Hyperlipidemia, inflammation and atherosclerosis : roles of apolipoprotein C1 and cholesteryl ester transfer protein

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CHAPTER 1

General Introduction

Contents

Cardiovascular disease (CVD) is the first cause of death in the Western world and its prevalence is increasing in Eastern Europe and developing countries. The main cause of CVD is atherosclerosis.[1-3] Atherosclerosis is a chronic inflammatory disease of the vessel wall that occurs in the large and medium-sized arteries of the body. Although risk factors for atherosclerosis include hyperlipidemia, dietary habits, hypertension, cigarette smoking, and physical inactivity, genetic factors also contribute to the development of atherosclerosis.[1-3] The function of lipoproteins, apolipoproteins, enzymes, and lipid transfer factors that play a role in lipoprotein metabolism, inflammation, and atherosclerosis will be outlined in more detail in this introduction.

1. Lipoproteins and lipid metabolism

Cholesterol and triglycerides (TG) are the most important lipids in the circulation. Cholesterol is a constituent of cellular membranes and is essential for the synthesis of steroid hormones and bile acids, whereas TG is a precursor of free fatty acids (FFA) that are used as an energy source in skeletal muscle or heart, or stored as TG in adipose tissue. For distribution of these lipids through the body, they are packaged in water-soluble lipoproteins, which consist of a lipid core rich in cholesteryl esters (CE) and TG, surrounded by a phospholipid (PL)-rich shell that is stabilized by unesterified cholesterol and one or more apolipoproteins.[4] Apolipoproteins are involved in the generation of lipoproteins, and modulate the activity of plasma enzymes and transfer factors involved in lipoprotein metabolism. Based on their density, lipoproteins are divided into five classes: chylomicrons, very-low-density-lipoproteins (VLDL), intermediate-density-lipoproteins (IDL), low-density-lipoproteins (LDL), and high-density-lipoproteins (HDL) that exhibit different lipid contents and apolipoprotein composition. The characteristics of the various lipoproteins are listed in Table 1.[4-6] Lipoproteins mediate lipid transport, which can be divided into three different pathways: the exogenous pathway, the endogenous pathway, and reverse cholesterol transport.

Table 1. Physical properties and composition of human plasma lipoproteins.

	Chylomicron	**VLDL**	**IDL**	**LDL**	**HDL**
Source	Intestine	Liver	VLDL	VLDL	Liver+intestine
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Density (g/mL)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Mobility*	Origin	Pre-ß	slow pre-ß	ß	α
Composition**					
Triglycerides	88	55	23	6	4
Phospholipids	7	18	20	22	30
Cholesteryl esters	3	13	31	41	14
Free cholesterol	1	6	7	9	5
Protein	1-2	8	19	22	47
Apolipoproteins	AIV	AV	-	-	AI,AII,AIV,AV
	B48	B100	B100	B100	-
	CI,CII,CIII,CIV	CI,CII,CIII,CIV	CI,CII,CIII,CIV	-	CI,CII,CIII,CIV
	E	E	E	-	E

* According to the electrophoretic mobility of plasma α- and β-globulins on agarose gel electrophoresis.
**Expressed as percentage of total weight. Modified from Wasan and Cassidy.[4]

1.1. Exogenous pathway

The exogenous pathway involves the uptake of lipids by the intestine and their transport to the liver in chylomicrons. Cholesterol and TG are the most common dietary lipids. In the intestinal lumen, TG is hydrolyzed by pancreatic lipase into monoacylglycerol and FFA, upon which they are absorbed by enterocytes in the intestinal wall, and re-esterified into TG. After microsomal TG transfer protein (MTP)-mediated lipidation of apolipoprotein (apo)B48, the resulting particles, termed chylomicrons, are secreted into the lymph.[8;9] These nascent chylomicrons consist mainly of TG (88%), the remainder being phospholipids (PL), cholesterol, cholesteryl esters (CE), apoB48, apoAI, apoAII, and apoAIV. After secretion into the lymph, chylomicrons enter the circulation, where apoAI, apoAII, and apoAIV are exchanged with HDL, and apoCI, apoCII, apoCIII, apoCIV and apoE are acquired from circulating lipoproteins. Concomitantly, TG from chylomicrons undergo lipolysis catalyzed by lipoprotein lipase (LPL),[10] that is associated with heparan sulfate proteoglycans (HSPG) on the luminal site of vessels.[11] LPL hydrolyzes TG, thereby delivering liberated FFA to adipose tissue for storage, to skeletal muscle and heart as an energy source, and to the liver for storage or generation of lipoprotein particles.[12] In addition, TG from chylomicrons are exchanged for CE from HDL via the activity of cholesteryl ester transfer protein (CETP).[13] CETP is a transfer factor, that is expressed in humans, yet not in mice.[14] As a result, chylomicron remnants that are reduced in size and relatively enriched in CE in their core are formed. During lipolysis, excess surface PL are transported to HDL via the activity of PL transfer protein (PLTP).[15] Then, these remnants possibly associate first with the scavenger receptor BI (SR-BI) or HSPG on the liver,[16] or are directly taken up via apoE-recognition receptors, such as the LDL receptor (LDLr) and the LDLr related protein (LRP).[17;18] In addition, HSPG on the liver can internalize chylomicron remnants.[19]

1.2. Endogenous pathway

The endogenous pathway involves lipid transport from the liver to peripheral tissues. The liver produces VLDL particles that consist of cholesterol and TG derived from chylomicron remnants or de novo synthesis.[20] MTP mediates lipidation of apoB100 (and in mice also apoB48) with TG and cholesterol,[21;22]

resulting in the generation of nascent VLDL particles that contain also small amounts of apoCI, apoCII, apoCIII, apoCIV, apoE,[23] and apoAV.[24] VLDL is then secreted into the circulation. Similar to chylomicrons, VLDL is enriched with apoCI, apoCII, apoCIII, apoCIV, and apoE in the circulation, and TG from VLDL are exchanged for CE from HDL via the activity of CETP.[13] Like chylomicrons, VLDL can undergo lipolysis catalyzed by LPL,[10] resulting in the formation of FFA for storage in adipose tissue or use as an energy source for skeletal muscle or heart.[12] During the lipolysis of VLDL, PL are transported to HDL via the activity of PLTP,[15] whereas TG are also exchanged with CE via the activity of CETP.[13] These processes result in the formation of IDL, which is partly cleared by the liver via apoE-recognizing receptors LDLr and LRP,[17;18;25] and probably also HSPG.[19;26] The remainder is processed more extensively by LPL and hepatic lipase (HL), and becomes depleted of many apolipoproteins, resulting in relatively CE-rich LDL with apoB100 as its sole apolipoprotein. LDL can be recognized and taken up by the LDLr on the liver and peripheral tissues, using apoB100 as its ligand.[25] In the liver, LDL undergoes lysosomal hydrolysis and cholesterol can be re-esterified into CE via acyl CoA:acyl transferase 2 (ACAT2),[27] or converted into bile acids or vitamin D.[28;29] The peripheral tissues that can take up LDL include adrenals, testes, and ovaries where LDL-derived cholesterol serves as precursor for steroid hormones.[30]

In addition, LDL can be modified in the circulation by for example oxygen radicals, resulting in the formation of minimally modified LDL (mmLDL) and oxidized LDL (oxLDL) that both can induce endothelial activation and monocyte to macrophage differentiation in the vessel wall. Subsequently, mmLDL/oxLDL can be taken up by the scavenger receptor A (SRA) or CD36 in macrophages.

1.3. Reverse cholesterol pathway

To maintain cholesterol homeostasis, excess cholesterol in extrahepatic tissues is returned to the liver via HDL, to be eventually excreted via the bile into the faeces, which is known as the classical reverse cholesterol pathway.[31] In addition, recent findings suggest that cholesterol is secreted from the circulation directly into the intestine and that the liver is not involved in this process.[32] The first step of HDL formation is represented by the synthesis of

apoAI by the liver and intestine, and secretion of apoAI into the circulation. Then, ATP binding cassette (ABC)A1 transporter in the liver and the intestine mediate cholesterol and PL efflux to apoAI, and small discoidal HDL_3 particles are formed.[33;34] These particles also contain apoE, apoCs, apoAII, apoAIV, apoAV,[24] and are further lipidated via ABCA1-mediated cholesterol and PL efflux and ABCA7-mediated PL efflux from macrophages, resulting in mature HDL_2 and HDL_1 particles.[33;35] HDL_2 and HDL_1 are good acceptors for subsequent cholesterol efflux from macrophages, as mediated by ABCG1 and SR-BI.[36] The enlargement of the HDL particles is facilitated by PLTP-mediated transfer of PL from chylomicrons, VLDL, and IDL. PLTP is also involved in the transfer of PL between the different subforms of HDL.[15] During the maturation of HDL_3 into HDL_1, the acquired cholesterol is esterified into CE in the core of HDL, mediated by lecithin:cholesterol acyl transferase (LCAT). Therefore, the core of HDL_1 mainly consists of CE.[36] Subsequently, CE from HDL is delivered to the liver. Several receptors and transfer proteins are involved in this process. In mice, that do not express CETP,[14] the majority of HDL-CE is selectively taken up via the SR-BI.[37] In addition, CE from apoE-rich HDL_1 can be recognized and taken up by the liver via the LDLr.[38;39] In humans, HDL-CE can be exchanged for TG from apoB-containing lipoproteins, mediated by CETP.[40] As a result, in humans the majority of HDL-CE is taken up by the liver via apoB-containing lipoproteins.[41] In addition, it has been postulated recently that hepatocyte-associated CETP itself can take up HDL-CE, independent of the SR-BI and the LDLr.[42;43]

Both in mice and humans, the lipolysis of TG in HDL is efficiently catalyzed by hepatic lipase (HL),[44] while endothelial lipase (EL) catalyzes the lipolysis of PL from HDL.[45] CE that have returned to the liver are hydrolyzed and used for VLDL or HDL formation. Alternatively, cholesterol is secreted into the bile either as neutral sterols or bile salts, which requires the cholesterol transporters ABCG5 and ABCG8, that form an obligatory heterodimer.[46] The exogenous, endogenous, and reverse cholesterol pathways in lipoprotein metabolism are summarized schematically in Figure 1.

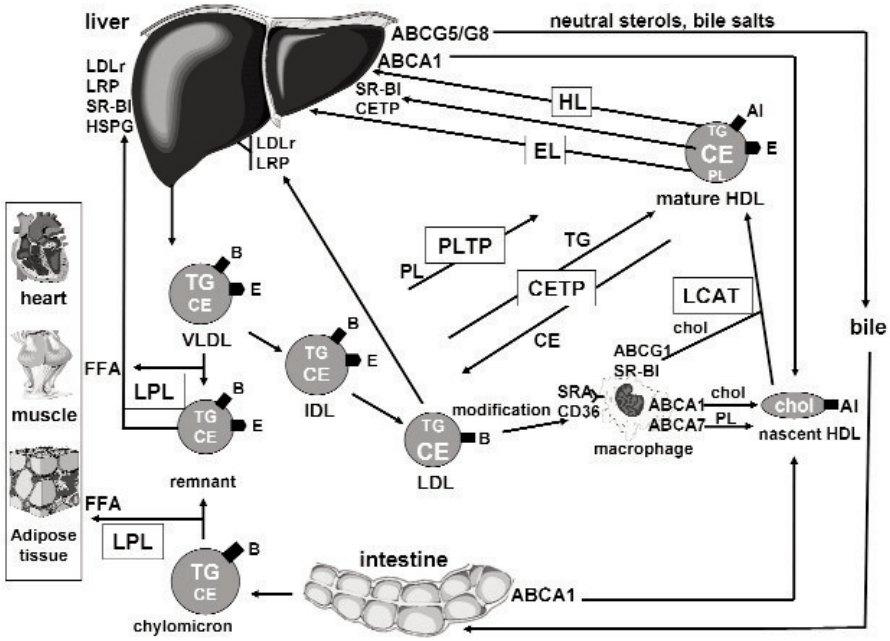

Figure 1. Schematically overview of pathways involved in lipoprotein metabolism. Explanation of pathways is described in text. Enzymes and transfer factors are boxed. LDLr, low-density-lipoprotein receptor. LRP, LDLr-related-protein. SR-BI or SRA, Scavenger receptor BI or A. FFA, free fatty acids. EL/HL/LPL, endothelial, hepatic, or lipoprotein lipase. TG, triglycerides. CE, cholesteryl esters. AI/B/E, apolipoprotein AI/B/E. VL/I/HDL, very-low-/intermediate-/high-density-lipoprotein. ABCA1/G1/G5/G8, ATP binding cassette transporter A1/G1/G5/G8. LCAT, lecithin:cholesterol acyl transferase. CETP/PLTP, cholesteryl ester or phospholipid transfer protein.

2. Atherosclerosis

Hyperlipidemia is an important risk factor for atherosclerosis. Atherosclerosis is driven by both increased VLDL/LDL-cholesterol levels and increased TG, often accompanied by low HDL-cholesterol levels.[1] Other risk factors for atherosclerosis involve inflammation and hypertension.[1] Atherosclerosis is a disease of the vessel wall and occurs principally in large and medium-sized elastic and muscular arteries.[3] This section describes how atherosclerotic lesions develop, from the healthy vessel wall to eventual plaque rupture and the formation of a thrombus that causes acute clinical events, such as myocardial infarction and stroke, by occluding the vessel lumen leading to ischemia of underlying tissue. In addition, this section describes treatment of atherosclerosis, and mouse models used to study atherosclerosis development.

2.1. Pathogenesis of atherosclerosis

The intact vessel wall consists of three layers: the intima, media, and adventitia. The intima is formed by a layer of endothelial cells at the luminal side of the vessel, and is separated from the media by an elastic membrane, the internal elastic lamina. The media is a layer of smooth muscle cells (SMC) that are embedded in an interstitial matrix, containing elastin and collagen, and separated from the adventitia by the external elastic lamina, composed of collagen, elastin fibres, and fibroblasts.[1;3] In addition, a fourth layer has been described consisting of proteoglycans, glycoproteins, and absorbed plasma proteins, termed the glycocalyx, that is present on the luminal side of the vessel, on the intima.[47]

The initial step in the development of atherosclerosis is injury of the endothelial cell layer. Most likely, this occurs at specific sites of arteries, such as branches, bifurcations, and curvatures, where alterations of blood flow decrease shear stress, making these sites more prone to endothelial cell damage.[3] Inflammatory molecules, that come into the circulation as a result of a bacterial infection, mmLDL or oxLDL induce endothelial activation, resulting in an inflammatory response, the start of the development of an atherosclerotic lesion.[3] The endothelial cells start to express selectins (P- and E-selectin), and intracellular and vascular adhesion molecules (ICAM-1 and VCAM-1), resulting in adhesion of monocytes and T-cells to the endothelial wall, and increased permeability of the endothelial cell layer.[3] The glycocalyx is likely to serve as a barrier for monocyte adhesion,[47;48] thus might be atheroprotective, however its role in atherosclerosis is still subject of ongoing research. The monocytes start rolling on the endothelium, and migration of monocytes and T-cells into the arterial wall is stimulated, in part by oxLDL that induces the expression of chemotactic molecules by endothelial cells, such as monocyte chemoattractant protein 1 (MCP-1).[49] Also the receptor for MCP-1, chemokine receptor 2 (CCR2), is upregulated on monocytes upon hypercholesterolemia,[50] leading to entry of monocytes in the subendothelial space, where they start to multiply and differentiate into macrophages. Monocyte to macrophage differentiation depends on factors such as (granulocyte-) macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), tumour necrosis factor α (TNFα), and interferon γ (IFNγ), secreted by SMCs, endothelial cells, and other leukocytes.

These processes are associated with upregulation of scavenger receptors and Toll-like receptors on macrophages.[2]

Macrophages subsequently take up modified forms of LDL, mediated by the scavenger receptors SRA and CD36,[3] followed by degradation in the lysosome. The liberated cholesterol is then esterified with fatty acids into cholesteryl esters, catalyzed by the enzyme ACAT1.[51] By excessive storage of cholesterol as CE, these macrophages develop into foam cells, which, together with some T-cells, form an initial lesion called the fatty streak.[2] The macrophage can release its cholesterol through efflux, which involves several pathways. Free cholesterol can be fluxed out of the cell, concomitant with apolipoproteins, as described for apoE.[52] Active cholesterol and PL efflux occurs via ABCA1 and PL efflux also via ABCA7, mainly to small, HDL_3 particles, thereby causing maturation of HDL into HDL_2 and HDL_1.[35;53] ABCG1 and SR-BI constitute the pathways that mediate cholesterol efflux to HDL_2 and HDL_1,[53;54] as described in section 1.3.

The Toll-like receptors present on foam cells are activated upon binding of molecules with pathogen-like molecular patterns, such as heat shock protein 60 and oxLDL.[2]

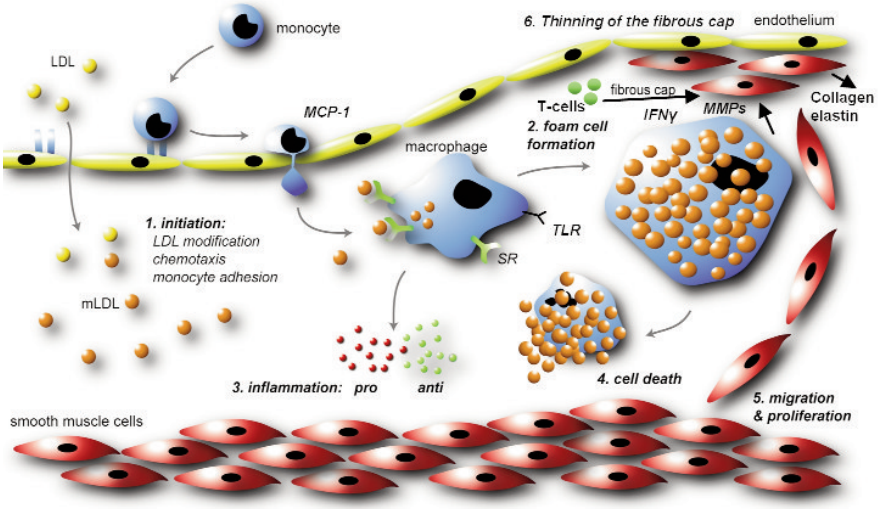

Figure 2. Pathogenesis of atherosclerosis.

For explanation see text. The development of an atherosclerotic lesion is schematically presented, from the monocyte adhesion to the thinning of the fibrous cap. mLDL, modified low-density-lipoprotein. MCP-1, monocyte chemoattractant protein-1. SR, Scavenger receptor. TLR, Toll-like receptor. IFNγ, interferon γ. MMP, matrix metalloproteinase. Figure kindly provided by Menno de Winther, and minimally modified.

In addition, Toll like receptors are activated by exogenous ligands that come into the circulation as a result of an infection with Gram-negative bacteria. Upon multiplication or lysis, these bacteria secrete lipopolysaccharide (LPS) that activates the Toll like receptor 4 (TLR4). Subsequently, signal cascades are initiated, involving the nuclear factor κB (NFκB) and the MAP kinase pathway,[55] leading to the secretion of cytokines.[2] Among these cytokines are IL-1 and TNFα that, next to increasing monocyte-to-macrophage differentiation continuously, induce the migration of SMCs from the media into the intima and the subsequent pro- liferation of SMCs.[2] SMCs can also take up lipids, thereby contributing to foam cell formation. In addition, SMCs start to synthesize and secrete connective tissue matrix, and also collagen, thereby forming a protective layer over the fatty streak, the fibrous cap.[2] Activated T-cells that differentiate into type 1 helper T (Th1) effector cells secrete IFNγ, that augments the synthesis of IL-1 and TNFα leading to SMC proliferation.[2] As the accumulated foam cells within the lesions continue to take up modified LDL, a lipid core is formed. Once cholesterol cannot be efficiently stored anymore, it becomes cytotoxic, and the cells go either in necrosis or apoptosis, resulting in formation of a necrotic core with extracellular lipid accumulation in the form of cholesterol crystals. As long as the fibrous cap is stable, the atherosclerotic plaque cannot release its content into the circulation. However, collagen production by SMCs can be inhibited by for example IFNγ secreted by Th1 cells.[56] When the fibrous cap is poor in SMCs and collagen, it becomes thin and much more prone to rupture. In addition, these advanced plaques contain a large necrotic lipid pool and an abundancy of macrophages,[57;58] that secrete matrix metalloproteinases (MMPs), such as collagenases and elastases that can degrade the extracellular matrix synthesized by SMCs in the fibrous cap. After rupture of the fibrous cap, the underlying necrotic core is exposed to the blood flow. Massive tissue factor expression by cells in the necrotic core results in activation of the coagulation cascade and accumulation of platelets at the site of rupture. This leads to the formation of a thrombus that may occlude the vessel entirely, resulting in an abrogated oxygen supply to underlying vital tissue. In fact, thrombus formation is the major cause of acute clinical events, such as myocardial infarction and stroke.[2] The processes involved in the development of atherosclerosis are summarized in Figure 2.

2.2. Treatment of atherogenic dyslipidemia

Treatment of atherogenic dyslipidemia nowadays is mainly focused on decreasing pro-atherogenic VLDL/LDL-levels, as increased VLDL/LDL-cholesterol and TG are important risk factors for atherosclerosis and may be an initial sign of susceptibility to atherosclerosis.

Statins are most widely used to treat atherosclerosis. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that catalyzes the rate-limiting step in cholesterol biosynthesis in the liver, leading to reduction of VLDL production. The liver compensates for reduced cholesterol biosynthesis by increasing LDLr expression, and subsequently LDL levels in the circulation decrease.[25] In addition, statins have been shown to increase HDL levels to some extent,[59] and exert anti-inflammatory properties, which may add to their anti-atherosclerotic action.[60;61] Meta-analysis of 70.000 subjects in 25 studies showed that statins reduce LDL-cholesterol levels up to 40%, thereby decreasing CVD mortality by 23%,[62] whereas no results on HDL-cholesterol were reported.

Next to statins, fibrates decrease VLDL and LDL-cholesterol; however their mode of action is mainly aimed at decreasing hypertriglyceridemia. Fibrates activate peroxisome proliferator-activated receptor α (PPARα), causing stimulation of LPL-mediated TG catabolism via increased hepatic LPL mRNA expression and decreased hepatic apoCIII expression.[63-65] In addition, fibrates reduce VLDL production.[66] Both processes lead to reduction of pro-atherogenic hypertriglyceridemia. Furthermore, fibrates increase HDL cholesterol levels through transcriptional induction of apoAI by PPARα,[66;67] and exert anti-inflammatory effects.[61] A meta-analysis of 16.802 subjects in 53 trials showed that fibrates decrease TG by 36%, decrease LDL-cholesterol by 8%, and increase HDL-cholesterol by 10%, thereby decreasing CVD mortality by 25%.[67] Despite the marked reductions in VLDL and LDL levels by statins, and TG reduction and HDL increase by fibrates, these drugs still do not prevent all the cases of CVD. As a low HDL level is the primary lipid abnormality in ~50% of men with coronary heart disease,[68] and prospective epidemiological studies have shown a strong inverse correlation between HDL cholesterol levels and CVD,[69] recent interests to treat CVD are aimed at increasing HDL levels. As described in the sections 1.3 and 2.1, HDL induces cholesterol efflux from macrophages.

In addition, HDL neutralizes inflammatory molecules and may decrease the oxidation level of LDL. Inhibition of CETP constitutes a novel approach in order to increase anti-atherogenic HDL with concomitant decrease of pro-atherogenic VLDL/LDL levels.[70] Two experimental CETP inhibitors, JTT-705 and Torcetrapib, have been shown to increase HDL-cholesterol in humans.[71-74] Torcetrapib (120 mg, daily) increased HDL-cholesterol by 60% in individuals with low HDL-cholesterol,[72] and decreased atherosclerosis development in rabbits. In phase I and II clinical trials, its only side effect seemed to be the induction of high blood pressure. Unexpectedly, recent phase III trials had to be terminated prematurely, since the combination of Torcetrapib and atorvastatin was found to induce more deaths in people with CVD than atorvastatin alone.[75] The question whether this effect was compound-specific or related to CETP inhibition in general is under current investigation, as well as the effects of other CETP inhibitors on atherosclerosis.

2.3. Mouse models for atherosclerosis

Wild-type mice are not suitable for atherosclerosis research because they express, in contrast to humans, low total cholesterol levels (~2 mM versus ~5 mM) with high levels of anti-atherogenic HDL and low levels of pro-atherogenic VLDL. Therefore, to study the effects of genes involved in atherogenesis and the effects of experimental anti-atherogenic drugs on atherosclerosis, several mouse models have been developed. Nowadays, the hyperlipidemic *apoe*$^{-/-}$, *ldlr*$^{-/-}$, and *APOE*3-Leiden* mice are the most common used to study atherosclerosis.

The *apoe*$^{-/-}$ mouse is prone to spontaneous atherosclerosis development on chow diet because of the largely abolished remnant uptake by the liver, via the HSPG, LRP, and LDLr,[76-78] and because of the largely reduced cholesterol efflux from macrophages, as apoE is a potent cholesterol acceptor.[79;80] The *ldlr*$^{-/-}$ mouse is less prone to atherosclerosis development as it is only devoid of clearance of apoB-containing particles via the LDLr, resulting in less elevated VLDL/LDL-cholesterol levels on a chow diet (~6 mM) as compared to *apoe*$^{-/-}$ mice (~10 mM). However, the *ldlr*$^{-/-}$ mouse develops atherosclerosis on a cholesterol-rich diet.[81;82]

Also the *APOE*3-Leiden* mouse has been generated to study atherosclerosis. These mice express a human mutant of the *APOE3* gene, as first discovered in hyperlipidemic patients,[83;84] resulting in the attenuated hepatic clearance of *APOE*3-Leiden* containing lipoproteins via the LDLr.[85] Female *APOE*3-Leiden* mice exhibit a human-like cholesterol distribution over their lipoproteins on a cholesterol-rich diet, and are susceptible to atherosclerosis development on this diet.[85] Their VLDL-cholesterol levels are highly responsive to the cholesterol levels in their diet,[85;86] and, in contrast to *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice, they show a human-like response to statins and fibrates, with respect to lowering of apoB-containing lipoproteins.[87;88]

It should be realized that atherosclerosis development in these mouse models is not completely identical to atherosclerosis development in humans. Most models, except for the *ldlr*$^{-/-}$ mouse, do not express human-like LDL. In addition, unlike humans, none of these models express CETP, and clinical events as induced by plaque rupture do not seem to occur spontaneously in mice. However, mice are still well-suited to study the mechanisms underlying the pathogenesis of the onset and progression of atherosclerosis. The *APOE*3-Leiden* mouse may be the preferred mouse model, since the clearance of apoB-containing lipoproteins is attenuated yet not abrogated as in *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice, which is very similar to the clearance of these lipoproteins in humans. The choice for the optimal mouse model should thus be based on the expected mechanism of action of the gene and drug of interest on atherosclerosis development.

In all of these mouse models, the development of atherosclerosis is not only related to induced levels of apoB-containing lipoproteins and HDL, yet also to chronic inflammation, which is a risk factor *per se* for atherosclerosis development.[2] As outlined in section 2.1., Gram negative bacteria, such as *Chlamydia pneumoniae*, secrete lipopolysaccharide (LPS) upon multiplication or lysis. Inoculation with *C. pneumoniae* accelerates the development of atherosclerosis in *APOE*3-Leiden* transgenic, *apoe*$^{-/-}$, and *ldlr*$^{-/-}$ mice.[89-91] In addition, chronic treatment with LPS accelerates atherosclerosis in *apoe*$^{-/-}$ mice.[92] Studies with LPS, administrated in a peri-adventitial cuff, showed that the effects of LPS on atherosclerosis are largely dependent on the TLR4, as the intimal lesion formation was dramatically decreased in the absence of TLR4.[93] It would be interesting to unravel other genetic factors that affect this process.

3. Roles of LPL, CETP, and apolipoprotein CI in lipoprotein metabolism and atherosclerosis

As outlined above, lipoprotein receptors, plasma enzymes, transfer factors, and apolipoproteins are involved in the production, processing, and clearance of lipoproteins and the development of atherosclerosis. This section will give more insights in the role of the enzyme LPL, the transfer factor CETP, and apoCI in lipoprotein metabolism and atherosclerosis development.

3.1. Lipoprotein lipase

As mentioned in section 1.1 and 1.2, LPL hydrolyzes VLDL and chylomicrons in order for TG-derived FFA to be taken up into tissues.[10;12] LPL is expressed in almost all tissues, yet most abundantly in adipose tissue, skeletal muscle, and heart.[94-96] The regulation of LPL is tissue-specific and dependent on the nutritional status, reflecting FA requirements of the respective tissues at a specific time point, and LPL thus functions as a gatekeeper for FA entry into tissues.[12;97-99] Postprandial LPL activity is high in adipose tissue, whereas in the fasted state, LPL activity is high in muscle.[12;97;100] LPL secretion from cells to the capillary surface is mediated by the VLDL receptor (VLDLr) that functions as an intracellular chaperone protein.[101] Upon secretion, LPL binds to HSPG at the luminal side of the capillary blood vessels.[97] Catalytically active LPL consists mainly of a homodimer of two non-covalently linked glycoproteins of equal size.[102;103] This LPL dimer is in equilibrium with the LPL monomer that can undergo a conformational change thereby losing its catalytic activity, and has lower affinity for HSPG as compared to dimeric LPL. Monomeric LPL can travel with lipoproteins and enhance their binding to the LDLr,[104;105] LRP,[106-108] VLDLr,[101;109] and HSPGs,[110;111] thereby exerting a bridging function in the clearance of those lipoproteins.
The catalytically active dimer of LPL, requires, in order to catalyze lipolysis of chylomicrons or VLDL, its co-factor apoCII.[112;113] After lipolysis, the generated FFA are taken up by the underlying tissues either by passive diffusion or by active transport,[114;115] via fatty acid transporters including CD36,[116;117] FA transporter protein (FATP),[118] and plasma membrane FA-binding protein (FABPpm).[119;120]

Next to apoCII, other apolipoproteins have been shown to affect LPL activity. ApoAV has been shown to activate LPL *in vitro* and in mice *in vivo*,[121] however, it is not clear as yet whether apoAV also activates LPL in humans and as such contributes to lowering of TG. In addition to LPL activators, inhibitors of LPL have also been described. ApoCIII seems to be the most prominent LPL-inhibitor, and apoCIII levels are consistently associated with plasma TG levels in humans.[122;122;123] In addition, apoE has been reported to inhibit LPL.[124;125] Recent observations from our laboratory have identified apoCI as an LPL inhibitor.[123] Overexpression of human apoCI in mice results in hypertriglyceridemia as a result of LPL inhibition.[123] However, it is still unclear whether physiological expression levels of endogenous apoCI can also affect LPL activity.

Decreased LPL expression has been associated with increased VLDL-TG levels and decreased obesity in mice. Mice deficient for LPL have extremely elevated VLDL-TG and show reduced subcutaneous fat stores in their short lifespan of 18 h,[126] and adipose tissue-specific *lpl*$^{-/-}$ mice show reduced obesity on the *ob/ob* background.[127] In addition, reduced LPL expression as a consequence of deficiency of the VLDLr, the intracellular chaperone protein for LPL, resulted in decreased diet-induced obesity.[128] As the lipolysis of TG is hampered in the absence of the VLDLr,[129] and less FFA were taken up by adipose tissue, reduced LPL activity probably plays a role in the reduction in obesity in *vldlr*$^{-/-}$ mice. Also mice transgenic for the LPL inhibitor apoCI were protected against diet-induced obesity.[130] Inversely, deficiency for the LPL inhibitor apoCIII resulted in increased diet-induced obesity.[131]

With respect to atherosclerosis, LPL activity in plasma and LPL in the vessel wall differently affect this disease in mice.[132] Whereas systemic LPL overexpression has been associated with decreased atherosclerosis on the *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ background,[133;134] LPL overexpression in macrophages of the vessel wall resulted in increased atherosclerosis in *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice.[135-137] The anti-atherogenic effect of plasma LPL is probably related to decreased hyperlipidemia by enhanced VLDL-TG lipolysis, while the pro-atherogenic effect of LPL in the vessel wall may result from enhanced local lipoprotein uptake due to its bridging function.

In humans, the mutations in LPL D9N and N291S, that occur in up to 5% of the general population, are associated with decreased lipolytic activity of LPL, elevated TG, decreased HDL and a higher incidence of cardiovascular

disease (CVD).[138-145] In contrast, the gain of function mutation S447X leads to decreased TG, increased HDL, and meta-analyses of association studies between the mutation and CVD indicate an overall lower incidence of CVD.[146-149] Nevertheless, there are also indications for increased CVD, and the mechanisms as how this polymorphism sorts its effects are still under investigation.[147]

3.2. Cholesteryl ester transfer protein

The glycoprotein CETP mediates the exchange of CE and TG between apoB-containing lipoproteins and HDL.[13] Not all mammalian species express CETP.[14;150] For example, rats and mice do not express CETP, whereas humans, monkeys, rabbits, and hamsters do.[14;150] In humans, *CETP* mRNA is expressed predominantly in adipose tissue, liver, and spleen, with lower expression levels in the small intestine, adrenal gland, kidney, skeletal muscle, and heart.[150;151] To evaluate the contribution of CETP to plasma lipid metabolism and atherosclerosis development, the *CETP* gene has been introduced in mice. These *CETP* transgenic mice express CETP predominantly in the liver yet also in adipose tissue and spleen.[152-154] CETP expression is mainly regulated by the liver X receptor (LXR).[155] As a consequence, CETP expression is highly upregulated in mice fed a hypercholesterolemic diet,[153;156] because the levels of oxysterols, the endogenous ligands for LXR,[157] increase in the liver. The introduction of CETP in mice shifts the cholesterol distribution from HDL to VLDL.[152] Feeding LXR agonists to the CETP-expressing species hamsters and Cynomolgus monkeys induced a similar shift,[158] and increased the shift in CETP-expressing mice.[159] CETP thus determines the ratio of VLDL/LDL cholesterol to HDL cholesterol to a great extent.[13]

As a result of mediating the exchange between CE from HDL and TG from VLDL/LDL, plasma CETP might be anti-atherogenic by facilitating the transport of HDL-cholesterol via apoB-containing lipoproteins to the liver, where they are taken up by the SR-BI, LDLr, and LRP. Recent results indicate that hepatocyte-associated CETP itself can also take up CE from HDL, independently of the SR-BI and the LDLr/LRP.[42;43] On the other hand, CETP expression might be pro-atherogenic, because of increased levels of pro-atherogenic VLDL/LDL concomitant with decreased HDL levels.

The role of CETP in lipoprotein metabolism and atherosclerosis has been studied in several mouse models. In the traditional mouse models for studying atherosclerosis development, *i.e.* in *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice, CETP expression appeared to be pro-atherogenic.[160] Other atherosclerosis studies, in *APOC3* transgenic and *LCAT* transgenic mice, revealed that CETP expression is anti-atherogenic.[161;162] *APOC3* mice accumulate particularly TG-rich VLDL as a result of LPL inhibition, enabling a massive flux of TG from apoB-containing lipoproteins to HDL,[163] which may result in an accelerated clearance of TG-rich HDL particles via HL. As a consequence, smaller, cholesterol-poor HDL particles are formed,[163] that may have higher anti-atherogenic potential by more efficiently inducing cholesterol efflux. *LCAT* transgenic mice accumulate apoE-rich HDL_1, that is not efficiently cleared by the liver and are, therefore, more susceptible to atherosclerosis.[161] CETP expression in *LCAT* mice provides an extra pathway of delivering HDL-cholesterol to the liver, resulting in normalization of the HDL particle size[161] and presumably increasing its cholesterol-accepting potency. However, it was shown recently that HDL from CETP deficient humans, which shares similarities with HDL from *LCAT* transgenic mice, is a potent cholesterol acceptor.[164] Taken together, studies in mice show conflicting results regarding the role of CETP in atherosclerosis development. On the other hand, CETP inhibition in rabbits that naturally express CETP has been consistently associated with decreased atherosclerosis.[165-167]

The role of CETP in atherosclerosis in humans is also still undergoing debate. In humans, the main quantity of HDL-CE is exchanged for TG from apoB-containing lipoproteins, mediated by CETP,[40] indicating that this HDL-CE is cleared via the LDLr/LRP pathway.[41] Humans that are deficient for CETP show increased atherosclerosis despite increased HDL levels.[168;169] However, more recently, decreased atherosclerosis has been reported in CETP-deficient subjects, although the effects were not significant.[170] As HDL isolated from these individuals is still a potent cholesterol acceptor *in vitro*,[164] it is likely that the observed increased atherosclerosis is not the consequence of the reduced cholesterol efflux accepting potency of HDL in plasma. In contrast, the C629A promoter polymorphism,[171] and the C629A,G971A,C1337T promoter polymorphism are associated with increased CETP levels concomitant with decreased HDL-cholesterol.[172] In addition, the C629A promoter is also associated with increased progression of CVD.[171;173] Collectively, studies in

mice and humans show that CETP expression can be either pro- or anti-atherogenic and more research regarding the role of CETP in atherosclerosis is warranted, before CETP inhibition can be used as a therapeutic approach to treat CVD in humans. With respect to the human situation, it would be interesting to investigate the effect of CETP on atherosclerosis in the *APOE*3-Leiden* mouse model that is a mouse model with a humanized lipoprotein profile, as described in section 2.3.

3.3. Apolipoprotein CI

ApoCI is present on chylomicrons, VLDL, and HDL, and circulates in the plasma at a concentration of ~6 mg/dl.[122] It is the smallest member of the apolipoprotein family (6.6 kDa), consists of 57 amino acids, and contains two amphipathic class II α-helices in the N-terminus (residues 7-29) and C-terminus (residues 38-52), connected by a unordered linker (residues 30-37).[122;174] ApoCI has a high content of lysine (16 mol%), and its isoelectric point is the highest of all the apolipoproteins (6.5).[175-177] It readily exchanges between particles due to self-association in an aqueous environment.[122] The structure of apoCI is represented in figure 3. ApoCI is expressed in the liver, brain, adipose tissue, lung, and spleen.[122;178] The apoCI expression is highest in the liver, and the expression in the lung and the spleen is probably due to the presence of resident macrophages in these tissues.[178]

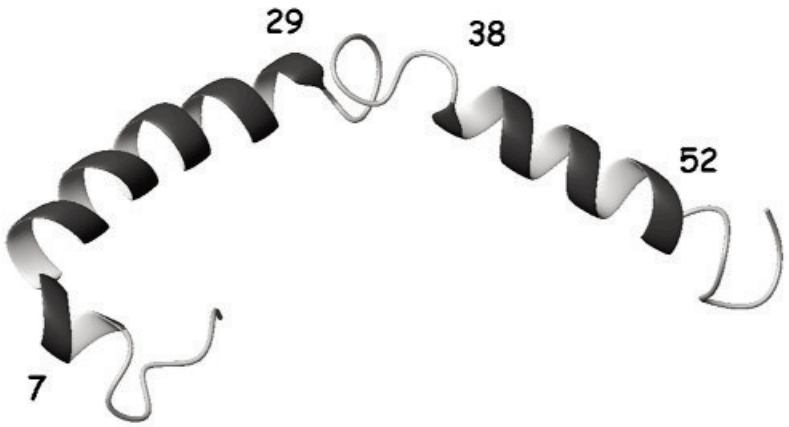

Figure 3. Structure of human apoCI. ApoCI consists of two α-helical structures, residues 7-29 (with a mobile hinge region involving residues 12-15) and residues 38-52, which are linked by a structurally unordered region (residues 30-37). Figure kindly provided by Jimmy Berbée.

The *APOC1* gene is part of the *APOE/APOC1/APOC4/APOC2* gene cluster on chromosome 19, which is regulated by the hepatic control region (HCR) in the liver[179-181] and by an LXR response element in macrophages.[182] In the liver, *APOC1* mRNA expression is upregulated after activation of a DR1 motif of the HCR by the TR4 orphan nuclear receptor,[179] whereas in macrophages, *APOC1* mRNA expression is upregulated by the formation of an obligatory heterodimer between LXRα/β and the retinoic X receptor (RXR).[182] Since the apoCI propeptide contains a signal peptide that is cleaved co-translationally in the endoplasmatic reticulum (ER), newly synthesized apoCI is not retained intracellularly but is secreted from cells.[183]

ApoCI affects the activity of several enzymes and receptors involved in VLDL metabolism. It inhibits the apoE-mediated binding of IDL and VLDL to LRP and the LDLr *in vitro*,[184-186] and the apoE-mediated binding of VLDL to the VLDLr *in vitro*.[187] Mice overexpressing human apoCI hemizygously (*APOC1*$^{+/0}$ mice) and homozygously (*APOC1*$^{+/+}$ mice) show elevated levels of plasma cholesterol and TG, which correlate positively with the expression level of the transgene.[188;189] These increased lipid levels have initially been explained by the apoCI-mediated inhibition of apoE-mediated clearance of IDL and VLDL by the LDLr and LRP, also *in vivo*.[188] However, these mice showed a predominant hypertriglyceridemia, which is not observed in *apoe*$^{-/-}$ mice, and more recent results showed that in the absence of apoE, VLDL-TG and VLDL-cholesterol are still elevated in *APOC1*$^{+/0}$ mice, indicating that additional mechanism(s) should contribute to the hyperlipidemia in *APOC1*$^{+/0}$ mice.[123;190] Indeed, we recently showed *in vitro* and *in vivo* that apoCI is a potent inhibitor of LPL,[123] which can fully explain the combined hyperlipidemia in *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice. However, whether physiological endogenous apoCI expression affects VLDL metabolism in normolipidemic and hyperlipidemic mice by modulating LPL activity has not been addressed in detail.

In addition, apoCI affects plasma factors involved in HDL metabolism. It activates LCAT, at least *in vitro*, yet to a lower extent than apoAI, the main activator of LCAT.[191] As compared to apoAI, apoCI activates LCAT by 10-45%, dependent on the lipid substrate. Also apoE2 and apoE3 are LCAT activators, albeit weaker than apoAI.[122;174;192] In addition, it has been shown that apoCI inhibits hepatic lipase (HL) *in vitro*.[190;193] More apolipoproteins exert this effect, as also apoAI, apoAII, apoCIII, and apoE inhibit HL *in vivo*.[193]

HDL-cholesterol levels are decreased in *apoc1*$^{-/-}$ mice,[194] and increased in wild-type mice that were injected with a human apoCI adenovirus (C.C. van der Hoogt *et al.*, unpublished observations). These data suggest that apoCI also modulates HDL metabolism *in vivo*, presumably by activation of LCAT and/or inhibition of HL.

Next to its effects on VLDL and HDL metabolism separately, apoCI inhibits CETP that affects both VLDL and HDL metabolism described in section 3.2.[175;195] A first study in dyslipidemic baboons showed that a naturally occurring N-terminal peptide of apoCI that contained the first 38 amino acids (4 kDa, pI=7.1) inhibited CETP activity *in vitro* and *in vivo*.[196] In humans, of all the apolipoproteins on HDL, apoCI appeared to be the only apolipoprotein that reduces CETP activity.[175] Interestingly, a recent study using human CETP showed that the CETP-inhibitory effect of human apoCI was mainly due to its C-terminal peptide (residues 34-54).[197] The effect of apoCI on CETP activity has also been studied *in vivo* in *CETP* transgenic mice.[195;198] Interestingly, endogenous apoCI expression appeared to inhibit CETP effectively,[195] reflected by decreased VLDL cholesterol and increased HDL cholesterol. In *APOC1*$^{+/0}$*.CETP* mice, apoCI also effectively inhibited CETP, as measured in endogenous CETP activity assays, however, this was not reflected by an increased HDL and a decreased VLDL.[193;198] It appeared that the effect of apoCI on CETP inhibition was completely overruled by the aforementioned effect of apoCI on LPL and the apoE-recognition receptors on the liver resulting in hyperlipidemia.[198] As a consequence of the hyperlipidemia in *APOC1*$^{+/0}$ mice, LXR-mediated transcription of CETP in the liver increased, leading to increased total CETP levels in plasma.[198] As a result, the inhibition of CETP by apoCI did not translate in expected changes of VLDL and HDL levels. Thus, in *APOC1*$^{+/0}$ mice, yet not in *apoc1*$^{-/-}$ mice, the CETP-inhibitory effect of apoCI is secondary to the VLDL clearance-inhibiting effects of apoCI with respect to the overall effect of apoCI on plasma lipid levels.

Not many apoCI polymorphisms are known in humans. In American Indian and Mexican humans, one polymorphism has recently been found that involves the substitution of the tyrosine on position 45 for serine.[199] Though studies with synthesized apoCI suggested that the variant had a higher preference for VLDL and a lower preference for HDL,[199] the functionality of this apoCI mutation still needs to be determined.

Furthermore, the *HpaI* polymorphism in the promoter region of *APOC1* has been described, resulting in increased expression of *APOC1* and elevated TG.[200;201] However, the correlation between apoCI levels and TG levels is not very strong, and the effects of *APOC1* on TG levels are in linkage disequilibrium with *APOE2* and *APOE4*, yet not *APOE3*.[201] Collectively, it still needs to be determined whether apoCI is a causal factor in determining VLDL-TG levels in humans.

The role of apoCI in LPL inhibition probably relates to the finding that *APOC1*$^{+/+}$ mice are protected against the development of obesity, either diet-induced, or on the *ob/ob* background, as described in section 3.1.[130] Next to protection against obesity, *APOC1*$^{+/+}$ mice exhibit cutaneous abnormalities, including sebaceous and meibomian gland atrophy and lack of sebum production, resulting in excessive hair loss.[202] Although these mice exhibit elevated levels of FFA in plasma and decreased levels of wax esters and TG in their skin, the mechanism behind the cutaneous abnormalities has not been elucidated yet.

In addition to its roles in lipoprotein metabolism, we have recently demonstrated that apoCI binds to lipopolysaccharide (LPS), thereby disaggregating LPS, and increasing the inflammatory response to LPS by macrophages *in vitro* and in mice *in vivo*.[203] Increased apoCI expression in mice (*APOC1*$^{+/0}$ versus wild-type versus *apoc1*$^{-/-}$) thus resulted in an apoCI dose-dependent increase in the inflammatory response against intropulmonal infection with the Gram-negative bacterium *Klebsiella pneumoniae*. In fact, by effectuating a more efficient inflammatory response, apoCI increased the anti-bacterial attack and protected against *K. pneumoniae*-induced septic death.

Though many roles of apoCI in lipoprotein metabolism and also inflammation have now been described, it is not clear yet whether apoCI plays a role in atherosclerosis. *APOC1* mRNA expression is highly induced during monocyte to macrophage differentiation (85-fold),[204] and 25-fold upregulated in macrophages treated with 1 μM of the LXR agonist T013017,[182] indicating that apoCI might indeed play a role in macrophage foam cell formation and thus atherosclerosis development. *In vitro* data show that apoCI induces ABCA1-mediated cholesterol efflux, at least from human epithelial (Hela) cells.[205] Cholesterol efflux from macrophages is considered anti-atherogenic. In addition, apoCI activates LCAT,[191] and increased LCAT activity was shown to be anti-atherogenic in *apoe*$^{-/-}$ mice.[206;207] Furthermore, apoCI

inhibits CETP,[175;195-197] which might be considered anti-atherogenic. On the other hand, apoCI inhibits LPL,[123] and systemic LPL inhibition in plasma might be considered pro-atherogenic by increasing VLDL/LDL levels.[133;134] In fact, human *APOC1* overexpression increases atherosclerosis in *apoe*$^{-/-}$ mice, which was presumably the consequence of dramatically increased levels of VLDL-TG and VLDL-cholesterol.[190] Other studies have indicated that apoCI increases the apoptosis of aortic smooth muscle cells *in vitro* via recruiting neutral sphingomyelinase, a condition that might accelerate plaque rupture *in vivo*, and thus be pro-atherogenic.[208] As apoCI increases the LPS-induced inflammatory response in macrophages *in vitro* and in mice *in vivo*,[203] and LPS accelerates atherosclerosis in *apoe*$^{-/-}$ mice *in vivo*,[92] apoCI expression may also be pro-atherogenic in the context of chronic inflammation. Overall, the role of endogenous apoCI in atherosclerosis development, related to hyperlipidemia and inflammation, requires more extensive research.

4. Outline of Thesis

In this thesis studies were performed to elucidate the mechanisms behind the effects of apoCI and CETP on plasma lipoprotein metabolism and atherosclerosis development.

Although the role of apoCI in lipoprotein metabolism has been quite extensively studied in mice, these studies mainly addressed the effects of human apoCI overexpression instead of the effects of physiological endogenous apoCI expression. Therefore, in **chapter 2**, the role of endogenous apoCI on lipoprotein metabolism in *apoe*$^{-/-}$ mice was investigated. The *apoe*$^{-/-}$ background was used to exclude potential effects of apoCI on lipoprotein metabolism that are sorted via inhibition of the apoE-mediated remnant clearance. In **chapter 3**, studies were performed to investigate the mechanism(s) behind the observation that *APOC1*$^{+/+}$ mice are protected against obesity and the cutaneous abnormalities of these mice, since clues have been found that these effects may not simply be explained by apoCI-induced LPL inhibition.

Apart from these systemic effects of apoCI on lipoprotein metabolism, it has also been suggested that apoCI affects local lipid homeostasis in macrophages, as its expression is highly upregulated during monocyte to

macrophage differentiation and upon incubation of macrophages with agonists of the lipid sensor LXR. As macrophages play an important role in several stages of atherosclerosis development, these findings suggest that apoCI might affect atherosclerosis. The effects of apoCI expression on atherosclerosis development are described in **chapter 4**. In addition to its role in lipoprotein metabolism, apoCI increases the LPS-induced response *in vivo* and in macrophages *in vitro*. Therefore, the effect of apoCI on LPS-induced atherosclerosis was investigated in **chapter 5**.

The role of CETP in atherosclerosis is still undergoing debate and a human-like mouse model to study the effect of CETP modulation on plasma lipoprotein metabolism and atherosclerosis development is lacking. In **chapter 6**, human *CETP* transgenic mice were cross-bred with the humanized *APOE*3-Leiden* mouse, and the effect of CETP expression on plasma lipid metabolism and atherosclerosis development was investigated.

The results obtained from these studies and the implications of these studies for future research are described in **chapter 7**.

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