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

Pouwer, M.G.


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General discussion and future perspectives

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Cardiovascular disease (CVD) is currently globally the major cause of mortality and morbidity, and 85% of all CVD deaths are caused by the formation of atheromatous plaques in the vessels, leading to ischemic heart disease, ischemic stroke and peripheral arterial disease (1). The build-up of an atherosclerotic plaque is a slow process that starts with accumulation of low-density lipoproteins (LDL) into the intima and subsequent the recruitment of inflammatory cells (2). Chronic exposure to cardiovascular risk factors, such as hypertension, smoking, dyslipidemia and diabetes (3), can increase the rate and severity of atherosclerosis. Primary prevention of CVD is achieved through early identification and modification of 'lifestyle risk factors', eventually in combination with interventions to reduce plasma lipids or blood pressure (4). These strategies slow disease progression but do not heal, shifting CVD into a chronic disease. This thesis described a variety of studies that aimed to reduce CVD risk by (I) evaluation of novel lipid-lowering interventions to prevent or regress atherosclerosis development, (II) identification of CV side-effects of registered drugs and an environmental pollutant, (III) development of a novel animal model combining dyslipidemia and diabetes, and (IV) evaluation of the cytokine oncostatin M (OSM) as potential target for CVD.

Mouse models have been extensively used for the study of CVD and permit experimental conditions to be controlled. Moreover, preclinical models enable the investigation of molecular and pathophysiological mechanisms and provide platforms for the development and evaluation of novel pharmaceuticals. Disadvantages are differences in lipoprotein metabolism between commonly used mouse models and man (5), which hamper the translation of preclinical findings to valuable clinical applications. All studies described in this thesis used the APOE*3-Leiden(.CETP) mouse as model for diet-induced hyperlipidemia and experimental atherosclerosis. These mice were initially developed as an animal model for Familial Dysbetalipoproteinemia (FD) or type III hyperlipoproteinemia, and were generated by the introduction of a DNA-construct containing the human *APOE*3LEIDEN* and *APOC1* genes (6,7). Subsequent insertion of the *CETP* gene (8), encoding for cholesteryl ester transfer protein (CETP) that transfers cholesteryl esters from high-density lipoprotein (HDL) to apolipoprotein-B (apoB)-containing lipoproteins in exchange for triglyceride(TG), generated an animal model with a lipoprotein metabolism representative for the human situation with a delayed clearance of apoB-containing particles and CETP expression (8). These mice have been widely used for the evaluation of lipid-lowering interventions and consistently demonstrated their translatable value (9–11).

Proprotein convertase subtilisin kexin 9 (PCSK9) was discovered in 2003 (12) as the major down-regulator of the LDL-receptor and to date, PCSK9 inhibition is among the most powerful strategies to target LDL-C. Two monoclonal antibodies against PCSK9, evolocumab and alirocumab, are currently available in the clinic, and several innovative strategies to modulate PCSK9 levels are under development (12). One approach is

activation of the immune system to eliminate endogenous circulating PCSK9 using PCSK9-peptide-based vaccines. In **Chapter 2** we evaluated such a vaccine and found that immunization induces a strong and long-lasting immune response resulting in reduced plasma levels of PCSK9, total cholesterol (TC) and non-high-density lipoprotein-cholesterol (non-HDL-C), as well as markers of systemic inflammation. Furthermore, atherosclerotic lesion progression and vascular inflammation was reduced. Preliminary data in healthy subjects showed that immunization was safe and well-tolerated. More than 90% of the subjects developed a PCSK9 specific antibody response with a mean LDL-C reduction of 13.3% at week 70 (13). This novel vaccine may have a future role in lowering LDL-C beginning in early adulthood to reduce lifetime risk of CV events, since Mendelian randomization studies have suggested that prolonged exposure to lower LDL-C beginning early in life is associated with a substantially greater reduction in the risk of CVD than the current practice of lipid-lowering beginning later in life (14). The advantage of vaccination over chronic treatment with antibodies to achieve long-term LDL-C reductions is the less frequent application which may enhance tolerability, drug adherence and cost-effectiveness (15). Another advantage is the potential to combine the anti-PCSK9 epitope with epitopes of different potential targets for LDL-C lowering, for instance ANGPTL3, apoC3 or lipoprotein(a). Preventive immunization against viruses/bacteria have been successfully used for decades and is widely accepted, and numerous therapeutic cancer vaccine strategies have been developed or are currently under development (16). Also, two vaccines for hypertension and hyperglycemia are under development (17). These advances demonstrate the possibilities of immunization, which might become an important approach in future preventive medicine. Importantly, regarding the more permanent approach of active immunization, it is crucial to exclude side-effects to ensure a safe application of the vaccine in the future.

Most preclinical studies evaluated novel lipid-lowering interventions in a progression setting, including our study with the PCSK9 vaccine. However, most patients start their treatment when atherosclerosis has already developed and therefore, strategies focusing on regression of pre-existing lesions are warranted. In **Chapter 3** we evaluated whether gradual and aggressive reduction of cholesterol in both LDL and remnant lipoproteins by antibodies against PCSK9 (alirocumab) and/or angiopoietin like 3 protein (ANGPTL3) (evinacumab) on top of atorvastatin could regress experimental atherosclerosis. In this study, alirocumab and evinacumab similarly reduced non-HDL-C levels and fully blocked atherosclerosis progression when administered on top of atorvastatin. In addition, plaque stability was improved, as evidenced by a decrease in macrophages and an increase in collagen content. When administered in triple combination (alirocumab + evinacumab + atorvastatin) non-HDL-C levels were reduced to 1 mmol/L and atherosclerotic lesions regressed beyond the baseline level. This is the first intervention study in a well-established, translational mouse model for hyperlipidaemia and atherosclerosis showing that high-intensive cholesterol-lowering triple treatment with atorvastatin, alirocumab and

evinacumab regresses lesion size, diminishes macrophage accumulation through reduction of proliferation and improves plaque stability.

Recently, Mendelian randomization studies have demonstrated that the CV risk reduction of TG-lowering *LPL* variants (e.g. ANGPTL3) is similar to the CV risk reduction of LDL-C lowering *LDLR* variants (e.g. PCSK9) per unit apoB change (18). These findings correspond with our observation that alirocumab and evinacumab equally block lesion progression. Clinical trials, including the IMPROVE-IT (ezetimibe) (19), ODYSSEY OUTCOMES (alirocumab) (20,21), and FOURIER (evolocumab) (22) trials, demonstrate that the combination of statin therapy with other non-statin agents has a significantly improved clinical benefit over statin treatment alone. Also, these studies demonstrated that long-term (3 years), high-intensive cholesterol lowering with anti-PCSK9 antibodies on top of atorvastatin did not adversely affect new-onset of diabetes, diabetes worsening, hepatic disorders and neurocognitive disorders (23). The present data in APOE*3-Leiden.CETP mice provide evidence that combined lowering of LDL and remnant lipoproteins on top of a statin further reduce CV risk. The efficacy and safety of this combination strategy should be confirmed in clinical trials. Alirocumab is approved by the FDA and EMA for heterozygous Familial Hypercholesterolemia (FH) patients or those with clinical atherosclerotic CVD who require additional lowering of LDL-C as an adjunct to diet and maximally tolerated statin therapy (24). Evinacumab is currently being evaluated in phase II trials for patients with severe hypertriglyceridemia (NCT03452228) and persistent hypercholesterolemia (NCT03175367) and in phase III trials for patients with homozygous FH (NCT03399786 and NCT03409744).

Unexpected cardiovascular toxicities in patients receiving approved anti-cancer treatments are common and have been observed during active treatment as well as in cancer survivors (25). In **Chapter 4**, we explored the etiology of the toxic cardiovascular side-effects of BCR-ABL1 tyrosine kinase inhibitors (TKIs), used for the treatment of chronic myeloid leukemia (CML) patients. While the first line TKI imatinib has proven to be effective and safe, the second and third line nilotinib and ponatinib, respectively, increase the prevalence of myocardial infarction, peripheral arterial occlusive disease and ischemic cerebrovascular events pointing to pro-atherosclerotic, pro-thrombotic or combined effects (26–28). Using APOE*3-Leiden.CETP mice, we found that nilotinib and ponatinib enhance mRNA expression of coagulation factors of both the contact activation (intrinsic) and tissue factor (extrinsic) pathways and increase plasma levels of FVII (ponatinib) and activity of FVIIa (nilotinib), important players in the pathogenesis of atherothrombotic events. Also, we observed a reduction in plasma lipids and atherosclerosis development with imatinib and ponatinib. In **Chapter 5** we investigated the mechanism behind the observed lipid alterations and found that imatinib decreased plasma TC and TG levels by reduction of the very-low-density-lipoprotein (VLDL)-apoB-particle production and cholesterol ester content of the VLDL particles, while ponatinib reduced plasma TC levels by lowering intestinal lipid absorption. Our findings correspond with the lipid-modulating

effects (29–32) and improved cardiovascular outcome (33) of imatinib. In addition, our data provide evidence that nilotinib and ponatinib do not enhance atherosclerosis, but increase coagulability. Patients that suffered from cardiovascular side-effects upon nilotinib and ponatinib treatment commonly presented cardiovascular risk factors (27). Therefore, we propose that the pro-thrombotic effects of nilotinib and ponatinib as found in our study may aggravate a pre-existing atherothrombotic condition. In addition to our findings on coagulation, several reports using *in vivo* or *ex vivo* approaches found pro-thrombotic properties of nilotinib (34,35) and ponatinib (36) via other mechanisms (e.g. platelet aggregation, increased expression of von Willebrandt factor, thrombus growth). Moreover, hematological malignancies increase plasma tissue factor levels (37,38), which further potentiates the pro-thrombotic state. These observations underline the importance to select and monitor CML-patients that have the potential to develop atherothrombotic cardiovascular disease before application of the drugs, to improve therapy decision and patient care.

In addition to unexpected post-market safety events of registered drugs, environmental pollutants like perfluorooctanoic acid (PFOA) may increase CV risk. Before being phased-out, PFOA was widely used as an emulsifier in the manufacture of fluoropolymers, and as it is extremely stable, it persists in the environment (39). Epidemiological studies have reported positive associations between serum PFOA and total and non-HDL-C (40–46). However, the modest association observed in studies of general populations is inconsistent with the weaker associations reported in more highly exposed workers (47–54). In addition, there is no increased risk for coronary artery disease in these populations when compared to internal reference cohorts (55–57). Therefore, in **Chapter 6** we evaluated the effects of three different doses PFOA, representing environmental, occupational and toxicological exposure, on plasma lipid levels and lipoprotein metabolism using APOE*3-Leiden.CETP mice. We found that PFOA did not alter plasma lipid levels or lipoprotein metabolism at environmentally or occupationally relevant exposure levels. However, when mice were exposed to a toxicological PFOA dose, plasma TC, non-HDL-C and TG levels were decreased and HDL-C levels were increased. In the latter mice, PFOA decreased VLDL production and increased VLDL clearance by the liver, leading to a reduction of plasma non-HDL-C levels. Moreover, HDL-C levels increased through reduction of CETP activity and changes in gene expression of proteins involved in HDL metabolism. The majority of these changes were mediated by activation of peroxisome proliferator-activated receptor (PPAR)α. Our data correspond with the reduced plasma TC levels observed in a phase I trial in patients that received high doses of PFOA as an antitumor agent (58). In contrast, our findings do not support the increase in cholesterol as found in some observational epidemiological studies, thereby indicating that the reported associations between plasma cholesterol and PFOA exposure are associative rather than causal.

The number of patients with type 2 diabetes is rising and among these patients, cardiovascular complications are the leading cause of morbidity and mortality. Cardiovascular

safety and efficacy of anti-diabetic drugs received increased attention since the FDA and EMA mandated all new diabetes drugs to demonstrate cardiovascular safety (59,60). Preclinical models are used for the development and evaluation of novel drugs, and translational models combining diabetes and cardiovascular disease are required. In **Chapter 7** we described the characteristics of the APOE*3-Leiden.Glucokinase$^{+/-}$ (E3L.GK$^{+/-}$) mouse model, which was generated by cross-breeding the hyperlipidemic APOE*3-Leiden mouse with the hyperglycemic glucokinase knockout (GK$^{+/-}$) mouse. E3L.GK$^{+/-}$ mice had plasma lipid levels comparable to E3L mice and plasma glucose levels comparable to GK$^{+/-}$ mice, leading to enhanced atherosclerosis progression in E3L.GK$^{+/-}$ mice relative to E3L mice, which was predicted by glucose exposure. Since the E3L mouse model responds similarly as humans do to lipid-lowering agents (61–70) and GK$^{+/-}$ mice to anti-diabetic drugs at doses corresponding to therapeutic drug levels in man (71,72), we propose that the E3L.GK$^{+/-}$ mouse is a promising novel diet-inducible disease model for investigation of the etiology and evaluation of drug treatment on diabetic atherosclerosis. Examples of these applications are the evaluation of novel anti-diabetic and anti-atherosclerotic agents and combinations, investigation of the pathophysiological mechanisms behind the cardiovascular adverse (73–75) and beneficial (76,77) effects of some anti-diabetic agents, and the etiology of statin-induced risk for diabetes (78).

The role of cytokines in the initiation and progression of atherosclerosis is increasingly recognized and consequently, novel therapies targeting cytokines (79), including IL-1β with the anti-IL-1β antibody canakinumab (80), are being developed. In **Chapter 8**, we evaluated the role of the cytokine OSM in the initiation of atherosclerosis and found that OSM induced endothelial activation *in vitro* using human endothelial cells from different vascular beds, and *in vivo* using APOE*3-Leiden.CETP mice. Since endothelial activation is an initial step in atherosclerosis development, we proposed that OSM may play a role in the initiation of atherosclerotic lesion formation. However, remarkably, long-term exposure of APOE*3-Leiden.CETP mice to OSM reduced atherosclerotic lesion size and severity, despite enhanced plasma E-selectin levels and monocyte adhesion to the activated endothelium of the aortic root (**Chapter 9**). These findings correspond to our observation that higher serum OSM levels in humans are associated with post-incident coronary heart disease and overall survival probability in the AGES Reykjavik Study, suggesting a protective cardiovascular effect. Interestingly, knockout of the OSMβ receptor in APOE$^{-/-}$ mice also attenuated atherosclerotic lesion size (81). Similar contradictions have been reported regarding the pro- and anti-inflammatory effects of OSM. OSM is associated with inflammatory diseases including lung inflammation, rheumatoid arthritis and multiple sclerosis. Moreover, intradermal injection of, and intranasal exposure to OSM induces accumulation of inflammatory cells. On the other hand, OSM suppresses inflammation in mouse models of inflammatory bowel disease, arthritis, autoimmune encephalomyelitis and multiple sclerosis (82), and it has been suggested that administration of OSM has favorable effects on the metabolic syndrome (82,83). Given the confusing effects of OSM

and its involvement in many biological processes, including tumorigenesis, hematopoiesis, bone and fat turnover, central nervous system development, liver regeneration and inflammatory responses in several tissues (84), further research is required to ensure safe application of potential OSM-related therapies.

Today, we understand better how to treat CVD, but despite these advances, many patients remain at increased cardiovascular risk. In this thesis, we discussed several strategies that may contribute to further risk reduction in the future. The novel lipid-lowering strategies (e.g. vaccination, combination therapy) that have been evaluated in our studies provide evidence that further LDL-C/non-HDL-C lowering and subsequent cardiovascular risk reduction is achievable, which has to be confirmed in clinical trials. Furthermore, we unraveled (part of) the etiology of the cardiovascular safety issues of the TKIs nilotinib and ponatinib and the mechanistic insights provided by our data may contribute to safer application of the drugs to CML-patients. Serum PFOA in environmental and occupational exposed adults had been found to be associated with increased plasma cholesterol, but our data demonstrate that this association is associative rather than causal . Looking forward, we described a novel mouse model, the E3L.GK$^{+/-}$ mouse, that can be used for the study of diabetic macrovascular complications and the evaluation of anti-diabetic drugs. Shifting towards the role of inflammation in atherosclerosis, we evaluated the potential of the cytokine OSM as new target for CVD. In contrast to our hypothesis and evidence provided by the literature, administration of OSM decreased atherosclerotic lesion size, and this confusing observation has to be elucidated before further development of OSM-related treatment strategies.

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