme HMG-CoA reductase (HMGCR) is the rate-limiting step. The key structural protein component of chylomicrons and VLDL is apolipoprotein (apo) B. When chylomicrons and VLDL arrive via the circulation in metabolically active tissues, their TG are hydrolysed by lipoprotein lipase (LPL) into FA and glycerol.[9] These FA are taken up by the skeletal muscle and heart for use as an energy source, by brown adipose tissue for thermogenesis, and by white adipose tissue for storage.[10] Upon lipolysis, chylomicrons and VLDL become so called remnants enriched in CE and acquire ApoE.[11, 12] These remnants are cleared by the liver predominantly via the ApoE-LDL receptor (LDLr) pathway, although the LDLr-related protein-1 (LRP1) is also involved.[13] The VLDL remnants can also be further lipolysed and processed in the circulation to generate LDL.[9, 14] LDL is virtually depleted of TG and rich in CE. LDL can be taken up via the LDLr by the liver, but also by extra-hepatic tissues that need cholesterol.[15, 16]

HDL

The main function of HDL in lipid metabolism is to acquire excess cholesterol from peripheral tissues and transport it to other lipoproteins or back to the liver (so called reverse cholesterol transport). ApoA1, the most abundant apolipoprotein of HDL, is synthesized in the liver and the intestine. After being released into the circulation, ApoA1 is lipidated with phospholipids via the ATP binding cassette transporter A1 (ABCA1), to form nascent discoidal HDL. This HDL particle can take up cholesterol from various peripheral tissues. The acquired cholesterol is esterified by lecithin-cholesterol-acyltransferase (LCAT) and accumulates in the core of the HDL particle. The HDL becomes a more mature spherical HDL particle and acquires additional apolipoproteins from the circulation. The maturation also results in an increased affinity for ATP binding cassette transporter G1 (ABCG1) and scavenger receptor-BI (SR-BI), to increase the cholesterol efflux from tissues.[17, 18] Subsequently, CE in HDL are selectively taken up by the liver,[19] and can be used for storage, assembly of VLDL, or for excretion into the intestine as neutral sterol or bile acids.[20] Alternatively, in humans and some other

species, CE in HDL can be transferred to ApoB-containing lipoproteins by cholesteryl ester transfer protein (CETP) in exchange for TG.

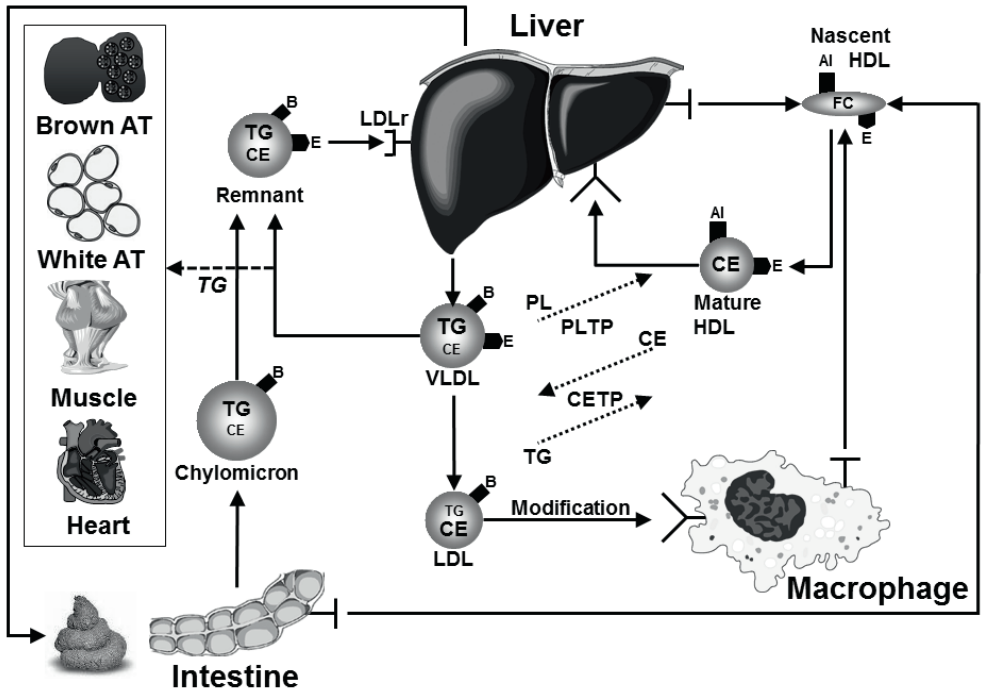


Figure 1: Schematic overview of lipoprotein metabolism
AT, adipose tissue; TG, triglycerides; LDLr, LDL receptor; CE, cholesteryl ester; E, ApoE; B, ApoB; AI, ApoAI; FC, free cholesterol; C, cholesterol; PL, phospholipids; PLTP, phospholipid transfer protein; CETP, cholesteryl ester transfer protein.

CETP as a modulator of lipoprotein metabolism

At least two lipid transfer proteins can be found in human plasma; phospholipid transfer protein (PLTP) and CETP. PLTP transfers phospholipids from TG-rich lipoproteins to HDL during their lipolytic conversion by LPL, thereby enabling maturation of HDL. In this thesis, we will focus on CETP that promotes the exchange of CE and TG between plasma lipoproteins. CETP is a 74 kDa glycoprotein that is expressed by several species, including humans, monkeys, rabbits, hamsters and pigs, but not by rats and mice.[21-23] In humans, expression of CETP is described in the liver and adipose tissue, but also to some extent in spleen, heart, small intestine, adrenal gland, kidney and skeletal muscle.[21, 22, 24, 25] CETP expression is regulated by various factors, among which are sterol regulatory element binding protein (SREBP), the liver-X-receptor (LXR) and farnesoid-X-receptor (FXR).[24-30] Albeit that CETP seems to be expressed by multiple organs, the relative contribution of these organs to whole-body CETP production, and the cellular origin are still under debate. The protein structure of CETP

reveals a curved molecule with N- and C- terminal cavities and a tunnel spanning the entire length of the protein, which can accommodate neutral lipids such as CE and TG.[31] CETP is secreted into the plasma where it binds to HDL. The net-effect of CETP activity is a transfer of CE from HDL to chylomicrons/VLDL in exchange for TG.[32]

Dyslipidaemia and atherosclerosis development

As mentioned above, dyslipidaemia is an important risk factor for the development of atherosclerosis. LDL is considered to be pro-atherogenic and HDL to be anti-atherogenic. The role of CETP in the development of atherosclerosis is currently under debate.

The development of an atherosclerotic plaque starts with the infiltration of atherogenic lipoproteins such as LDL or lipoprotein remnants into the vessel wall. Thus, increased levels of these lipoproteins are obvious causes for the increase of atherosclerosis development. The infiltrated lipoproteins undergo modification (e.g. oxidation and/or aggregation) resulting in a signal for the activation of endothelial cells and the recruitment of immune cells (neutrophils, T- and B-cells, and monocytes). Infiltrating monocytes differentiate into macrophages and start to phagocytose the modified lipoproteins, turning the macrophages into lipid-laden “foam cells”. These foam cells are the first markers for atherosclerosis development.

HDL has a dual anti-atherogenic role. Firstly, HDL scavenges the cholesterol from “foam cells” in the atherosclerotic plaque. This cholesterol is esterified by LCAT into CE and transported by HDL to the liver where it can be excreted as neutral sterol or as bile acid. This is generally called reverse cholesterol transport (RCT). Studies have shown that increasing RCT reduces the development of atherosclerosis.[33] Secondly, in addition to its role in cholesterol metabolism, it is proposed that HDL has a variety of anti-inflammatory, anti-microbial and anti-oxidant properties,[34-37] contributing to the anti-atherogenic properties of HDL.

CETP activity decreases HDL-C levels and is considered to be pro-atherogenic and indeed in several mouse models, including C57Bl/6J, Ldlr$^{-/-}$, Apoe$^{-/-}$ and APOE*3-Leiden mice, CETP expression aggravates the development of atherosclerosis.[38-40] Genetic variants of the CETP gene, that are associated with decreased plasma CETP concentration and activity, are associated with increased HDL-C levels. Moreover, homozygous CETP deficiency results in decreased plasma LDL-C and ApoB levels.[41] This suggests that reduced CETP concentration and activity beneficially affect lipoprotein metabolism and possibly the development of atherosclerosis. However the relation between CETP deficiency and CVD risk in humans is controversial CVD.[42, 43] Moreover, a meta-analysis showed that CETP polymorphisms associated with decreased CETP activity are associated with a decrease in CVD risk.[44] However, other studies find that CETP polymorphisms, despite raising HDL-C, do not alter CVD risk[42, 45] or even increase CVD risk.[46]

Commonly used wild-type mice (C57Bl/6) have a very rapid clearance of ApoB-containing lipoproteins. To mimic the slower clearance observed in humans, a transgenic mouse model has been developed expressing a dominant mutation in APOE, called APOE*3-Leiden mice.[47] Patients carrying the APOE*3-Leiden gene have increased levels of lipoprotein remnants, and increased susceptibility to atherosclerosis. The E3L mice have been intercrossed with human CETP-expressing mice (APOE*3-Leiden.CETP mice)[40] and both APOE*3-Leiden and APOE*3-Leiden.CETP mice have an attenuated clearance of TG-rich lipoproteins and increased TG level.[48] Similar to patients carrying the APOE*3-Leiden variant, in APOE*3-Leiden and APOE*3-Leiden.CETP mice, a major part of plasma cholesterol is contained in the VLDL (remnant) particles, so called β-VLDL particles, which further increase after cholesterol feeding. The APOE*3-Leiden.CETP mouse model, unlike Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice, responds in a human-like way to the lipid lowering effects of statins,[49] fibrates,[50] niacin,[51, 52] torcetrapib[53] and anti-PCSK9mabs,[54] with respect to both direction and magnitude of the change. In conclusion, APOE*3-Leiden.CETP mice have a more human-like lipoprotein metabolism when compared to C57Bl/6, Apoe$^{-/-}$ or Ldlr$^{-/-}$ mice.

MACROPHAGES

White blood cells, or leukocytes, are a diverse group of cells that are crucial to the body's immune response. They circulate through the blood and are recruited to sites of inflammation and damage. The different types of leukocytes have a common origin in hematopoietic stem cells and develop along distinct differentiation pathways. Two types of common progenitor cells exist, common lymphoid progenitor cells (that give rise to T-, B-, and natural killer cells) and common myeloid progenitor cells (that give rise to granulocytes, erythrocytes and monocytes). Common myeloid progenitor cell-derived monocytes give rise to a large variety of macrophages throughout the body, as well as dendritic cells and osteoclasts.

Tissue-resident macrophages

Macrophages are equipped with a range of pathogen-recognition receptors that make them efficient in phagocytosis and that induce the production of inflammatory cytokines.[55] Macrophages have frequently been grouped into two functionally different classes using the 'M1-M2 paradigm'.[56] M1 macrophages, derived from the pro-inflammatory monocytes, exhibit anti-microbial properties and promote an interleukin-1 and -12 mediated T-helper 1 response. On the other hand, M2 macrophages support an anti-inflammatory T-helper 2 response and play a role in the resolution of inflammation.

The majority of tissues contain tissue-resident macrophages, e.g. brain (microglia), skin (Langerhans cells), spleen (marginal zone macrophages), and liver (Kupffer cells). Tissue-

resident macrophages are a heterogeneous population of macrophages that fulfil tissue-specific functions. These range from dedicated homeostasis, such as clearance of cellular debris (e.g. apoptotic cells), growth factor production and iron processing, to central roles in tissue immune surveillance and the resolution of inflammation. According to the 'M1-M2 paradigm', tissue-resident macrophages are classified as M2-macrophages, which relates to their role in maintenance of homeostasis and the resolution of inflammation.[57, 58]

Kupffer cells are one of the largest populations of tissue macrophages[59] and were first observed by Karl Wilhelm von Kupffer in 1876.[60] He described them as "specialized endothelial cells that line the sinusoids of the liver and form part of the reticuloendothelial system".[60] Von Kupffer called these cells 'sternzellen' ("star cells"). They are predominantly distributed in the lumen of hepatic sinusoids and are a component of the innate[61] and the adaptive[62, 63] immune system. The main role of Kupffer cells is to eliminate pathogens from blood,[64, 65] in some extent to regulate liver regeneration[66, 67] and bilirubin metabolism.[65, 67] In addition, Kupffer cells are known to play a role in the pathogenesis of various liver diseases.

Macrophages and atherosclerosis development

Monocytes and macrophages play an important role in the development and stability of an atherosclerotic plaque. Invading monocytes differentiate into macrophages and start to engulf the infiltrated and modified lipoproteins via scavenger receptor A (SRA) and CD36. Unlimited uptake turns them into lipid-laden "foam cells" that are the first markers of a 'fatty streak' in the vessel wall. These fatty streaks or mild plaques consisting of primarily foam cells mostly cause no clinical symptoms and can reverse. Progression of mild plaques into more severe plaques is the consequence of the infiltration of additional immune cells and the production of pro-inflammatory cytokines and chemokines by activated endothelial and immune cells. In response, smooth muscle cells proliferate and migrate towards the endothelium to form a fibrous cap. If this cap is strong enough, it stabilizes the plaque, preventing the plaque from rupture. However, necrosis of the foam cells and/or smooth muscle cells, resulting in a necrotic core, destabilizes the plaque, and might cause rupture. Thus, plaque stability is determined by the composition of the plaque. Stable plaques have a thick fibrous cap and a low number of foam cells, whereas vulnerable plaques have a thin fibrous cap and a high number of foam cells and/or a necrotic core. Rupture of the plaque might lead to coagulation and thrombus formation, causing an infarction or stroke.[4, 68, 69]

PHARMACOLOGICAL INTERVENTION FOR CARDIOVASCULAR DISEASE

The standard treatment for dyslipidaemia and to halt and even reduce atherosclerosis development, thereby reducing cardiovascular risk, is statin therapy aimed at reducing plasma (V)LDL-cholesterol.

Statins

Statins are inhibitors of HMGCR. As mentioned above, HMGCR is the rate limiting enzyme in the cholesterol biosynthesis pathway. Statins block the binding of 3-hydroxy-3-methylglutaryl-coenzyme A to HMGCR and thereby the formation of mevalonate, a precursor of cholesterol.[70, 71] The reduced cholesterol production results in a reduction of VLDL secretion and thereby less LDL formation,[72] and less atherosclerosis development.[73] Statin treatment not only reduces the cholesterol content of the liver, but also upregulates the hepatic LDL receptor,[74-76] further reducing the plasma (V)LDL-C. A pleiotropic mechanism which is thought to also play a role in the reduction of CV risk is the anti-inflammatory properties of statins.[77] It has been shown in experimental and clinical studies, that statins decrease inflammation,[78] decrease monocyte adherence to the plaque[79, 80] and reduce the inflammatory biomarker C-reactive protein (CRP).[81-84] These effects are largely independent of lowering (V)LDL-C in the plasma.[83]

Intervention trials provide ample evidence that lowering of LDL-C contributes to a reduction in CVD risk.[5, 85-87] Although a substantial CVD risk remains and some patients do not reach the recommended LDL-C target, statin treatment remains the most effective treatment for CVD.[6, 87] However, this residual CV risk has prompted the search for secondary treatment targets.[5, 6] Already in the 1970-80s, Castelli *et al*[7, 8] showed in the Framingham Heart Study that subjects with low levels of HDL-C have similar risk for CVD as compared to those with high levels of LDL-C. These observations, and prospective epidemiological studies, have indicated that raising-HDL-C may be a suitable potential secondary target for the treatment of CVD.[88] The inverse association of HDL-C with CVD risk and the fact that CETP plays a critical role in HDL metabolism has made CETP an important therapeutic target to modulate HDL-C levels. In addition, mutations that cause CETP deficiency or reduce CETP mass and/or activity lead to increased HDL-C levels.[89-94] This has led to the development of several CETP-inhibitors, e.g. torcetrapib, dalcetrapib, anacetrapib and evacetrapib.

CETP inhibitors

Torcetrapib, although reducing (V)LDL-C up to 25% and increasing HDL-C up to 72%, failed in a phase III clinical trial (ILLUMINATE).[95] Despite improving the lipoprotein profile, torcetrapib increased the risk of CVD events and mortality. The detrimental effects were ascribed to off-target effects that included a blood pressure raising effect a decrease in serum potassium, and increases in serum sodium, bicarbonate, and aldosterone.[95] However, post-hoc studies showed that the raise in blood pressure could not explain the increased CV mortality.[95] Studies in APOE*3-Leiden mice showed that torcetrapib also induced a pro-inflammatory plaque phenotype and failed to reduce atherosclerosis development beyond atorvastatin.[53] A second CETP phase III clinical trial with dalcetrapib (dal-OUTCOMES) was also prematurely terminated. Although dalcetrapib increased HDL-C up to 40%, no additional clinical benefit

was observed beyond statin treatment, most probably due to the minimal reduction in LDL-C.[96] Also no adverse blood pressure effect was observed in the dal-OUTCOMES trial.[97] A third CETP phase III clinical trial with evacetrapib (ACCELERATE) was also recently stopped due to insufficient efficacy.[98] Nonetheless, the effects of the more potent CETP inhibitor anacetrapib in patients on standard statin treatment on CV outcome is currently being evaluated (REVEAL), and results are to be expected in 2016-17.[99] In phase II clinical trials, anacetrapib decreased LDL-C up to 40% and increased HDL-C up to 130%[100, 101] without any indication for an off-target blood pressure effect[100, 102, 103] as observed with torcetrapib.[104]

In addition, the effects of two CETP inhibitors, DRL-17822, TA-8995 (DEZ-001) are still being tested in phase II and III clinical development. Next to that, dalcetrapib is being reinvestigated after a genetically distinct patient population demonstrated a significant reduction in cardiovascular events.[105]

THESIS OUTLINE

The overall aim of this thesis was to gain insight in the mechanism underlying the effects of CETP on atherosclerosis. To this end, we examined the cellular origin of CETP and gained insight in the effect of CETP inhibition on lipid metabolism and the development of atherosclerosis.

After a general introduction (**chapter 1**) we addressed the cellular origin of CETP, both in humans and APOE*3-Leiden.CETP transgenic mouse in **chapter 2**. Previous studies have indicated that adipose tissue and the liver are the two major sources of CETP. However, our data show that the liver and more specifically Kupffer cells are the principal source of CETP. In **chapter 3** we further characterized the specific Kupffer cell subset responsible for CETP production. And in **chapter 4** we investigated the effect of intraperitoneal lipopolysaccharide injection on hepatic macrophage activation, CETP expression, and plasma lipid and lipoprotein levels.

In addition, we set out to evaluate whether CETP can serve as a target for treatment of atherosclerosis. We examined the effect of inhibiting CETP activity by anacetrapib on the development of atherosclerosis in the APOE*3-Leiden.CETP mouse model (**supplementary chapter 1**). In addition, we examined the effects of anacetrapib on HDL function and the possible additive/synergistic effects of anacetrapib to atorvastatin on plasma lipid levels and atherosclerosis prevention. In **chapter 5**, the mechanism by which anacetrapib decreases (V)LDL-C was elucidated.

Finally, in **chapter 6** the major results and implications of this thesis are discussed.

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