Novel pharmaeutical interventions in experimental atherosclerosis and myocardial infarction

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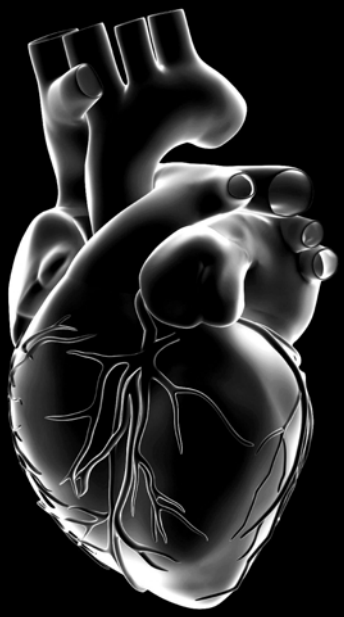

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General Introduction

Cardiovascular disease is the number one cause of death globally and is projected to remain the leading cause of death in the future. Estimates of the World Health Organization[1] show that 17.5 million people died from cardiovascular disease (CVD) in 2005, which represented 30% of all global deaths. It was estimated that if appropriate action is not taken, by 2015, 20 million people will die from CVD every year, mainly from myocardial infarction (MI) and stroke. The extensive frequency of CVD in the industrialized countries has been observed for decades. Therefore, in 1976 CVD risk equations were developed by the investigators of the Framingham Heart Study (FHS)[2], enabling clinicians to predict the development of coronary disease in individuals free of disease. The FHS is a longitudinal study, which started follow-up of healthy residents of Framingham (Massachusetts, USA) in 1948 and has included subsequent generations ever since. In a 12-year follow-up of a defined cohort of the FHS, the Framingham risk score was developed. It provides a 10-year hazard ratio for CVD based on sex, age, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein (HDL)-C, blood pressure, diabetes and smoking habits[3,4].

The European Society of Cardiology initiated the development of a European risk score system (SCORE) and used data from 12 European cohort studies (n=205,178) covering a wide geographic spread of countries at different levels of cardiovascular risks[5]. The SCORE was even calibrated for the different countries; the one for The Netherlands is presented in **figure 1**. These new SCORE risk estimates of cardiovascular death are based on the same factors as the Framingham risk score with exception of diabetes. With a relative risk of approximately five in women and three in men, the impact of diabetes on CVD appeared to be much greater in these European studies. Therefore it was not included in the SCORE estimate but identified as an independent risk factor.

The single most important contributor to the growing burden of CVD is atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. The pathophysiology of this key problem has been studied extensively in the past century. Nowadays we consider atherosclerosis as a multifactorial disease in which lipids and inflammation play major roles[6,7]. The approach to primary prevention of atherosclerosis and CVD is founded on the public health approach that calls for lifestyle changes, including (I) reduced intakes of saturated fat and cholesterol, (II) increased physical activity, and (III) weight control. The clinical approach emphasizes preventive strategies for higher-risk persons. The major risk factors for CVD development and the general therapeutical options will be outlined in this chapter.

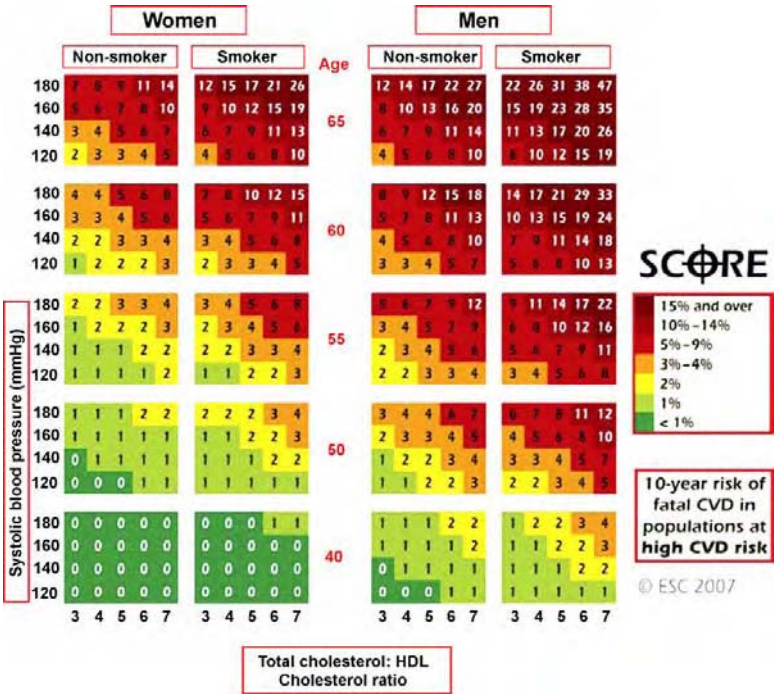

Figure 1 The SCORE estimate for The Netherlands providing a 10-year hazard ratio for fatal CVD[5]

Risk Factors for the development of CVD

Cholesterol

Identified as an important risk factor in SCORE and FHS, also the current guidelines to treat CVD from the Adult Treatment Panel III[8], the American Diabetes Association[9] and American Heart Association[10] emphasize targeting primarily LDL-C. HMG-CoA reductase inhibitors (statins) are widely used to lower LDL-C. In intervention trials using statins substantial reductions in major cardiovascular events in the treated groups were observed[11]. Furthermore, the magnitude of the reduction in events is a function of the amount of LDL-C, with each decrease of 1.0 mmol/L in LDL-C corresponding to a 23% reduction in major cardiovascular events[11]. However, in all the statin trials, substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL-C [11-14]. This indicates that additional treatment is required.

Clinical studies have shown that HDL-C levels, independently of LDL-C, are inversely correlated with the risk of CVD (**figure 2**)[15-18]. In statin treated patients this relationship was also observed among patients with very low LDL-C levels (<1.8 mmol/L) [19]. In the FHS, HDL-C level was more potent as a risk factor for CVD than was the level of LDL-C[20]. An analysis of data from four large studies concluded that each increase of 0.03 mmol/L in HDL-C is associated with a decrease of 2 to 3% in the risk of future CVD[16]. These findings have shifted the attention towards strategies for targeting HDL-C as adjunctive therapy to prevent and treat CVD.

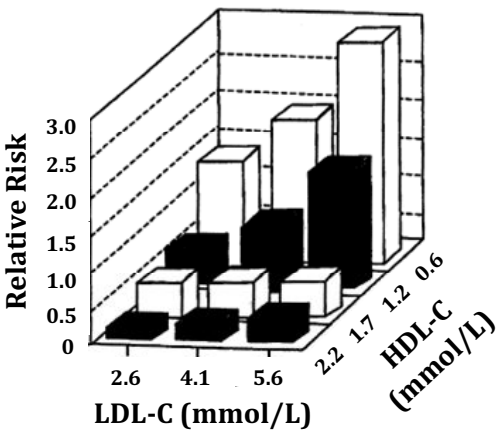

Figure 2 For any given level of LDL-C in the Framingham population, the relative risk of CHD decreases with increasing serum concentrations of high-density lipoprotein cholesterol (HDL-C) [15,21].

Hypertension

Hypertension is considered a 'traditional' risk factor for developing atherosclerosis, which entails a threefold risk over that of normotensive persons of the same age[22]. Hypertension *per se* might facilitate atherosclerosis development by the pressure-induced stretching of the arterial wall, which is a major determinant of arterial mass transport. Therewith it could enhance LDL-C accumulation in the inner media of the vessel wall[23], inducing endothelial activation and vascular inflammation[7]. However, evidence is also accumulating that hypertension may be just a marker and that the underlying mechanism is the risk factor for the development of atherosclerosis. A central role herein is considered for angiotensin II, a key molecule of the renin-angiotensin-aldosterone-system (RAAS), which regulates blood pressure[24-26]. However, in the fast majority of cases no single reason can be found for a patient causing the hypertension, indicating that hypertension is a very complex multifactorial disease, in which different mechanisms are involved.

Diabetes

Diabetes has been identified as a risk factor for CVD since many years and has gained the interest of research because of its increasing prevalence[8,10,27]. Estimates of current and future diabetes prevalence predict more than a doubling of the global burden of diabetes within 25 years from now[28]. In 75% of these subjects with type 2 diabetes mellitus

(T2D) an atherogenic triad will be observed, whereas it also is a common characteristic of patients with insulin resistance and abdominal obesity[29]. The atherogenic lipid triad comprises raised plasma triglycerides (TGs), reduced HDL-C, and a predominance of small dense (sd) LDL, all of which are associated with an increased risk in CVD[27].

Besides inducing dyslipidemia, insulin resistance itself also affects other pathophysiologic mechanisms, which may increase the risk on CVD. Though not all mechanisms are clarified yet, insulin resistance is thought to contribute to the development of hypertension[29,30], to impair thrombolysis[30,31], to case endothelial dysfunction[30] and to induce systemic and vascular inflammation[32], all contributors to the development of atherosclerosis and CVD[7,33].

Atherothrombosis

The main CVD events are MI and stroke, which occur when an atheromatous process precipitates thrombosis that prevents blood flow through the coronary or cerebral artery. Platelets, essential for primary hemostasis and repair of the endothelium, play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events by triggering the acute onset of arterial thrombosis when atherosclerotic lesions rupture. In addition, they participate in the process of forming and extending atherosclerotic lesions. As atherosclerosis is a chronic inflammatory process, inflammation is an important component of acute coronary syndromes[7]. The relation between chronic and acute vascular inflammation is unclear, but platelets are a source of inflammatory mediators, which once activated, are able to activate vascular cells[34,35]. The activation of platelets by inflammatory triggers may be a critical component of atherothrombosis[36].

Pharmaceutical therapies

Cholesterol

The current armamentarium of lipid-lowering drugs includes inhibitors of hydroxy-3-methyl-glutaryl-CoA reductase (statins), PPARα agonists (fibrates), niacin (nicotinic acid), all directly or indirectly inhibiting lipid synthesis in the liver, and selective cholesterol absorption inhibitors (e.g. ezetimibe) and bile acid sequestrants (anion exchange resins), which work in the intestine by inhibiting the cholesterol absorption from food and bile. Next to lipid lowering, statins and fibrates also reduce inflammation via inhibition of NF-κB pathways, whereas lowering LDL-C *per se* also has anti-inflammatory effects[7,37,38].

Statin therapy may be considered as the 'standard' therapy to decrease (V)LDL-C, which has been shown to be very effective by lowering LDL-C by almost 30% in numerous studies and which may increase HDL-C modestly by a few percents[11].

Together with their pleiotropic effects statins are very potent in reducing CVD endpoints[11,39,40]. Fibrates potently reduce VLDL-TG (approx. -40%) and mildly increase HDL-C (approx. +10%) and seem to have most pronounced effect on CVD in obese and diabetic patients[18,41,42]. Niacin is a very powerful (V)LDL-TG lowering compound (approx. -40%) and the strongest HDL-C raising compound currently available (+ 15-30%)[18]. However, due to its side-effect, severe flushing, it is not very well tolerated.

The cholesterol uptake inhibitor ezetimibe mildly lowers LDL-C (approx. -20%)[43], but in combination with a low dose of statin the compounds strongly reduces LDL-C levels (approx. -55%). Bile acid sequestrants also lower LDL-C mildly, which is to a similar extent as ezetimibe, however, these compounds tend to increase TG[18,44].

Future therapies aiming at increasing HDL-C are cholesteryl ester transfer protein (CETP) inhibitors, GPR109A ('niacin receptor') agonists, selective cannabinoid type I receptor (CB1) antagonists, ApoAI mimetics and intravenous infusion of HDL[18]. These latter two therapies with a transient increase of HDL aim at an increased cholesterol efflux from the vessel wall and additionally a reduced the vessel wall inflammation[39].

Hypertension

The RAAS plays an important role in the regulation of blood pressure and body fluid and electrolyte homeostasis and may therefore be targeted to treat hypertension. The synthesis of angiontensin II, the main regulator molecule of RAAS, or the binding of angiotensin II to its receptor can be inhibited by angiotensin converting enzyme (ACE) inhibitors or angiotensin II type I receptor blockers (ARBs), respectively both are frequently used anti-hypertensive treatments. The RAAS also interacts with inflammatory pathways and its inhibition has clear anti-inflammatory effects[26,45]. Vasoconstriction can also be inhibited by blocking the calcium transport into the vascular smooth muscle cells by selective calcium channel blockers (CCBs). Other regularly used anti-hypertensive drugs are β-blockers and diuretics. Collective data of numerous prospective trials showed that anti-hypertensive treatment with any commonly-used regimen reduces the risk of total major cardiovascular events, whereby larger reductions in blood pressure produce larger reductions in risk [46].

Diabetes and insulin resistance

The most prescribed and effective insulin sensitizers are the thiazolidinediones, also referred to as the glitazones, and metformin. The latter compound has been used internationally for decades. Its primary mechanism of action is to suppress gluconeogenesis and to increase glucose uptake in the liver[47]. The glitazones increase peripheral utilization of insulin by acting as ligands of the peroxisome proliferator-activated receptor gamma (PPARγ). This receptor is found in high concentrations in adipose tissue and in the vessel wall, and is involved in the regulation of genes that

control glucose homeostasis, lipid metabolism, and adipose tissue[48]. In order to increase the plasma levels, insulin can be administered, whereas it is also possible to stimulate its secretion by the use of sulfonylureas and meglitinides. However, these compounds exhibit adverse effects of hypoglycemia and weight gain[49]. New and future therapies to improve insulin sensitivity enclose the endocannabinoid system (CB1 receptor antagonists) and gut-hormone regulated routes.

Anti platelet therapies

The anti-platelet drug acetylsalicylic acid (aspirin) has been proven to prevent myocardial infarction and stroke in patients with CVD[50]. However, the major adverse side effect, bleeding, and the large prevalence of aspirin resistance (5-45%) are drawbacks of this drug[51,52]. Another class of anti-platelet agents are the thienopyridines, of which clopidogrel is a member, which act by blocking the adenosine diphosphate (ADP)-mediated pathway of platelet activation. Clopidogrel is at least as effective as aspirin in preventing ischemic stroke, myocardial infarction and vascular death[53]. However, combining the two does not significantly decrease cardiovascular events and may even increase major bleedings[54]. The clinical efficacy of aspirin is based on inhibition of the platelet cyclo-oxygenase-1 (COX-1), inhibiting the generation of platelet thromboxane A_2 (TxA_2), which binds to the thromboxane-prostanoid endoperoxide (TP) receptor and thereby activates the platelet[55]. A third therapeutic route to inhibit platelet activation therefore may very well be direct inhibition of TxA_2 or its TP-receptor, which is present on platelets. No TP-receptor antagonist is currently available; however a new compound terutroban (S 18886) has been developed and is currently in phase III of development.

This thesis will present and discuss a variety of novel pharmaceutical interventions in experimental CVD as new ways to treat elevated lipid levels, blood pressure and conditions with increased risk of atherosclerosis. To study the effect of pharmaceutical intervention therapies on lipid metabolism, atherosclerosis and CVD we used suitable 'humanized' mouse models for hyperlipidemia and atherosclerosis: APOE*3Leiden and APOE*3Leiden.CETP transgenic mice.

Experimental model for hyperlipidemia, atherosclerosis and myocardial infarction

Wild-type mice are resistant to atherosclerosis as a result of high levels of anti-atherosclerotic HDL and low levels of proatherogenic LDL and VLDL, making them not useful for atherosclerosis research. All of the current mouse models for atherosclerosis are therefore based on modulations of lipoprotein metabolism through dietary or genetic manipulations. Among the most widely used mouse models are apolipoprotein

E–deficient mice (apoE$^{-/-}$ mice), the LDL receptor- deficient mice (LDLr$^{-/-}$ mice) and the APOE*3Leiden transgenic mice.

*Apolipoprotein E- deficient (*apoE$^{-/-}$*) mice*

The targeted deletion of the *apoE* gene of the homozygous apoE$^{-/-}$ mice results in a pronounced increase in the plasma levels of LDL and VLDL attributable to the failure of LDLr- and LDLr-related protein (LRP) mediated clearance of these lipoproteins[56,57]. Even on a chow diet they exhibit severe hypercholesterolemia (about 9 mmol/L), which, together with the reduced apoe-mediated cholesterol efflux from macrophages, leads to spontaneous lesion development especially in the aortic arch[58]. Over time these lesions become quite complex, progressing well beyond the fatty streak and they resemble human lesions. This model is suitable to study cellular aspects of lesion development and has been used for years to that end. However, one of the major drawbacks of this model is the lack of responsiveness to pharmaceutical and/or nutritional lipid lowering therapy[64]. This makes the model less suitable for the evaluation of therapeutic interventions in atherosclerosis. The apoE$^{-/-}$ mice may be considered as a severe model for atherosclerosis. Additionally hampering the HDL clearance by cross breeding apoE$^{-/-}$ mice with HDL receptor scavenger receptor class B, type I deficient mice (generating apoE$^{-/-}$/SR-BI$^{-/-}$mice), results in extreme hypercholesterolemia and a dramatically accelerated atherosclerosis, which even leads to spontaneous lipid- and fibrin-rich occlusive coronary arterial lesions, multiple myocardial infarctions, and cardiac dysfunction[59]. These apoE$^{-/-}$/SR-BI$^{-/-}$ mice die prematurely at about 6 weeks of age and can be considered as the most extreme model for CVD.

*LDL receptor- deficient (*LDLr$^{-/-}$*) mice*

The LDLr$^{-/-}$ mice display a modest hypercholesterolemia on a chow diet (about 5 mmol/L), with the cholesterol mainly confined to the LDL. Atherosclerosis develops slowly and is enhanced when these mice are fed a lipid-rich diet[60]. Interestingly, LDLr$^{-/-}$ mice cross bred with ApoB mRNA editing catalytic polypeptide-1 deficient mice (generating LDLr$^{-/-}$/ApoBEC$^{-/-}$ mice)[61] or with human ApoB100 transgenic mice (generating LDLr$^{-/-}$; Tg(ApoB$^{+/+}$) mice)[62] show a large increase in plasma LDL-C and develop atherosclerosis on a low-fat diet. The LDLr$^{-/-}$ mouse represents a more moderate model than the apoE$^{-/-}$ mouse, mainly because of the lower degree of hyperlipidemia. However, their responsiveness to lipid-lowering therapies is not optimal or might even be absent[64].

*APOE*3Leiden transgenic mouse*

A milder model is the APOE*3Leiden transgenic mouse, which develops atherosclerosis upon cholesterol feeding, and is more sensitive to lipid-lowering drugs than apoE$^{-/-}$ and LDLr$^{-/-}$ mice[63,64]. Hyperlipidemic APOE*3Leiden transgenic mice were generated by

introducing a human APOE*3Leiden gene construct, which also contained the APOC1 gene and a promoter element regulating the expression of APOE and APOC1 genes, into wild-type C57Bl/6 mice[63,65]. Although APOE*3Leiden mice still express endogenous apoE protein, the clearance of apoE-containing lipoproteins is impaired, albeit less dramatically than in apoE$^{-/-}$ mice. APOE*3Leiden mice show significant elevations of plasma cholesterol and TG on a regular chow diet and are, in contrast to wild-type mice, highly responsive to fat-, sugar-, and cholesterol-containing diets. This results in a lipoprotein profile similar to that of patients with familial dysbetalipoproteinemia in whom the elevated plasma cholesterol and TG levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction[63]. Plasma lipid levels can easily be adjusted to a desired concentration by titrating the amount of cholesterol and sugar in the diet. As compared with other hyperlipidemic mouse models (e.g. apoE$^{-/-}$ and LDL$^{-/-}$ mice), APOE*3Leiden mice represent a milder mouse model for hyperlipidemia (cholesterol levels on chow are about 2-3 mmol/L and do not exceed 25 mmol/L on a western-type high-cholesterol diet). The development of atherosclerosis strongly correlates with the plasma cholesterol levels and the duration of cholesterol elevation (**figure 3**), and consists of lesions with all the characteristics of human vascular pathology, varying from fatty streak to mild, moderate, and severe lesions[66].

The APOE*3Leiden mice are a suitable model to study the (V)LDL metabolism and, in contrast to apoE-/- and ldlr-/- mice, they respond in a human-like manner to treatment of CVD (e.g. statins, calcium channel blockers, fibrates, angiotensin II receptor blockers, and cholesterol uptake inhibitors [67-73])[64]. However, APOE*3Leiden mice do not respond to HDL raising therapies. This is the consequence of the lack of CETP expression in mice, an important factor in the human HDL metabolism. CETP mediates the transfer of cholesteryl ester from HDL particles to the apoB-containing lipoproteins ((V)LDL) in exchange for triglycerides.

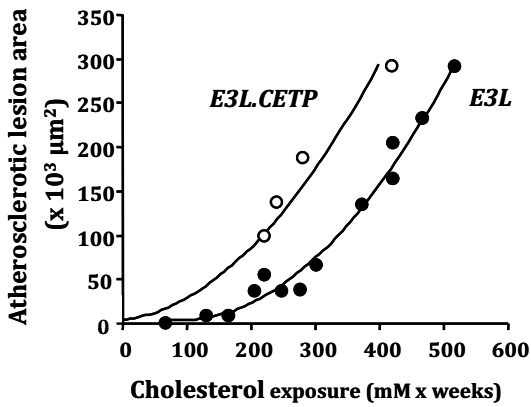

Figure 3 The strong correlation between cholesterol exposure (plasma cholesterol levels in mmol/L times the duration in weeks) and the atherosclerosis development in APOE*3Leiden (black circles) and APOE*3Leiden.CETP (open circles) transgenic mice. Unpublished observations of H.M.G. Princen and P.C.N. Rensen.

Therefore, APOE*3Leiden mice were recently cross-bred with mice expressing the human CETP gene under control of its natural flanking regions, resulting in APOE*3Leiden.CETP mice[74]. These mice display an elevated basal cholesterol level and

a human-like lipoprotein profile. CETP expression in APOE*3Leiden mice shifts the distribution of cholesterol from HDL toward VLDL/LDL, and strongly (7-fold) increases atherosclerosis development (**figure 3**)[74]. Their responsiveness to lipid modulating therapies was even increased, since these mice also respond to the HDL-raising effects of fenofibrate[75], atorvastatin[76], torcetrapib[77] and niacin[78].

Outline of the thesis

This thesis describes a variety of novel pharmaceutical interventions in experimental CVD of APOE*3Leiden mice.

Chapter 2 reviews the effect of current 'standard' therapies for the treatment of two separate risk factors for CVD, *i.e.* hyperlipidemia with atorvastatin and hypertension with the calcium channel blocker amplodipine. Additionally, scientific evidence is collected to determine the possible advantage of combining these two kinds of treatments, potentially leading to additive or synergistic effects in the prevention of CVD.

Although compounds have proven their benefit in the clinic, their exact working mechanism is not always clarified. Niacin (vitamin B3) is one of those compounds. In the early 1950s it was known that niacin decreases LDL-C and concomitantly increases HDL-C. The underlying mechanism however, remained to be elucidated. In **Chapter 3** the mechanism of the HDL-C raising effect of niacin is explored in the APOE*3Leiden.CETP transgenic mouse model, which was proven in this study to be a very suitable model to investigate HDL-raising therapies.

Modulating plasma lipid levels using compounds like niacin, PPARα agonists, or cholesterol uptake inhibitors is of significance to obtain an indication about their effect on atherosclerosis development. In **chapter 4** we evaluate the effect of a new cholesterol uptake inhibitor AVE5530 with regard to its VLDL/LDL-C lowering capacity as well as its anti-atherosclerotic effects in APOE*3Leiden mice. Ezetimibe, a cholesterol uptake inhibitor already used in the clinic, has been used as a reference compound. A major difference between AVE550 and ezetimibe is that nearly 100% of ezetimibe is absorbed in the intestine whereas AVE5530 is not.

As described in chapter 2, combination therapy targeting two risk factors of CVD might have synergistic or additive effects in the prevention of CVD and atherosclerosis. An example of such a study is presented in **chapter 5** where APOE*3Leiden mice are treated with either the anti-hypertensive angiotensin receptor blocker olmesartan or the antihyperlipidemic drug pravastatin, alone or with the combination of both compounds.

Nowadays compounds are developed in order to target two independent risk factors for CVD simultaneously, for instance the dual PPARα/γ agonist tesaglitazar

treating both hyperlipidemia and insulin resistance/diabetes. In **chapter 6** the effect of tesaglitazar is investigated in APOE*3Leiden.CETP with pre-existing atherosclerotic lesions. Such a study design is of more clinical significance, since in humans lesions have already been developed before treatment is started.

A similar design is used for the study presented in **chapter 7** where APOE*3Leiden mice had developed mild lesions before cholesterol-lowering and anti-platelet therapy with a thromboxane prostanoid (TP) receptor antagonist S18886 was started. The effects of thromboxane and its receptor on platelet function and peripheral tissue are not fully clarified yet. However, evidence is accumulating that it interacts with inflammatory pathways and affects atherosclerosis and CVD endpoints. It has been observed that selective cyclooxygenase-2 (COX-2) inhibition by rofecoxib is associated with increased risk of cardiovascular events. We hypothesized that this could be due to a disrupted local TXA_2-PGI_2 balance, which could be prevented by concomitant treatment with TP-receptor antagonist S18886 that might ameliorate possible negative effects. This is investigated in **chapter 8** in APOE*3Leiden mice with ischemia reperfusion injury of the myocardium.

The results obtained in these studies and their clinical relevance are discussed in the **General Discussion.**

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