Efficacy, safety and novel targets in cardiovascular disease : advanced applications in APOE*3-Leiden.CETP mice

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General introduction

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Atherosclerosis

Prevalence

Cardiovascular disease (CVD) is the number 1 cause of death globally and the annual number of deaths from CVD is predicted to rise from 17.5 million in 2012 to 22.2 million by 2030 (1). Currently, 31% of all deaths worldwide is caused by CVD and low and middle-income countries are now most affected. Its major clinical manifestations include ischemic heart disease, ischemic stroke and peripheral arterial disease, all caused by the formation of atheromatous plaques in the vessels, and comprises 85% of all CVD deaths (1).

Development of atherosclerotic plaques

The build-up of an atherosclerotic plaque is a complex and slow process, which in humans begins in early childhood, and becomes clinical relevant after many decades. Atherogenesis begins with the recruitment of inflammatory cells into the intima. As response to irritative stimuli (e.g. dyslipidaemia, hypertension or pro-inflammatory mediators) endothelial permeability increases, the composition of the extracellular matrix beneath the endothelium changes, and the arterial endothelial cells express leukocyte adhesion molecules (2). As a result, blood monocytes are captured on the endothelial surface, and cholesterol-containing low-density lipoproteins (LDL) and remnant particles enter and accumulate in the arterial wall and are oxidized (2). Oxidized LDL (oxLDL) promotes monocyte adhesion and also binds to scavenger receptors on macrophages which triggers uptake of oxLDL leading to the formation of foam cells (type I and II lesions). These cells produce pro-inflammatory mediates, reactive oxygen species and tissue factor pro-coagulants, that amplify the inflammatory process and further increase endothelial permeability (2,3). Smooth muscle cells (SMCs) migrate from the media into the intima, proliferate, and produce extracellular matrix molecules, e.g. interstitial collagen, proteoglycans and elastin, to form a fibrous cap that overlies the lipid-laden foam cells (2,3). The subendothelial proteoglycans entrap LDL and subsequently extra-cellular lipids accumulate (type III lesions). Several plaque factors, including excessive inflammation, oxidized lipids and cholesterol, trigger macrophage cell death (4) leading to the formation of a pool with accumulated cellular debris and extracellular lipids, called the necrotic core of the plaque (type IV lesions). Also, as the result of necrosis, calcium deposits develop (type V lesions). In the advanced type IV and V lesions, thick layers of fibrous connective tissue cover the lipid-rich necrotic core. Activated macrophages and type 1 T-helper cells produce metalloproteinases and cytokines that weaken the tensile strength of the collagen cap (4). Consequently, lesions may rupture thereby releasing their fatty core into the lumen which triggers thrombus formation (type VI lesions) (5). Plaque rupture and subsequent thrombus formation can be clinically silent as they may heal, but can also induce CV ischaemic events through partial or total occlusion of the affected artery.

Risk factors

Several risk factors contribute to the initiation and progression of atherosclerosis development and can be divided in non-modifiable and modifiable risk factors. Non-modifiable risk factors include personal history of CVD, family history of CVD, age and gender. Modifiable risk factors include hypertension, obesity, diabetes mellitus, and elevated plasma glucose, LDL-cholesterol, and triglyceride (TG) levels, and lifestyle variables (poor dietary patterns, smoking, physical inactivity and harmful use of alcohol). Moreover, genetic disorders in the lipoprotein metabolism, e.g. familial dysbetalipoproteinema or type III hyperlipidemia, add to CVD risk. As lifestyle variables and metabolic perturbations are closely linked to each other, patients with CVD commonly present a cluster of risk factors. Estimated odds ratios of these risk factors demonstrate that abnormal plasma lipids are a major risk factor for atherosclerosis (**Table 1**) (6), and therefore, this thesis mainly focuses on the role of the lipoprotein metabolism in atherosclerosis development and CV safety. In addition, the contribution of diabetes and inflammation to CV risk will be discussed.

Table 1 Risk of acute myocardial infarction associated with risk factors in the overall population

Risk factor	Odds ratio (99% CI) adjusted for all risk factors
Current smoking	2.87
Current and former smoking	2.04
Diabetes	2.37
Hypertension	1.91
Abdominal obesity (2 vs 1)†	1.12
Abdominal obesity (3 vs 1)†	1.62
Vegetables and fruit daily	0.70
Exercise	0.86
ApoB/ApoA1 ratio (2 vs 1) §	1.42
ApoB/ApoA1 ratio (3 vs 1) §	1.84
ApoB/ApoA1 ratio (4 vs 1) §	2.41
ApoB/ApoA1 ratio (5 vs 1) §	3.25

The relation between the individual risk factors and first myocardial infarction is indicated. In total 15152 cases and 14820 controls from 52 countries representing every continent, were enrolled. †Top two tertiles vs lowest tertile. §Second, third, fourth, or fifth quintiles vs lowest quintile. Data are extracted from reference (6).

Lipoprotein metabolism

Lipids are transported within the plasma in the form of lipoprotein particles, and, depending on their density are classified as chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL, and high-density lipoprotein (HDL). LDL and HDL predominantly transport cholesterol, whereas chylomicrons and VLDL are enriched in TGs. The metabolism of these lipoproteins is divided into two pathways, the exogenous pathway and the endogenous pathway (7) and lipids are removed from the peripheral tissues by reverse cholesterol transport (**Figure 1**).

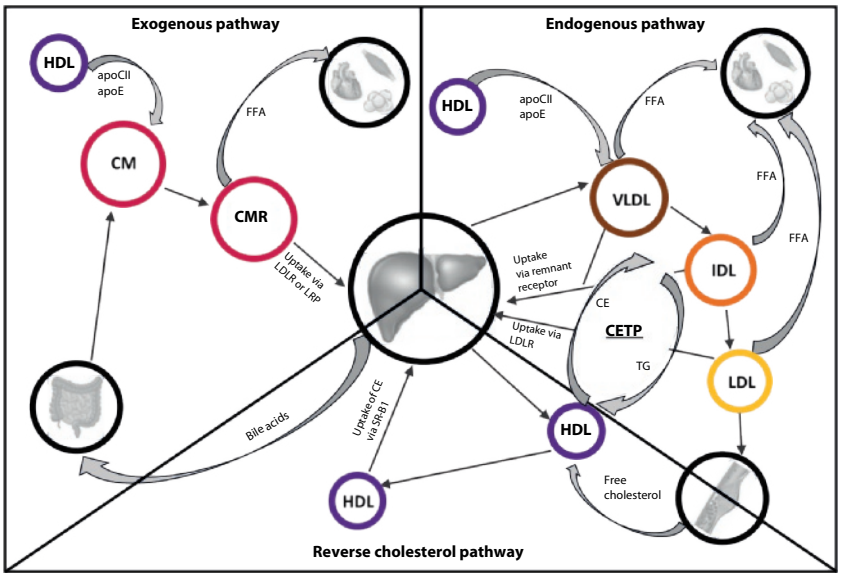

Figure 1 Pathways of lipoprotein metabolism. The liver plays a central role in the exogenous and endogenous pathway of lipid transport. HDL facilitates reverse cholesterol transport. CM, chylomicron; CMR, chylomicron remnant; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FFA, free fatty acids; apoCII, apolipoprotein C-II; apoE, apolipoprotein E; CE, cholesterol ester; TG, triglycerides; CETP, cholesteryl ester transfer protein; LDLR, low-density lipoprotein receptor, SR-B1, scavenger receptor class B type 1.

The exogenous pathway

The exogenous pathway refers to the absorption of dietary lipids by the enterocytes in the intestine, where they are assembled with apolipoprotein(apo)B48 into chylomicrons and enter the blood stream via the lymphatic vessels. In the blood, the chylomicrons receive apoCII and apoE from HDL-particles. ApoCII binds and activates lipoprotein lipase (LPL),

an enzyme attached to the luminal surface of endothelial cells in capillaries of adipose, heart and skeletal muscle tissue. Upon binding, TGs from the chylomicron particles are hydrolysed into glycerol and fatty acids and the remnant particles are cleared by the liver through binding of apoE to the LDL receptor (7).

The endogenous pathway

The liver plays a central role in the endogenous pathway. Triacylglycerols and cholesterol esters (CE) are assembled with apoB100 into VLDL, and when they reach the blood stream they receive apoCII and apoE from HDL particles. Like chylomicrons, the TGs from the VLDL particles are hydrolysed by endothelial LPL and consequently transform into IDL. IDL particles are taken up by the liver through binding of the remnant and LDL receptor with apoE or apoB100, or are further hydrolysed into LDL. LDL particles contain a relatively high cholesterol content and transfer lipids to the peripheral cells or are cleared by the liver through LDLR-apoB100 interaction (7). However, more importantly with respect to atherosclerosis, LDL can enter the arterial wall, in contrast to the larger VLDL and chylomicrons, where they are oxidative and proteolytically modified and contribute to the formation of atherosclerotic lesions.

Reverse cholesterol transport

HDL is the main lipoprotein involved in the reverse cholesterol transport pathway, which starts with the formation of nascent HDL by the liver and intestine. HDL particles acquire free cholesterol and phospholipids that are effluxed from cells in the peripheral tissues, including the vessel wall, a process mediated by ABCA1 resulting in the formation of mature HDL. The HDL particles transport the cholesterol to the liver either directly by interacting with hepatic scavenger receptor B1 (SR-B1), or the CEs in HDL are exchanged for TGs from VLDL or LDL particles through cholesterol ester transfer protein (CETP) (7). When remnant particles and LDL are taken up by the liver, unesterified cholesterol can be secreted into the bile, or is converted into bile acids.

The contribution of LDL-C, HDL-C and TGs to CVD risk

1. LDL-C

LDL-C is recognized as a primary causal risk factor in CVD as evidenced from many experimental, epidemiological and genetic studies (8,9). In addition, intervention trials with statin therapy confirm a reduced incidence of coronary heart disease as a consequence of cholesterol-lowering in LDL (10,11), and recent trials indicate that intensive lipid-lowering with statins may be more beneficial in risk reduction than less intensive (or standard) therapy (12). According to results from the latter meta-analysis, every 1 mmol/L (39 mg/dL) reduction in LDL-C is associated with a 23% reduction in the risk of major vascular events (12) suggesting that a 2–3 mmol/L reduction in LDL-C would correspond with a 40–50% reduction in events.

2. HDL-C

Epidemiological studies consistently report an inverse association between coronary heart disease risk and HDL-C: results from 4 prospective epidemiologic studies indicate that an increase of 1 mg/dL (0.03 mmol/L) in HDL-C is associated with a 2–3% reduction in risk (13).

Besides its major role in reverse cholesterol transport, HDL has also been described to have anti-inflammatory, anti-oxidant, anti-platelet and vasodilatory properties and may therefore have a protective role in coronary heart disease (14). Several therapeutic approaches aimed at raising HDL-C levels have since been investigated. However, undisputed proof for causality of low HDL-C in CVD is lacking and clinical trials aimed at raising HDL-C to prevent disease (AIM-HIGH, HPS2-THRIVE, ILLUMINATE, dal-OUTCOMES, ACCELERATE, REVEAL) have failed to meet their primary goals (15–17). In addition, data from Mendelian randomization studies show that HDL-C and myocardial infarction risk are not causally related (14,18). A systematic review and meta-analysis of relevant preclinical studies and clinical trials on the contribution of non-HDL-C/LDL-C lowering versus HDL-C raising concluded that the protective role of lowering LDL-C and non-HDL-C is well-established (19). However, the contribution of raising HDL-C on inhibition of atherosclerosis and the prevention of CVD remains undefined and may be dependent on the mode of action of HDL-C-modification. Similar outcome data were found for the prevention of clinical events in randomized controlled trials and on inhibition of atherosclerosis in relevant, CETP-expressing, animals emphasizing the validity/translatability of these animal models to the human situation (19).

3. TGs and remnant cholesterol

Triglycerides are primarily carried by remnants, a combined term for IDL-, VLDL-, and chylomicron remnants (20). Because of the small size of remnants, they are able to penetrate the arterial wall, thereby promoting accumulation of cholesterol in the intimal space, foam cell formation, and atherosclerosis (21). It is most likely that the cholesterol content of remnants, and not TGs, causes atherosclerosis because most cells can degrade TGs but not cholesterol (20). However, the concentration of TGs is highly correlated with the cholesterol content of remnants (22) and Mendelian randomization analyses demonstrated that TG-lowering *LPL* variants and LDL-C lowering *LDLR* variants were similarly associated with lower risk of CVD per unit difference in apoB (23). As a result, targeting TGs has become an interesting approach to reduce CV events and several novel therapies that interfere with the LPL pathway are under development, including inhibition of apoCIII and angiopoietin-like protein 3 (ANGPTL3) (24–26). One of these agents, the ANGPTL3 antibody evinacumab, was evaluated in this thesis and is therefore discussed in the next section.

Lipid-lowering interventions

Primary prevention of CVD can be achieved by promoting healthy lifestyle behaviour to the general population and at the individual level, and by targeting CV risk factors, e.g.

increased blood pressure, plasma lipid and glucose levels (27). Lifestyle modifications to improve the plasma lipid profile include quit smoking, reduced intake of dietary unsaturated fat, saturated fat and cholesterol, increased intake of dietary fibre, vegetables and fruits, reduction of excessive body weight, and increased physical activity (6,27). Depending on the estimated total CV risk and plasma LDL-C levels, lifestyle modifications can be accompanied by lipid-lowering drugs. For patients that are at high risk, subjects with documented CVD, diabetes mellitus or markedly elevated plasma cholesterol, additional lipid lowering therapies should always be considered (27). **Table 2** summarizes the lipid lowering interventions currently available and their relative risk reduction for major vascular events. Two of these agents, statins and PCSK9 inhibitors, have been evaluated in this thesis and are therefore discussed below.

Table 2 Overview of lipid-lowering interventions currently available

Lipid-lowering intervention	Mechanism of LDL-C lowering	Relative risk reduction for major vascular events*1
PCSK9 inhibitors	Increased LDL-C clearance through upregulation of LDLR.	0.49
Ileal bypass	Reduced absorption of cholesterol by the intestine and restoration of the metabolic response to a meal.	0.65
Statins	Decreased cholesterol biosynthesis through inhibition of HMG-CoA reductase.	0.80
Bile acid sequestrants	Bind components of the bile in the intestine thereby preventing their reabsorption.	0.78
Dietary interventions	Reduced calorie/fat intake and binding of bile acids and cholesterol to fibers.	0.83
Fibrates	Activation of peroxisome proliferator-activated receptor α leading to decreased VLDL particle production and increased lipid clearance.	0.88
Ezetimibe	Reduced cholesterol absorption in the small intestine through blockage of the Niemann-Pick C1 like 1 transporter, essential for the sterol transport across the enterocytes.	0.94
Niacin	Increases clearance of VLDL particles.	0.94

*1 Data extracted from reference (12).

1. Statins

Statins are discovered in 1973 by Akira Endo who isolated the compound compactin from the fungus *Penicillium citrinum*, which was found to be a competitive inhibitor of HMG-CoA reductase, the rate-controlling enzyme in hepatic cholesterol synthesis (28). The first

commercially available statin based on this discovery was lovastatin, followed by 2 semi-synthetic statins (simvastatin and pravastatin) and 4 synthetic statins (fluvastatin, atorvastatin, rosuvastatin and pitavastatin) (28). Inhibition of HMG-CoA reductase lowers intracellular cholesterol concentration which results in a compensatory increased LDLR expression on the hepatocytes and consequently, increased LDL-C uptake and decreased plasma LDL-C levels. Statins reduce plasma LDL-C levels by 20 to 50%, depending on the type of statin and dose (27), and reduce CV risk by 23% per 1.0 mmol/L LDL-C reduction (12). Due to their proven efficacy, statins are among the most frequently prescribed drugs in the world, although there are some limitations. The response to statins is variable and despite maximally tolerated statin doses, a subgroup of patients does not reach their LDL-C goals and remain at significant residual risk. Also, meta-analyses demonstrate that further LDL-C lowering further reduces CVD risk (12), while an estimated 6% reduction of LDL-C is achieved per doubling of the statin dose, the so-called "6% rule" (29). Last, while statins are generally well-tolerated, "muscle complaints" have been reported (30,31) and are the primary reason for statin non-adherence and discontinuation (30). To overcome these limitations, additional therapeutic agents, including proprotein convertase subtilisin/kexin 9 (PCSK9) and ANGPTL3 inhibitors, have been introduced or are currently under development.

2. PCSK9 inhibitors

PCSK9 inhibitors are the most powerful cholesterol-lowering agents currently available. PCSK9 is an enzyme that binds to and shuttles the LDL receptor in the intracellular lysosomal degradation pathway in the liver and other cells thereby preventing the clearance of LDL-C from the plasma. Humans with loss-of-function mutations in the *PCSK9* gene exhibit extremely low levels of LDL-C and are protected from atherosclerosis, whereas gain-of-function mutations are associated with hypercholesterolemia (32). Consequently, antibodies (evolocumab and alirocumab) against PCSK9 have been developed. When administered on top of maximally tolerated doses of statins, these antibodies additionally reduce plasma LDL-C levels up to 60% and the risk of CV events by 15% (33,34). Evolocumab and alirocumab are FDA and EMA approved for subjects with heterozygous familial hypercholesterolemia or for those with clinical atherosclerotic CVD that do not reach their LDL-C goals despite maximally tolerated statin treatment.

3. ANGPTL3 inhibitors

ANGPTL3 is almost exclusively synthesized in the liver, and is an endogenous inhibitor of lipoprotein lipase (LPL), thereby reducing the hydrolysis of TGs in capillaries of adipose tissue and muscles (25). Genetic loss-of-function of *ANGPTL3* causes familial combined hypolipidemia, characterized by very low plasma TG, LDL-C and HDL-C concentrations, and decreased odds of atherosclerotic CVD (26). Pharmacologic antagonism of ANGPTL3 with the antibody evinacumab reduced atherosclerotic lesion area in dyslipidemic

APOE*3-Leiden.CETP mice, and dose-dependently reduced TG and LDL-C levels in healthy subjects evaluated in a phase I trial (26). This novel approach to reduce plasma lipids is particularly important for the treatment of patients with familial hypercholesterolemia with defects in the LDLR, as statins and PCSK9 inhibitors depend on functional LDLR, as well as for patients with the metabolic syndrome and type 2 diabetes, which are associated with elevated plasma TG levels (25). Evinacumab is currently being evaluated in a phase III trial for patients with homozygous familial hypercholesterolemia.

Diabetes and CVD risk

Type 2 diabetes is characterized by elevated blood glucose levels and insulin resistance, and is commonly associated with obesity and other components of the metabolic syndrome (**Table 3**) (35), including atherogenic dyslipidaemia, which consists of elevated plasma concentrations of both fasting and postprandial TG-rich lipoproteins, small dense LDL and low HDL-cholesterol (36). Consequently, CVD remains the leading cause of morbidity and mortality for patients with type 2 diabetes (36). Despite the close relation between hyperglycaemia and CVD, most studies that evaluated intensive glycaemic control in diabetic patients failed to show significant benefits in terms of CV morbidity and mortality (37), and some agents even increased adverse CV events, e.g. heart failure (38) and myocardial infarction (39,40). To establish the safety of new antidiabetic drugs, the FDA and EMA mandated all new diabetes drugs to demonstrate CV safety (41,42), of which the clinical trials with empagliflozin (EMPA-REG OUTCOME) (43), liraglutide (LEADER) (44) and piogliazone (45) were among the first that showed beneficial effects on CVD outcomes.

Table 3 Definition of the metabolic syndrome

Central obesity Plus any two:	
Raised TGs	>150 mg/dL (1.7 mmol/L) Specific treatment for this lipid abnormality
Reduced HDL-C	<40 mg/dL (1.03 mmol/L) in men <50 mg/dL (1.29 mmol/L) in women Specific treatment for this lipid abnormality
Raised blood pressure	Systolic >130 mmHg Diastolic >85 mmHg Treatment of previously diagnosed hypertension
Raised fasting plasma glucose	Fasting plasma glucose > 100 mg/dL (5.6 mmol/L) Previously diagnosed type 2 diabetes

Data extracted from reference (35).

Inflammation in atherosclerosis

The role of inflammation in atherosclerotic disease is well-established and it has been shown that inflammatory processes mediate all stages of atherosclerosis: from the initiation through progression and eventually, thrombotic complications (46). As a result, not only plasma lipid levels, but also plasma levels of the inflammatory biomarker C-reactive protein (CRP) are predictive for individual CVD risk (47). Important players in the inflammatory pathways are cytokines, that can be classified as pro- or anti-atherogenic. Examples of pro-atherogenic cytokines are tumour necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, whereas transforming growth factor -β (TGF-β), IL-10, and IL-35 are among the anti-atherogenic cytokines (48). Cytokines are expressed by a variety of inflammatory cells but also by other tissues including white adipose tissue, liver, vascular SMCs and the endothelium. Plasma levels of the pro-inflammatory cytokines IL-6, IL-5 and interferon- γ (IFN-γ) have been found to be associated with CVD risk (49). From a clinical perspective, targeting cytokines would be an interesting approach to reduce inflammation-driven atherosclerosis progression. As a result, several therapeutic approaches that modulate cytokine production have been developed or are under investigation (48). Examples are the anti-IL-6 antibody tocilizumab which has been shown to attenuate the inflammatory response after coronary angiography in patients with non-ST-elevation myocardial infarction (50), and the CANTOS trial with the anti-IL-1β antibody canakinumab that demonstrated a lower rate of recurrent CV events in patients with previous myocardial infarction, which was related to the magnitude of CRP reduction (51). In this thesis, the pro-inflammatory cytokine Oncostatin M (OSM) has been evaluated as potential therapeutic target for CVD.

OSM

OSM is a member of the IL-6 family cytokines and plays an important role in various biologic actions. There are two types of functional OSM receptors, the leukaemia inhibitory factor receptor (LIFR) and the OSM receptor (OSMR) (53). OSM signals through both receptors in humans, whereas only the OSMR is used in mice (53). OSM is synthesized in hematopoietic cells and in various inflammatory cells such as activated T-cells, neutrophils, eosinophils, and macrophages (54). OSM has been found to be upregulated in multiple chronic inflammatory diseases (55–57) and it is expressed at sites of atherosclerotic lesions (58). Epidemiological studies have shown that an elevated serum OSM level is positively correlated with the degree of coronary stenosis in patients with coronary artery disease (59). Moreover, development of atherosclerosis is attenuated in OSMR-β deficient APOE$^{-/-}$ mice (60), indicating the pro-atherogenic properties of OSM. Currently, there are no therapies available that target OSM.

Experimental atherosclerosis

This thesis describes (I) novel strategies to reduce plasma lipids and atherosclerosis development, (II) the (cardio)vascular off-target effects of registered drugs and an environmental pollutant, (III) a novel mouse model for diabetic atherosclerosis combining modifiable elevated plasma lipid and glucose levels, and (IV) the potential of OSM as novel pro-inflammatory CV target. In all these studies we used the APOE*3-Leiden(.CETP) mouse model, a humanized model for lipoprotein metabolism and atherosclerosis.

Mouse versus man

Conventional mouse strains used in preclinical biomedical and toxicological research, for example C57BL/6 mice or BALB/c mice, are considered not to be the most appropriate animal models to study modulation of lipoprotein metabolism, since lipolysis of TG-rich particles as chylomicrons and VLDL and their remnants and clearance of the apoB-containing (non-HDL) lipoproteins via the apoE-LDL-receptor pathway are fast processes as compared to humans (61). Consequently, the mice have relatively low plasma TG and cholesterol levels with low levels of the atherogenic VLDL and LDL, and the majority of cholesterol is contained in HDL (**Figure 2A-B**). Severe dietary regimens with saturated fat and high amounts of cholesterol and cholic acid are required to increase the amount of non-HDL-C to some extent, but still lower than in humans (62). As a result, these strains only develop small lesions with features of the earliest state of atherosclerosis, but do not develop complex atherosclerotic lesions (63) as seen in CVD patients.

In humans, lipolysis is slower and removal of apoB-containing lipoproteins is delayed (61). In addition, humans unlike mice possess an important player in lipoprotein metabolism, CETP, which transfers cholesterol from HDL to (V)LDL in exchange for triglycerides, thereby increasing (V)LDL-C levels and decreasing HDL-C. Due to these differences, in man cholesterol is contained mainly in the pro-atherogenic LDL and to a lesser extent in HDL (**Figure 2C-D**).

The APOE*3-Leiden.CETP mouse model

To develop a mouse model with a more human-like lipoprotein metabolism for pharmacological, nutritional and toxicological research, the APOE*3-Leiden transgenic mouse was generated by the introduction of a genomic human DNA construct carrying the mutant *APOE*3-LEIDEN* gene, the *APOCI* gene, and all known regulatory elements, obtained from a patient with familial dysbetalipoproteinemia (FD) (64). FD or type III hyperlipoproteinemia is characterized by elevated levels of plasma cholesterol and an increased ratio of cholesterol to TG in the VLDL and IDL fractions, resulting in the appearance of β-VLDL particles (65). These mice were cross-bred with mice expressing human CETP under control of its natural flanking regulatory DNA-sequences (66) to obtain the APOE*3-Leiden.CETP mouse, as a humanized model for FD and mixed dyslipoproteinemia (67). While normal wild-type mice have a very rapid clearance of apoB-containing

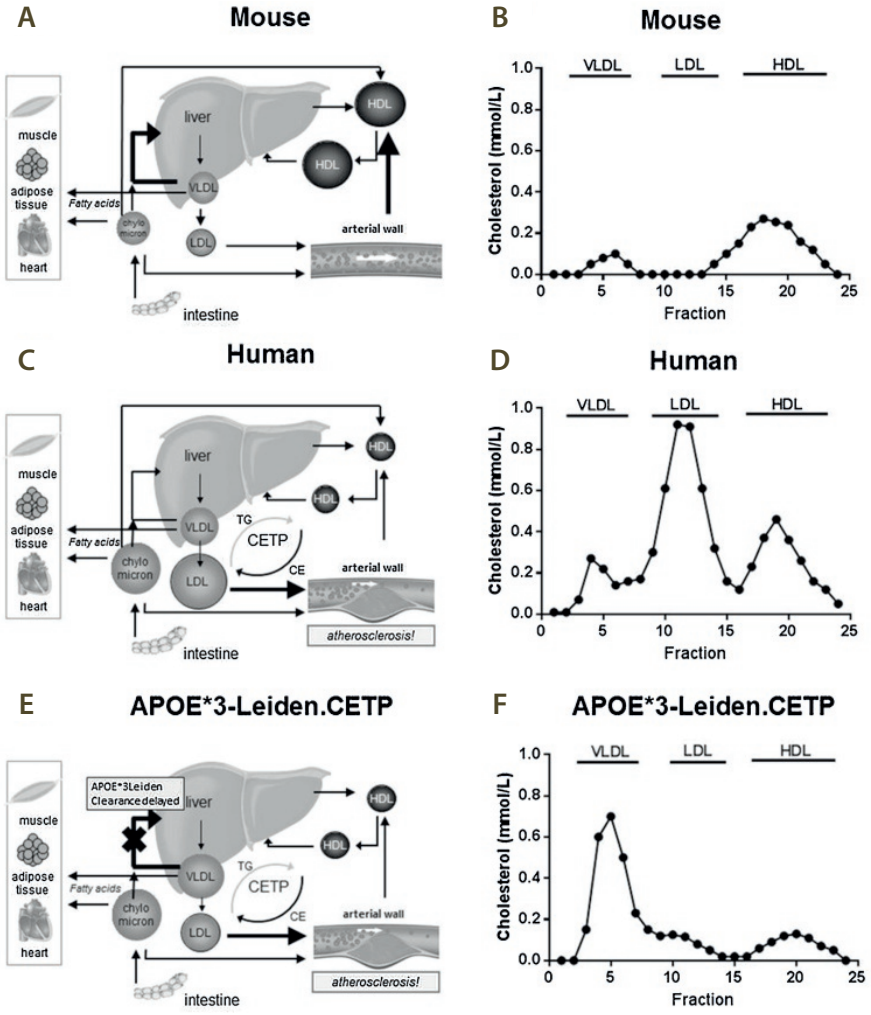

Figure 2 Mice have a fast clearance of apoB-containing lipoproteins and do not express CETP (A), as a result the majority of plasma cholesterol is confined to HDL (B), with TC and TG levels of 1.5–2.0 and 0.2–0.3 mmol/L in C57BL/6 mice. Humans have a slower clearance of apoB-containing lipoproteins and do express CETP (C) and normolipidemic man have TC and TG levels of <5.2 and 0.5–1.5 mmol/L, respectively, and cholesterol consists mainly of non-HDL-C (VLDL-C/LDL-C) (D). The APOE*3-Leiden. CETP mouse has a lipoprotein profile similar as in FD patients and a lipoprotein metabolism similar to that in man (E), and on a chow diet TC and TG levels are 3.0–4.0 and 2.5–3.0 mmol/L, mainly confined to the non-HDL-C fraction (F).

lipoproteins, APOE*3-Leiden(.CETP) mice have an impaired clearance and increased TG level, and are thereby mimicking the slow clearance observed in humans, particularly in patients with FD (61,65,68). Similarly as in FD patients, in APOE*3-Leiden and APOE*3-Leiden.CETP mice, the major part of plasma cholesterol is contained in the VLDL and VLDL-remnant particles, leading to formation of β-VLDL particles, which is further increased by cholesterol feeding (64,67) (**Figure 2E-F**). Consequently, APOE*3-Leiden.CETP mice develop advanced atherosclerotic lesions with characteristics of human pathology that can be histologically classified according to the American Heart Association (AHA) (5) (**Figure 3**).

Importantly, as compared to the widely used hyperlipidaemic and atherogenic apoE- and LDLR-deficient (apoE$^{-/-}$ and LDLR$^{-/-}$) mice, the APOE*3-Leiden(.CETP) mice possess an intact but delayed apoE-LDLR-mediated clearance, which is an essential characteristic of human lipoprotein metabolism and for the proper, human-like response on hypolipidemic drugs (69,70). APOE*3-Leiden.CETP mice respond well to dietary intervention using human-relevant (Westernized) diets with increases in plasma cholesterol and TG and these lipids can be titrated to levels mimicking those in humans. Therefore, APOE*3-Leiden.CETP mice are a translational and predictive animal model for the effect of drugs on lipoprotein metabolism and atherosclerosis. Also, the APOE*3-Leiden.CETP mouse model has proved to be a suitable model for investigation of the mechanism of action of off-target effects of drugs (71) and environmental pollutants (72). **Table 4** gives an overview of lipid-lowering interventions that have been evaluated in APOE*3-Leiden.CETP mice and compares the effects on plasma lipids and atherosclerosis with data in hyperlipidaemic and FD-patients. It should be noted that APOE*3-Leiden.CETP mice respond similarly as FD-patients to niacin and fibrates, whereas greater (V)LDL-C reductions are achieved in APOE*3-Leiden.CETP mice relative to hyperlipidaemic patients (73–75).

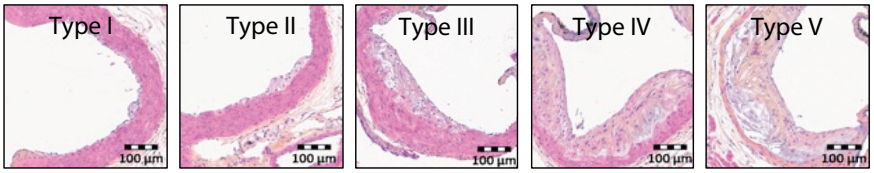

Figure 3 Atherosclerotic lesions in APOE*3-Leiden.CETP mice. Type I: early fatty streaks consist of ≤ 10 foam cells in the intima. Type II: regular fatty streaks consist of >10 foam cells in the intima. Type III: mild plaques consist of foam cells covered with a fibrotic cap. Type IV: moderate plaques consist of foam cells, often together with necrosis and cholesterol crystals, and severe disorganization of the intima. Inflammatory cells and foam cells infiltrate the media and intimal smooth muscle cells disarrange. Type V: severe plaques consist of foam cells, a fibrotic cap, necrosis, cholesterol crystals and calcium deposits. The media and adjacent adventitia may contain accumulations of lymphocytes, macrophages, and macrophage foam cells. Severe disarrangement of the media with disruption of the elastic fibers.

Table 4 Effects of lipid-lowering interventions in APOE*3-Leiden.CETP mice and humans

Lipid lowering intervention	APOE*3-Leiden.CETP mice		Humans		References
	Plasma cholesterol	Atherosclerosis	Plasma cholesterol	Cardiovascular risk	
HmgCoA reductase inhibitors/ statins					
Atorvastatin	↓	↓	↓	↓	(12,76–79)
Simvastatin	↓	↓	↓	↓	(12,73)
TG-lowering, HDL-raising drugs					
Niacin	↓/↑[*1]	↓	↓/↑[*1]	↔	(12,16,17,73,75,80)
Fibrates	↓/↑[*1]	↓[*2]	↓/↑[*1]	↓	(12,75,81–85)
HDL-modulating drugs					
Anacetrapib	↓/↑[*1]	↓	↓/↑[*1]	↓	(74,87,88)
Torcetrapib	↓/↑[*1]	↑	↓/↑[*1]	↑	(89,90)
PCSK9 inhibitors					
Alirocumab	↓	↓	↓	↓	(12,33,77)
Evolocumab	↓	↓	↓	↓	(69,91)
Miscellaneous					
Ezetimibe	↓	↓	↓	↓	(12,78)
Bile acid sequestrants	↓[*2]	nd	↓	↓	(12,86)
Evinacumab	↓	↓	↓	nd	(26)

[*1] HDL-C increased; [*2] In APOE*3-Leiden mice, unpublished data in APOE*3-Leiden.CETP mice; nd, not determined.

Outline of the thesis

This thesis describes a variety of studies on novel interventions and targets in lipid and lipoprotein metabolism and atherosclerosis, and on CV safety of anti-cancer drugs and a widely used industrial surfactant that persists in the environment. In all studies the APOE*3-Leiden(.CETP) mouse model was used as a well-established translational model for lipoprotein metabolism and atherosclerosis development.

In **Chapter 2** we evaluated whether a vaccine against PCSK9 could induce an effective immune response against PCSK9, thereby reducing plasma cholesterol levels and atherosclerosis progression. However, as most patients at CVD risk are treated after development of atherosclerosis, therapies that regress pre-existent lesions are warranted. It is known that the magnitude of regression is correlated with the percentage of LDL-C reduction, and therefore, **Chapter 3** evaluated if aggressive lipid-lowering interventions

using double and triple treatment with simple or combined inhibition of PCSK9 (alirocumab) and ANGPTL3 (evinacumab) on top of atorvastatin, could regress pre-existent lesions. **Chapter 4** describes the CV off-target effects of three generations tyrosine kinase inhibitors (TKIs), imatinib, nilotinib and ponatinib, respectively, that are being used for the treatment of patients with chronic myeloid leukaemia (CML). In contrast to the safe profile of imatinib, CV side effects have been reported in patients receiving nilotinib and ponatinib. Also, modulations in plasma lipids occur when CML patients are treated with these TKIs, therefore we investigated the mechanism of these lipid modulations in **Chapter 5**. The dose effects of perfluorooctanoic acid (PFOA) on lipoprotein metabolism are presented in **Chapter 6**. PFOA has been widely used as an emulsifier in the manufacture of fluoropolymers, is extremely stable and therefore persists in the environment. In addition to abnormalities in plasma lipids, diabetes can add to the CVD risk and the development of novel anti-diabetic drugs has shifted from solely glucose-lowering agents towards agents that additionally reduce CVD risk. This shift requires preclinical translational models that combine hyperlipidaemia and hyperglycaemia, and we therefore developed a mouse model with both features, the APOE*3-Leiden.glucokinase$^{+/-}$ mouse. The characteristics of this novel model are described in **Chapter 7.** The next two chapters describe the evaluation of the cytokine OSM as possible target to reduce endothelial inflammation, important in the initiation of atherosclerosis. In **Chapter 8** we evaluated the inflammatory response to OSM in different human vascular beds, and on markers of endothelial inflammation in plasma and the aortic root of APOE*3-Leiden.CETP mice. In **Chapter 9**, mice were prolonged exposed to OSM and atherosclerosis development was examined. In addition, we investigated possible associations between plasma OSM levels in CVD patients and survival from coronary heart disease.

The results obtained in these studies and future perspectives are discussed in **Chapter 10**.

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