Cardiovascular drug target discovery

In vitro and *in vivo* validation studies

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In vitro en *in vivo* validatie studies

(met een samenvatting in het Nederlands)

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CHAPTER 1

General introduction

HISTORY OF CARDIOVASCULAR DISEASE EPIDEMIOLOGY

The marked increase in life expectancy in industrialized countries in the beginning of the 20th century, which was predominantly the result of improved prevention and treatment of infectious diseases, came along with an increase in cardiovascular disease (CVD) mortality[1,2]. In the US, CVD death rate increased from less than 10% in 1900 to more than 25% in 1940 and to almost 40% in 1960 (reviewed in [3]), making it the leading cause of death[4]. Human cohort studies, such as the Framingham study, contributed to identification of non-modifiable risk factors including age, gender, ethnicity and family history and modifiable risk factors including body weight, diabetes, high blood pressure, high cholesterol levels, physical inactivity and smoking[5,6]. It also led to intervention studies and the development of treatments such as coronary bypass surgery and statin medication[1]. The increased understanding and treatment of the disease subsequently declined CVD mortality in developed countries[1]. Despite this success, CVD is still the primary cause of death in high-income countries[7]. Although it was previously considered as a Western Disease[8], the incidence of CVD is catching up in middle- and low-income countries[9], making it a global problem. In 2016, an estimated 17.9 million deaths (31%) were caused by CVD, making it the leading cause of death worldwide[7]. Besides its social impact, CVD is a huge economic burden as only in the United States, 396 billion dollars were spend on direct medical costs of CVD in 2012 and these costs are expected to rise to 918 billion dollars in 2030[10]. More than half of the CVD related deaths (61.6%) is caused by coronary heart disease or stroke[10], which both have atherosclerosis as underlying disease pathology[11–13]. Current therapies on primary prevention of atherosclerosis driven CVD like aspirin (relative risk (RR) = 0.90) and statins (RR = 0.75) lower the risk of atherosclerotic cardiovascular disease, whereas blood pressure lowering therapies reduce the risk of coronary heart disease and stroke (RR = 0.84 and 0.64 respectively)[14]. However, the risk reduction obtained by these therapies is limited and therefore, it is of great importance to develop novel therapies against atherosclerosis development[14].

ATHEROSCLEROSIS DEVELOPMENT

Atherosclerosis is a complex, lipid and inflammation driven disease[15,16], which is initiated with the formation of a fatty streak on the inside of a blood vessel[17,18]. These fatty streaks develop already at a young age and may even be present in fetal aortas and infants below 6 months of age[17,18]. These fatty streaks may evolve to atherosclerotic lesions[17,18] of which the amount[19] and severity[20] increases with age and further depends on the presence of other non-modifiable and modifiable risk factors[21–23]. The atherosclerotic lesions may become life threatening since thrombi may be formed on top of the plaque or due to plaque rupture[18]. These thrombi may occlude smaller blood vessels, which may, depending on the vessel that is affected, cause a myocardial infarction or stroke[18]. Roughly, there are three important, greatly intertwined processes that drive the initiation and progression of atherosclerosis; deviations in lipids and lipoprotein metabolism, inflammation and

endothelial dysfunction[24]. Of these, I focussed in this thesis mostly on factors that are involved in endothelial cell function.

The endothelium is mainly known for its barrier function between the blood and the underlying tissue. However, besides its selective permeability to fluids, solutes and cells, it also has an essential role in maintaining blood fluidity and regulating the vascular tone[25,26]. Atherosclerosis often occurs at predilection sites, where the endothelium is more likely to be dysfunctional[27] or activated[28]. Both, endothelial dysfunction and endothelial activation, impair the endothelial barrier integrity, upregulate the expression of adhesion molecules on the endothelial surface and enhance leukocyte adhesion to the endothelium[29,30]. Endothelial dysfunction distinguishes itself from endothelial cell activation by the reduced nitric oxide synthesis and release, thereby diminishing vasodilation, which is the clinical manifestation of endothelial dysfunction[22]. Endothelial cell activation, on the other hand, is often induced by cytokines[29,30]. These two processes not only share a lot of common features, but endothelial cell activation can also lead to endothelial dysfunction and *vice versa*[29]. In general, studies focussing on either endothelial cell activation or endothelial dysfunction do not exclude the simultaneous presence of the other process, which makes it difficult to assess if one of the processes has a more prominent role in atherosclerosis development. However, it is indisputable that the endothelial state has an significant role in the disease pathology.

The role of the endothelium is already highlighted in the initial stages of atherosclerosis development as human and mice studies showed that atherosclerotic lesions are mainly found in regions with low or oscillatory shear stress that is often present in arterial bifurcations, branch points and areas of vessel curvature, while atherosclerosis is often absent in areas of high shear stress[31–36]. The altered shear stress induces an increase in cytokine and adhesion molecule expression and a decrease in nitric oxide production in endothelial cells. Also, leukocyte adhesion to the endothelial barrier and endothelial permeability is increased (reviewed in [35]), which are all signs of endothelial cell activation and/ or dysfunction[29,30,37]. Due to the increased endothelial permeability, low-density lipoproteins (LDLs), which are lipid carriers delivering cholesterol and other lipids to cells[38], are able to cross the endothelial barrier and accumulate in the subendothelial space[13,39], thereby forming a fatty streak. In the arterial wall, the LDL becomes oxidized and proteolytically modified and aggravates the inflammatory response[40] that was initiated by the activated endothelial cells[27]. The oxidized LDL is taken up by tissue resident macrophages or by macrophages that are recruited by the endothelial cells through secretion of monocyte chemoattractant protein-1 (MCP-1)[39]. Selectins that are expressed on the surface of dysfunctional endothelial cells, slow down the recruited monocytes as they bind to proteins expressed on the monocytes. When the pace of the monocyte is decelerated, adhesion molecules on the endothelial cells such as intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1) are able to bind to the integrins on the monocytes. These are strong interactions that allow a firm adhesion between the two cell types and enable the monocyte to slowly crawl over the endothelial surface to find an appropriate place for transmigration through the endothelial barrier (reviewed in [41]). The involvement of adhesion molecules in atherosclerosis development was emphasized in mouse studies which showed that mice lacking P-selectin, E-selectin or ICAM-1 expression

develop smaller atherosclerotic lesions than their littermates that do express these adhesion molecules[42,43]. After transmigration, the monocyte differentiates into a macrophage[44,45], which is able to take up huge amounts of lipids[46]. The lipid accumulation and the induced inflammatory response is still reversible in the initial stages of the disease[40] as the accumulated lipids can be removed from the artery wall by high-density lipoproteins (HDLs), which are cholesterol carriers that transport cholesterol away from cells[38,39]. In addition, HDL has the ability to remove reactive oxygen species, thereby attenuating the inflammatory response caused by oxidized LDL particles[39,47].
However, if the process of atherosclerosis progresses and the disease aggravates over time, the reversible fatty streak evolves into an early plaque[19,20,40]. Increased uptake of oxidized LDL, increased cholesterol esterification and decreased cholesterol efflux trigger macrophages to differentiate into foam cells[46,48], thereby prolonging the inflammatory response by the release of additional cytokines and chemokines[46]. The inflammatory processes induce apoptosis of cells that are present within the atherosclerotic lesion, including vascular smooth muscle cells, endothelial cells and macrophages[49]. In early stages of the disease, these apoptotic cells can still be removed by macrophages, but in later stages this process becomes defective and the apoptotic cells give rise to the formation of a necrotic core[50,51]. The ongoing inflammatory state, the oxidized LDL and the hypoxia that is present in the necrotic core stimulate angiogenesis of vasa vasorum by the release of angiogenic growth factors, including vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α)[52–54]. At least part of the vasa vasorum are endarteries, which do not redirect the blood flow[54–56]. Furthermore, the newly formed vessels have a weak barrier integrity and hence, erythrocytes, leukocytes, platelets and plasma lipids can easily enter the plaque, thereby contributing to further plaque growth and instability[53].

Plaque vulnerability

Atherosclerotic plaques are roughly divided into two types; the stable and the vulnerable plaque. While the stable plaque is characterized by a small necrotic core, little macrophages and a thick fibrous cap, the vulnerable plaque has a large lipid core, many macrophages and a thin fibrous cap[57,58] and is therefore more prone to rupture[59]. When the plaque ruptures, the lipid content and matrix proteins (e.g. collagen) come into contact with the blood, which results in the formation of a thrombus[60]. Depending on the thrombus size and lumen diameter, the thrombus may occlude the artery[60]. Although most thrombi (60 – 70%) are linked to plaque rupture[61], they can also originate on top of a stable plaque[62]. Obstruction of a blood vessel by a thrombus or by an intact atherosclerotic plaque that is large enough to occlude a vessel blocks the blood supply to the underlying and effluent tissue, resulting in ischemic disease[60]. Appearance of clinical manifestations depend on the location of the obstruction. If a coronary artery is occluded, the patient will suffer ischemic heart disease, while occlusion of a cerebral artery results in ischemic stroke[63,64].

Current therapies

Current primary and secondary prevention therapy of cardiovascular disease primarily focusses on life style changes and medical lipid-lowering, anti-hypertension and anti-

coagulant treatment[65,66]. European and American guidelines recommend a lifestyle with regular exercise, a healthy diet (high in fibres, fruits and vegetables and low in sugar and salt), a healthy weight and without smoking and no or moderate alcohol consumption[65]. Those lifestyle guidelines apply to every individual, while medical lipid-lowering treatment is only recommended in individuals with elevated cholesterol levels[65], medical anti-hypertensive treatment only in individuals with an elevated blood pressure[65] and medical anti-coagulant treatment only in secondary prevention[66]. As adherence to a healthy lifestyle is difficult[67] and prevalence and mortality of cardiovascular disease remains high despite therapeutic advancements, there is need for novel cardiovascular therapies[68].

Since current therapies are aimed at lowering lipid levels and blood pressure and preventing thrombus formation, it might be useful to investigate therapies targeting inflammation or endothelial dysfunction. Recently, the results of the CANTOS trial were published, which showed that Canakinumab, a monoclonal antibody targeting Il-1β, reduces the occurrence of recurrent cardiovascular events by approximately 20%[69]. These findings suggest that it could be possible to develop a successful CVD therapy that primarily targets inflammation. But also targeting endothelial dysfunction might be a promising approach as many cardiovascular risk factors, including hyperlipidaemia, diabetes, smoking, hypertension and physical inactivity[21,22] all individually induce endothelial dysfunction[70–74]. Excessive circulating lipid levels, for example, directly result in endothelial dysfunction[75], as well as advanced glycation end products, which are elevated in type 2 diabetes patients[76], smokers[77] and in physically inactive people[78]. Additionally, smoking induces endothelial dysfunction by reducing the nitric oxide bioavailability[77] and hypertension enhances reactive oxygen species production, which also causes endothelial dysfunction[79]. Furthermore, endothelial dysfunction is present in patients with chronic inflammatory diseases and these patients also have accelerated atherosclerosis development[80]. The effects that cardiovascular risk factors have on endothelial dysfunction, combined with the proven involvement of endothelial dysfunction in atherosclerosis development[81–83] and the lack of therapies directly targeting endothelial dysfunction, make it worthwhile to investigate opportunities targeting this process for future drug development.

DRUG DEVELOPMENT

The development of a novel drug starts with a pre-clinical phase that focusses on understanding the disease pathology, identifying a potential target, selecting a compound that effectively modifies the target activity and pre-clinical validation of the target[84]. A potential target should be related to the investigated disease, safe to target and druggable[85]. Nowadays, a lot of information on diseases and potential targets comes from literature, patent information, genetic associations and gene expression and proteomics data, which has increased the identification rate of potential targets[85]. After identification of a potential target, the target needs to be validated[85]. This process often involves cell culture to validate if the potential target is indeed related to processes involved in the disease of interest and

to screen compound libraries to find a lead compound[84,86,87]. Furthermore, cell culture experiments can be performed to obtain information about the mechanism of action, activity and possible toxic effects of the compound[88]. Advances in cell culture from monolayers to three-dimensional and co-culture systems mimic the *in vivo* situation much better[86,88], but the use of preclinical animal models in drug discovery remains inevitable as they provide important information on safety and on potential reproduction, cardiac and hepatic toxicology risks[84]. Moreover, animal models are required to gain full understanding of a disease as often, multiple organs and/ or processes are involved in a disease process or therapy[89]. For example, mutations in the LDL receptor, which is mainly present in the liver[90], contribute to atherosclerosis development due to increased LDL cholesterol levels[91]. Likewise, statins reduce cholesterol biosynthesis in the liver[92] and thereby reduce atherosclerosis development in the arteries[93]. After the preclinical phase, the compound is tested in a clinical phase I study in which the safety and tolerability are tested and information is obtained on dose-plasma concentrations. This is often performed in healthy subjects. Next, the efficacy and clinical safety of the compound is tested in a diseased population in a phase II study and finally, efficacy is confirmed in a phase III study[84].

Drug development is a costly process as preclinical research and clinical testing take a lot of time and cost a lot of money[94]. Over the years, the developmental costs of one single drug have risen from 802 million dollars in the early 1980s until the early 1990s to 2.6 billion dollars in the 2000s until the early 2010s[95]. This increase is partially caused by the drop in success rate, which dropped from 21.5% to 11.8% in the latter study[95] as the development costs do not only include money spend on the specific drug, but also contain the costs spend on researched drugs that did not make it to the market[95]. The amount of abandoned drugs is high, as of all 24 targets that enter preclinical research, only 8 enrol in clinical trials and only 1 will result in a launched drug[96]. To identify the main reasons of failure, Astrazeneca reviewed its own projects that have taken place between 2005 and 2010 and concluded that most projects failed due to safety issues, followed by inefficacy of the tested drugs[97]. From their analysis, they identified the quality of target validation, the level of drug exposure in the target tissue, the safety margins, the patient population and the commercial potential as the 5 key factors that contribute to a successful study outcome[97]. These insights have changed Astrazeneca's strategy from a high-volume-based strategy towards a depth of understanding and project quality strategy, designated the 5R strategy, in which the researchers try to get a better understanding of each investigated component and try to fill the knowledge gaps of the investigated drugs[97]. This strategy has increased the success rate from candidate drug nomination to completion of phase III clinical trials from 4% in 2005-2010 to 19% in 2012-2016[98].

CARTARDIS

The work described in this thesis was performed as part of the CarTarDis project that was executed by a pan-European multi-partner consortium. CarTarDis adopted a non-academic approach and formed a 'virtual' pharmaceutical company that mirrored the R&D processes

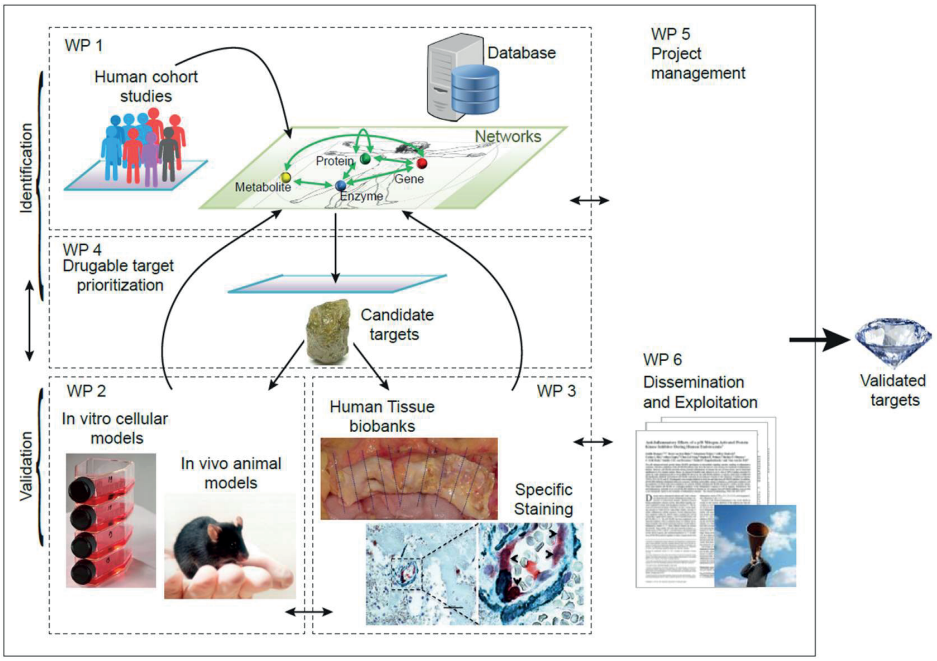

Figure 1. CarTarDis project workflow.

the industry would use (Figure 1). The aim was not only to identify and validate novel CVD drug targets and develop new tools, but also to increase the knowledge and competitive position of the participating partners in drug discovery. The following partners participated: TNO (the Netherlands, coordinator), Helmholtz Zentrum München (Germany), Karolinska Institutet (Sweden), AstraZeneca (Sweden), Icelandic Heart Association (Iceland), Leiden University Medical Center (the Netherlands), Quorics (the Netherlands), Polygene AG (Switzerland), Bioneer A/S (Denmark), Imabiotech SAS (France), Morphisto GMBH (Germany), Umeå University (Sweden) and Molecular Profiling Consulting (UK).

The research partners first mined available and emerging molecular data from three large independent cardiovascular cohorts and literature for statistical association of targets with CVD phenotypes with a preference for genetic associations. A database and target-selection workflow was designed to select and prioritise candidate CVD drug targets using selection filters that were inspired by the 5R strategy of AstraZeneca[97], scoring on druggability, novelty, genetic confirmation, feasibility and pathways. Targets with a high score were selected as candidate drug targets and were investigated in two parallel workflows: one focussing on mechanism of action in model systems (cellular, rodent models) and one focussing on clinical association using high quality biobanks and innovative molecular staining methodologies. Finally, all information was brought together per drug target to conclude on its suitability as attractive new drug target.

Druggability

The druggability score gives an indication of the likeliness of a drug to successfully modulate the identified target. Drug targets scoring high on druggability are targets repurposing existing drugs, as the efficacy and safety of these drugs have already been proven and easily accessible targets like receptors, which make up the largest group of drug targets[99]. Targets that are more difficult to reach on the other hand, like nuclear targets, score lower on druggability.

Novelty

The novelty score is based on the amount of Pubmed hits, patents and availability of existing compounds directed against the target. If multiple patents exist around an identified drug target, the target will receive a lower score, as it might restrict the freedom to operate and it implies that others are already trying to develop a drug against this target, making it less attractive for drug development. Also, an high amount of Pubmed hits results in a lower score. Although many publications on an identified target adds to the understanding of the target, it also indicates that a lot of research had been performed on this target and the lack of the existence of a drug for the specific target implies that it is difficult to target the specific protein.

Genetics

Genetics is a powerful tool in target identification as associations between common variables in the human genome and disease phenotypes help to identify genes that have a causal or protective relationship to disease probability and progression[100]. Moreover, the presence of a genetic link between a target and the disease of interest increases the success rate from 43% to 73% in phase II clinical trials[100]. We therefore assigned higher scores to targets that have a strong genetic link to CVD.

Feasibility

The feasibility score is related to knowledge, biobanks, techniques and *in vitro* and *in vivo* models that we were able to use to validate the role of candidate targets in CVD within the CarTarDis consortium. For our studies, we had access to human biologic material from BiKE (i.e. a biobank containing late stage/ advanced plaques and normal arteries as controls with microarray measured gene expression), SOCRATES (i.e. a biobank containing aortic wall patches obtained during kidney transplantation) and the AGES-Reykjavik study (i.e. a 50-year-long population-based study of deeply phenotyped elderly containing serum proteomics data). Our in-house techniques to visualize potential targets in atherosclerotic tissue included immunohistochemistry, *in situ* hybridization and *in situ* lipid mass spectrometry imaging. For our cell experiments, we had access to human endothelial cells that served as an *in vitro* model to study the effect of candidate targets on endothelial activation, which was assessed by examining the expression of cytokines and adhesion molecules and some basic functional testing including leukocyte adhesion. We also had access to optimized protocols to isolate human neutrophils and to THP1 monocytes to investigate the effect of candidate targets on the inflammatory response. To study the role of candidate targets *in vivo,* we had

access to the ApoE*3Leiden.CETP mouse model, which is a cross-breeding between the ApoE*3-Leiden mouse and the cholesteryl ester transfer protein (CETP) mouse[101]. The ApoE*3-Leiden mouse expresses the human APOE*3Leiden gene, the APOC1 gene and a promotor element regulating apolipoprotein E (apoE) and APOC1 expression[102]. ApoE mediates binding of plasma lipoproteins to specific cell-surface receptors, thereby clearing these lipoproteins from the plasma[103]. ApoE3*Leiden is a variant of apoE in which the binding of apoE to the LDL receptor is defective, which results in high plasma cholesterol and triglyceride levels due to impaired clearance of chylomicrons and very low density lipoprotein remnants[104]. The APOC1 gene and the promotor element were used as they are required to mediate apoE expression in the liver, which is the major source of apoE in humans[105,106] and contributes to the specific phenotype of the mice. Contrary to wild-type mice, ApoE*3-Leiden mice show elevated cholesterol and triglyceride levels on normal chow diet and on a Western-type diet[102]. Upon crossbreeding with the human CETP expressing mouse, the cholesterol distribution in mice shifts from HDL towards VLDL and LDL, which better represents the human situation[102].

Pathways

Pathway analysis was performed to obtain information on the pathway that our candidate targets are involved in to gain more insight into the mechanism of action behind each candidate target and to identify potential up- or downstream targets. This knowledge could shift our focus from a promising, but hardly druggable target towards a target in the same pathway that is better druggable. Moreover, pathway analysis can give an indication of the impact a certain compound has, as proteins that have many connections to other proteins are likely to have a higher impact than proteins with fewer interactions[107]. Of course, the predicted impact should be in line with the desired impact that is needed to treat the disease of interest[107]. Furthermore, pathway analysis could give insight into possible side-effects of targeting a potential target[108]. If pathway analysis predicts a high probability on clinically relevant side-effects, one should carefully consider if it is worth it to spend more time and money on this particular target[107].

After scoring each of the candidate targets on these five factors, we made a shortlist of candidate targets that were worth to investigate further. We tried to fill the existing knowledge gaps by searching and reading literature on the candidate targets, performing *in vitro* and *in vivo* experiments and histologically assessing the presence of the candidate targets and/ or their receptors or downstream targets in early and late stage/ advanced plaques. With this approach we hope to deliver promising novel druggable targets to treat CVD that can be picked up for drug development.

THESIS OUTLINE

This thesis provides an overview of three candidate targets that were investigated within the CarTarDis consortium; Oncostatin M, PLPP3 and CECR.

PART ONE - ONCOSTATIN M

Oncostatin M (OSM) was added to our shortlist of candidates based on the existing literature and on our own preliminary experiments with human endothelial cells. We hypothesized that OSM enhances atherosclerosis development by inducing endothelial activation, since endothelial cells express relatively high levels of OSM receptors. In **chapter 2**, we start testing this hypothesis by investigating the effect of OSM on endothelial cell activation in human endothelial cells, which were derived from multiple vascular beds, by quantifying cytokine and adhesion molecule mRNA and protein levels. In addition, we examined the effect of OSM on endothelial cell activation in ApoE3*Leiden.CETP mice, a mouse model for atherosclerosis, by quantifying adhesion molecule expression and monocyte adhesion to the endothelium in the aortic root area. In **chapter 3**, we investigated the possible role of OSM in human atherosclerosis development, by studying the presence of OSM, and it's receptors, OSMR and LIFR, in human atherosclerotic plaque material. Additionally, we quantified serum OSM levels in coronary heart disease patients to investigate if OSM levels correlate with survival probability. Also, we performed a 16-week atherosclerosis study in ApoE3*Leiden.CETP mice to investigate the effect of OSM on atherosclerotic lesion size, composition and severity. In **chapter 4**, we looked further into the possible role of OSM signalling in human atherosclerosis development by associating gene expression effecting common variants with atherosclerotic plaque vulnerability.

PART TWO - PHOSPHOLIPID PHOSPHATASE 3

Phospholipid Phosphatase 3 (PLPP3) was added to the shortlist of candidate targets mainly due to its strong genetic evidence to cardiovascular disease. We hypothesized that PLPP3 protects against atherosclerosis by inactivating lysophosphatidic acid (LPA), which is associated with enhanced atherosclerosis. In **chapter 5**, we start testing this hypothesis by evaluating the PLPP3 pathway as a novel therapeutic target in atherosclerosis. We first compared PLPP3 mRNA and protein expression in human atherosclerotic plaques with expression in normal arteries. Next, we investigated the correlation between plaque PLPP3 expression levels with event-free survival and finally, we investigated the effect of PLPP3 and its downstream targets, including LPA and its receptors, on endothelial cell activation.

PART THREE - CAT EYE SYNDROME CRITICAL REGION PROTEIN 1

Cat eye syndrome critical region protein 1 (CECR1) was added to the shortlist of candidate targets mainly due to its high score on novelty and pathways. We hypothesized that CECR1 plays a role in atherosclerosis development by regulating adenosine induced neutrophil activation, through its adenosine deaminase activity. In **chapter 6**, we evaluated the possible role of CECR1 in atherosclerosis. First, we investigated if CECR1 mRNA is expressed in human atherosclerotic lesions. Next, we explored if adenosine and inosine concentrations could possibly be altered by CECR1 in human atherosclerotic lesions. We tried to find out how CECR1 affects atherosclerosis development by stimulating isolated neutrophils from healthy donors with adenosine, CECR1 or the combination of the two and by stimulating THP1 monocytes with the conditioned medium of neutrophils stimulated with adenosine, CECR1 or the combination of the two. Finally, we investigated if circulating CECR1 levels are altered in atherosclerosis, myocardial infarction and coronary heart disease patients and if circulating CECR1 levels are associated with survival probability in humans.

Chapter 7 and **chapter 8** provide a general summary and discussion of the work presented in this thesis.

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PART ONE

ONCOSTATIN M

CHAPTER 2

Inflammatory cytokine oncostatin M induces endothelial activation in macro- and microvascular endothelial cells and in APOE*3Leiden. CETP mice

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ABSTRACT

Aims

Endothelial activation is involved in many chronic inflammatory diseases, such as atherosclerosis, and is often initiated by cytokines. Oncostatin M (OSM) is a relatively unknown cytokine that has been suggested to play a role in both endothelial activation and atherosclerosis. We comprehensively investigated the effect of OSM on endothelial cell activation from different vascular beds and in APOE*3Leiden.CETP mice.

Methods and Results

Human umbilical vein endothelial cells, human aortic endothelial cells and human microvascular endothelial cells cultured in the presence of OSM express elevated *MCP-1*, *IL-6* and *ICAM-1* mRNA levels. Human umbilical vein endothelial cells and human aortic endothelial cells additionally expressed increased *VCAM-1* and *E-selectin* mRNA levels. Moreover, ICAM-1 membrane expression is increased as well as MCP-1, IL-6 and E-selectin protein release. A marked increase was observed in STAT1 and STAT3 phosphorylation indicating that the JAK/STAT pathway is involved in OSM signaling. OSM signals through the LIF receptor alfa (LIFR) and the OSM receptor (OSMR). siRNA knockdown of the LIFR and the OSMR revealed that simultaneous knockdown is necessary to significantly reduce MCP-1 and IL-6 secretion, VCAM-1 and E-selectin shedding and STAT1 and STAT3 phosphorylation after OSM stimulation. Moreover, OSM administration to APOE*3Leiden.CETP mice enhances plasma E-selectin levels and increases ICAM-1 expression and monocyte adhesion in the aortic root area. Furthermore, *Il-6* mRNA expression was elevated in the aorta of OSM treated mice.

Conclusion

OSM induces endothelial activation *in vitro* in endothelial cells from different vascular beds through activation of the JAK/STAT cascade and *in vivo* in APOE*3Leiden.CETP mice. Since endothelial activation is an initial step in atherosclerosis development, OSM may play a role in the initiation of atherosclerotic lesion formation.

INTRODUCTION

The endothelium is involved in many processes including maintenance of the endothelial barrier function, prevention of spontaneous blood clot formation, inflammatory cell recruitment upon injury and regulation of the vascular tone[1–3]. Impairment of one or more of these functions is often referred to as endothelial dysfunction, and may lead to the development of atherosclerosis, angiogenesis in cancer, vascular leakage, infectious diseases or stroke[4].

Although endothelial dysfunction is often described as the inability to dilate vessels, endothelial dysfunction is also characterized by endothelial activation, which is marked by increased cytokine release, adhesion molecule expression and endothelial permeability. The released cytokines attract leukocytes to the site of the activated endothelium, where the leukocytes bind to the endothelial barrier, which is enabled by enhanced adhesion molecule expression. Firmly adhered leukocytes then migrate through the endothelial barrier into the underlying tissue[5].

The process of endothelial activation can occur both, locally on well-known predilection sites and systemically, and is often triggered by traditional cardiovascular risk factors such as hypercholesterolemia, hypertension, smoking or diabetes and is initiated by inflammatory cytokines. One such a cytokine, which was first discovered in the cancer field, is oncostatin M (OSM). This relatively unexplored cytokine is an interleukin-6 family member that can signal through the LIFR and the OSMR, which are both dependent on heterodimerization with the gp130 receptor to form a functional receptor complex[6]. OSM is upregulated in multiple chronic inflammatory diseases including periodontitis, rheumatoid arthritis and inflammatory bowel diseases and is known to induce angiogenesis and smooth muscle cell proliferation and migration, both processes that are involved in atherosclerosis development[7–16]. Other pro-inflammatory cytokines that promote angiogenesis, smooth muscle cell proliferation and endothelial activation, such as TNFα and IL-18, have already been proven to accelerate atherosclerosis[17–24]. Furthermore, OSM is found in human carotid atherosclerotic plaques and in the intima and media of atherosclerotic mice[16].

Based on these findings and on the knowledge that endothelial cells are very high expressers of OSM receptors[25], we hypothesized that OSM may be involved in atherosclerosis development partially by inducing endothelial activation as a first step in the development of atherosclerosis. In this study, we incubated human endothelial cells with OSM to investigate if OSM induces systemic or local endothelial activation. As the cell heterogeneity among endothelial cells is huge[26,27] and endothelial cells from different vascular beds show different responses/ behave different to physiological stimuli[28,29], we tested the effect of OSM in endothelial cells derived from multiple vascular beds, human umbilical vein endothelial cells (HUVECs), human aortic endothelial cells (HAECs) and human microvascular endothelial cells (HMEC-1). Of which HAECs are the most suitable endothelial cell type to study atherosclerosis development as atherosclerosis mainly affects the medium and large-sized arteries[30]. To validate our findings in cultured endothelial cells *in vivo*, we administered OSM to APOE*3Leiden.CETP mice, a translational mouse model for hyperlipidemia and atherosclerosis[31,32]. The mildly pro-inflammatory state that is present in this animal model

of hyperlipidemia makes it a suitable model to investigate the role of OSM in atherosclerosis prone conditions. We found that OSM induces endothelial activation in all different investigated human endothelial cell types and in mice after chronic administration and identified the JAK/STAT pathway as a key player in this process.

MATERIALS AND METHODS

Cell culture

2 different batches of pooled primary human umbilical vein endothelial cells (HUVECs, Lonza, the Netherlands), a single batch of primary human aortic endothelial cells from one single donor (HAECs, ATCC, Manassas, VA, USA) and a human dermal microvascular endothelial cell line (HMEC-1, ATCC, Manassas, VA, USA) were cultured in EBM®-2 medium (Lonza, Walkersville, MD) supplemented with EGM™-2 SingleQuots® (Lonza, Walkersville, MD) under normoxic conditions (21% O_2). Throughout the study, passage 6 was used for HUVECs and HAECs, while passage 27 was used for the HMEC-1 cell line. All experiments were performed in 70% subconfluent HUVECs, HAECs, or HMEC-1 cells. After each experiment, cells and conditioned medium were collected for subsequent RNA or protein analysis. Repetitive experiments were only started if the previous experiment had been finished.

***In vitro* RNA expression**

Human OSM (R&D systems, Minneapolis, MN) was added to HUVECs, HAECs and HMEC-1 cells in a concentration range from 0 – 20 ng/mL. After 3 or 6 hours, RNA was isolated with the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Isolated RNA (500 ng) was reverse transcribed into cDNA with the qSCript™ cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA) and analyzed by real-time fluorescence assessment of SYBR Green signal (iQ™ SYBR® Green Supermix, Bio-Rad, Hercules, CA) in the CFX96™ Real-Time Detection System (Bio-Rad, Hercules, CA). Each sample was measured in duplicates. Primers were designed for the human genes of interest, sequences are listed in Table 1. MRNA levels were analyzed and corrected for the housekeeping gene *ACTB*. Experiments were repeated 4-7 times.

***In vitro* cytokine release**

To determine the effect of OSM on endothelial activation, HUVECs, HAECs or HMEC-1 cells were incubated with 5 ng/mL OSM. 3h and 6h after OSM treatment, conditioned medium was collected. To investigate the effect of OSM on endothelial activation after siRNA knockdown of the LIFR and OSMR, siRNA transfected HUVECs were treated with 5 ng/mL OSM 48h post transfection. 6h after OSM treatment conditioned medium was collected. Conditioned medium was analyzed with the ProcartaPlex Mix&Match Human 6-plex (Thermo Fisher, Waltham, MA) according to the manufacturer's protocol and measured on the Bio-plex® 200 system (Bio-Rad, Hercules, CA) to determine the release of MCP-1, IL-6, soluble E-selectin, soluble P-selectin and soluble VCAM-1. Experiments were repeated 3-7 times.

Table 1. Primer sets for qPCR analysis.

Gene	Species	Direction	Primer sequence (5'-3')
MCP-1	Human	Forward	TGGAATCCTGAACCCACTTCT
		Reverse	CAGCCAGATGCAATCAATGCC
IL-6	Human	Forward	AGTGAGGAACAAGCCAGAGC
		Reverse	GTCAGGGGTGGTTATTGCAT
ICAM-1	Human	Forward	TTGAACCCCACAGTCACCTAT
		Reverse	CCTCTGGCTTCGTCAGAATCA
VCAM-1	Human	Forward	TGGGAAAAACAGAAAAGAGGTG
		Reverse	GTCTCCAATCTGAGCAGCAA
E-SELECTIN	Human	Forward	AAGCCTTGAATCAGACGGAA
		Reverse	TCCCTCTAGTTCCCCAGATG
ACTB	Human	Forward	GATCGGCGGCTCCATCCTG
		Reverse	GACTCGTCATACTCCTGCTTGC
Mcp-1	Murine	Forward	TTAAAAACCTGGATCGGAACCAA
		Reverse	GCATTAGCTTCAGATTTACGGGT
Il-6	Murine	Forward	CTATACCACTTCACAAGTCGGA
		Reverse	GAATTGCCATTGCACAACTCTTT
Icam-1	Murine	Forward	TCCGCTACCATCACCGTGTAT
		Reverse	TAGCCAGCACCGTGAATGTG
Hprt	Murine	Forward	TCAGGAGAGAAAGATGTGATTGA
		Reverse	CAGCCAACACTGCTGAAACA

Flow cytometry

5 ng OSM was added to HUVECs, HAECs, or HMEC-1 cells for 18h. Cells were washed with PBS and detached with accutase. Subsequently, cells were fixed with 1% PFA and incubated with 2.5 µL antibodies/ 1,000,000 cells against VCAM-1, ICAM-1, P-selectin and, E-selectin all obtained from Thermo Fisher (S1 Table). The experiment was repeated 3 times.

siRNA transfection

Knockdown of LIFR and OSMR was achieved by transfection with a mix of 4 specific siRNA sequences directed against the human mRNA sequence (SMARTpool siGENOME, GE Dharmacon, Lafayette, CO) in 70% subconfluent HUVEC cultures. Cells were incubated for 1 hour in a small volume of EGM-2 medium supplemented with DharmaFECT 1 (GE Dharmacon, Lafayette, CO) according to manufacturer's instructions. After 2 hours cells were supplemented with extra EGM-2 medium to complement medium volumes. As controls, HUVECs were transfected with a mix of 4 scrambled, non-targeting siRNAs (siSham Smartpool; GE Dharmacon, Lafayette, CO). siRNA transfected HUVECs were treated with OSM 48h after siRNA transfection.

Western blot

HUVECs were lysed with cOmplete™ Lysis-M, EDTA-free reagent (Sigma Aldrich, Saint Louis, MO) for 15 minutes on ice. Next, protein concentration was determined with the Pierce™ BCA protein Assay Kit (Thermo Scientific, Waltham, MA). The protein sample was treated with

NuPAGE™ Sample Reducing Agent (Thermo Scientific, Waltham, MA) and NuPAGE™ LDS Sample Buffer (Thermo Scientific, Waltham, MA). Subsequently, the solution was boiled at 70°C for 10 minutes. Samples were loaded on a Bolt™ 4-12% Bis-Tris Plus gel (Thermo Scientific, Waltham, MA), run for 50 minutes at 160V and transferred to an iBlot®2 PVDF Stack (Thermo Scientific, Waltham, MA) with the iBlot®2 Gel Transfer Device (Thermo Scientific, Waltham, MA). Blots were incubated with the primary antibody overnight at 4°C (S1 Table). Subsequently, blots were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for 1h at RT (S1 Table). Peroxidase labeled antibodies were detected with Chemiluminescent Peroxidase Substrate (Sigma, Saint Louis, MO).

Animals and treatments

Thirty-two female APOE*3Leiden.CETP transgenic mice (15-22 weeks of age) were used. The number of animals per group was calculated with Java Applets for Power and Sample Size [Computer software], from http://homepage.stat.uiowa.edu/~rlenth/Power /index.html using a one-way ANOVA with a probability of 0.05 and a Dunnett's correction, a SD of 20%, a power of 80% and a minimal expected difference of 35%. Mice were housed under standard conditions with a 12h light-dark cycle and had free access to food and water. Body weight, food intake and clinical signs of behavior were monitored regularly during the study. Mice received a Western type diet (WTD) (a semi-synthetic diet containing 15 w/w% cacao butter and 0.15% dietary cholesterol, Altromin, Tiel, the Netherlands). At T=0 weeks, after a run-in period of 3 weeks, mice were matched based on plasma total cholesterol levels, plasma triglyceride levels, body weight, and age in 4 groups of 8 mice. Two mice died during the diet intervention period, 1 in the 1μg/kg/day OSM group and 1 in the 10μg/kg/day OSM group. At T=7 weeks, an ALZET® Osmotic Pump Type 1004 (4-week release duration, Durect, Cupertino, CA) containing either 1, 3 or 10 μg/kg/day murine OSM (R&D systems, Minneapolis, MN) or PBS was placed subcutaneously in the flank. Doses were based on previous studies, which gave a single or double injection of 5-50 μg/kg OSM resulting in local increased permeability, oedema, swelling, infiltration of immune cells, increased serum VEGF levels and increased angiopoetin 2 expression[33–36]. All solutions, also PBS of control group, contained 1% mouse serum to prevent OSM from sticking to plastics. Prior to surgery, mice received the analgesic Carprofen (5 mg/kg s.c.) and were anesthetized with isoflurane (induction 4%, maintenance 2%). At T=10 weeks, mice were euthanized by gradual CO_2 inhalation (6 L/min in a 20 Liter container). CO_2 flow was maintained for a minimum of 1 minute after respiration ceased (as observed by lack of respiration and faded eye color). Death was confirmed by exsanguination (via heart puncture). Hearts were isolated for immunohistochemistry in the aortic root and aortas were isolated for RNA expression analysis. EDTA blood samples were drawn after a 4 hour fast at T=0 and T=10 weeks. All animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines. The care and use of all mice in this study was carried out at the animal facility of The Netherlands Organization for Applied Research (TNO) in accordance with the ethical review committee "TNO-DEC" under the registration number 3683. Animal experiments were approved by the Institutional Animal Care and Use Committee of TNO under registration number TNO-202.

Plasma parameters

Plasma cholesterol and triglycerides were measured spectrophotometrically with enzymatic assays (Roche diagnostics). The inflammatory markers, E-selectin and MCP-1 were measured with ELISA kits from R&D. Plasma ALT and AST were determined using a spectrophotometric assay (Boehringer Reflotron system) in group wise-pooled samples from sacrifice plasma. All assays were performed according to the manufacturer's instruction.

Histological assessment of vascular inflammation

Vascular inflammation was assessed in the aortic root area as reported previously by Landlinger *et al*[37] in control mice and mice receiving 10 μg/kg/day OSM. Briefly, the aortic root was identified by the appearance of aortic valve leaflets and serial cross-sections of the entire aortic root area (5 μm thick with intervals of 50 μm) were mounted on 3-aminopropyl triethoxysilane-coated slides and stained with hematoxylin-phloxine-saffron (HPS). Each section consisted of 3 segments (separated by the valves) and in 4 sections ICAM-1 expression and the number of monocytes adhering to the activated endothelium was counted after immunostaining with mouse monoclonal ICAM-1 antibody (Santa Cruz) and AIA 31240 antibody (Accurate Chemical and Scientific) respectively (S1 Table). One mouse from the control group was excluded from analysis due to a technical error, resulting in 7 and 8 mice per group.

RNA isolation murine tissue

To isolate RNA from aortic tissue, RA1 lysis buffer (Macherey-Nagel, Düren, Germany) containing 1% DTT was added to the tissue, which was cut in tiny pieces and subsequently minced. RNA was isolated with the RNeasy® Plus Micro Kit (Qiagen, Hilden, Germany) according to the RNeasy Fibrous Tissue Mini Kit protocol (Qiagen, Hilden, Germany). Isolated RNA (500 ng) was reverse transcribed into cDNA with the qSCript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA) and analyzed by real-time fluorescence assessment of SYBR Green signal (iQ™ SYBR Green Supermix, Bio-Rad, Hercules, CA) in the CFX96™ Real-Time Detection System (Bio-Rad, Hercules, CA). Each sample was measured in duplicates. Primers were designed for the murine genes of interest, sequences are listed in Table1. mRNA levels were analyzed and corrected for the housekeeping gene *Hprt*. RNA isolation was unsuccessful in one mouse from the 3μg/kg/day OSM group resulting in 6, 7 and 8 mice per group.

Statistical analysis

qPCR data was analyzed according to the ΔΔCt method, statistical tests were performed on ΔCt values. Two-way-anova was used to analyze *in vitro* data to take into account day-to-day variation of the experiments. Not normally (Gaussian) distributed parameters were transformed with the natural logarithm or in case of undetectable values analyzed with the appropriate non-parametric test. Dose-dependency was determined by a Pearson correlation. All statistical analyses were performed in SPSS statistics version 21.0. A two-tailed p-value of 0.05 was regarded statistically significant in all analyses. Graphs were made in GraphPad Prism version 7.02 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

RESULTS

OSM induces endothelial activation in human endothelial cells

To investigate whether OSM induces endothelial activation, we first examined cytokine mRNA expression in HUVECs, HAECs and HMEC-1 cells treated with 5 ng/mL OSM for 3 or 6 hours. OSM treatment was found to increase mRNA expression of the cytokines *MCP-1* ($p<0.01$) and *IL-6* ($p<0.001$) in HUVECs, HAECs ($p<0.001$) and HMEC-1 cells ($p<0.001$) at both 3h and 6h time points (Figure 1A-F). Since these cytokines are released by activated endothelial cells, we next measured MCP-1 and IL-6 protein concentrations in conditioned medium of OSM treated HUVECs, HAECs and HMEC-1 cells. Both MCP-1 ($p<0.05$) and IL-6 (<0.001) release were increased in OSM treated HUVECs, HAECs ($p<0.05$ and $p<0.01$ respectively) and HMEC-1 cells ($p<0.001$) at both time points (Figure 1G-L). Subsequently, we measured adhesion molecule expression, which is another feature of endothelial activation. *ICAM-1* mRNA expression was increased by OSM treatment in HUVECs ($p<0.001$) and HAECs ($p<0.01$) again at both 3h and 6h time points and in HMEC-1 cells 3h after addition of OSM ($p<0.01$)(Figure 2A-C). *VCAM-1* mRNA expression was upregulated in HUVECs at 3h ($p=0.008$) and in HAECs at both 3h and 6h ($p<0.001$)(Figure 2D-E). Moreover, we observed an upregulation in *E-selectin* mRNA expression in both HUVECs and HAECs at both 3h and 6h ($p<0.001$ and $p<0.05$)(Figure 2F-G), while *VCAM-1* and *E-selectin* mRNA levels were too low expressed in HMEC-1 cells. In addition, ICAM-1 membrane expression was increased in HUVECs ($p<0.05$), HAECs ($p<0.05$) and HMEC-1 cells ($p<0.05$) (Figure 2H-J), but not membrane expression of VCAM-1, P-selectin or E-selectin (Supplementary Figure 1). Since these adhesion molecules can also be shed upon endothelial activation[38], we measured P-selectin, E-selectin, soluble VCAM-1 and soluble ICAM-1 levels in conditioned medium. Soluble VCAM-1 was upregulated in conditioned medium of HUVECs 6h after OSM addition ($p<0.05$) and in HAECs at both 3h and 6h post OSM addition ($p<0.01$) (Figure 2K-L). Soluble VCAM-1 was not detectable in conditioned medium of HMEC-1 cells. Additionally, E-selectin levels were upregulated at both time points in conditioned medium of OSM treated HUVECs ($p<0.05$) and HAECs ($p<0.01$) and 6h post OSM addition in HMEC-1 cells ($p<0.05$) (Figure 2M-O). P-selectin levels were not detectable. Overall, these results indicate that OSM consistently induces endothelial activation *in vitro* in the different human endothelial cell types. Therefore, subsequent mechanistic studies were conducted in HUVECs.

JAK/STAT signaling is involved in OSM induced endothelial activation

IL-6 family members signal through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, a pathway that is often involved in cytokine and growth factor signaling[39–41]. Therefore, we investigated whether this pathway is also involved in OSM induced endothelial activation. STAT1 and STAT3 phosphorylation were markedly increased ($p<0.05$) (Figure 3) upon addition of OSM indicating that the JAK/STAT pathway is involved in OSM induced endothelial activation as well.

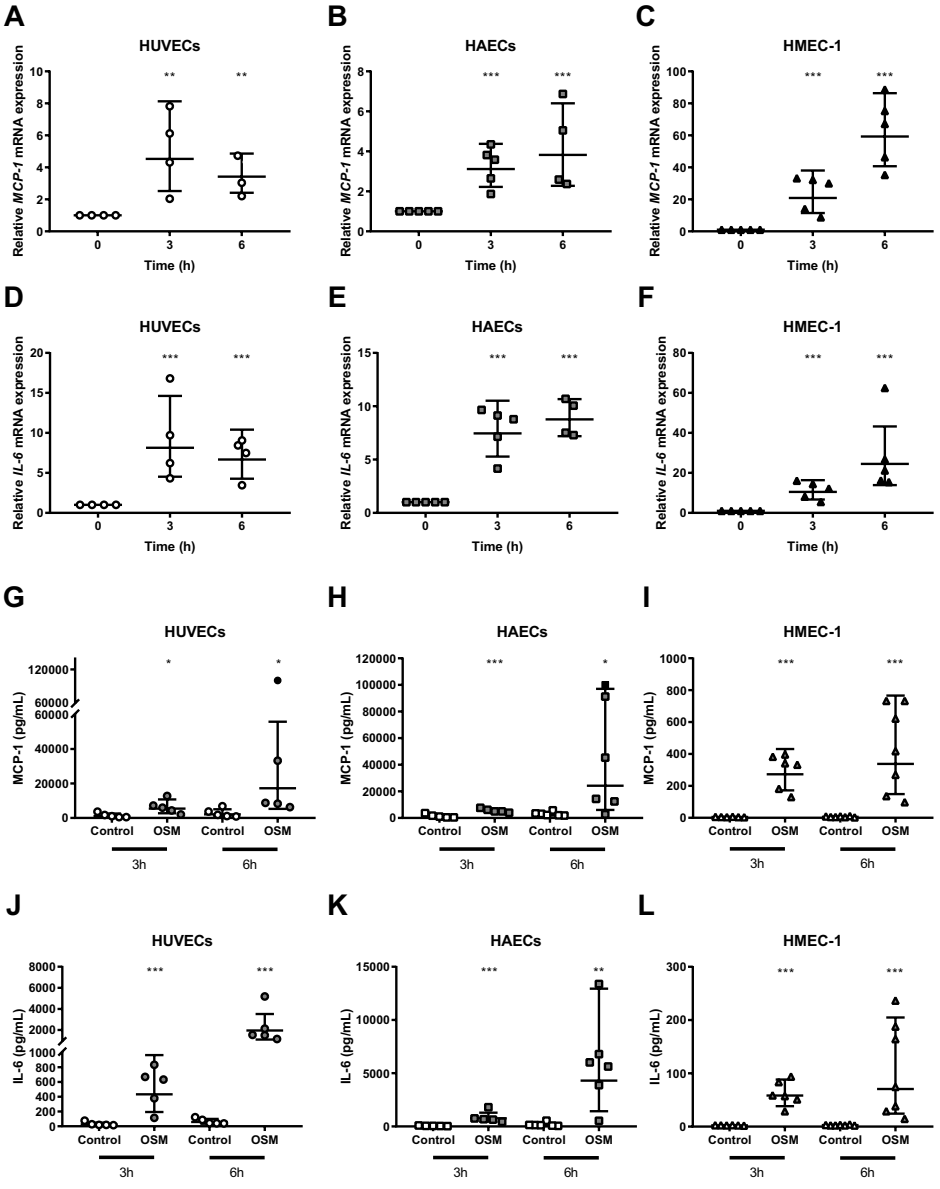
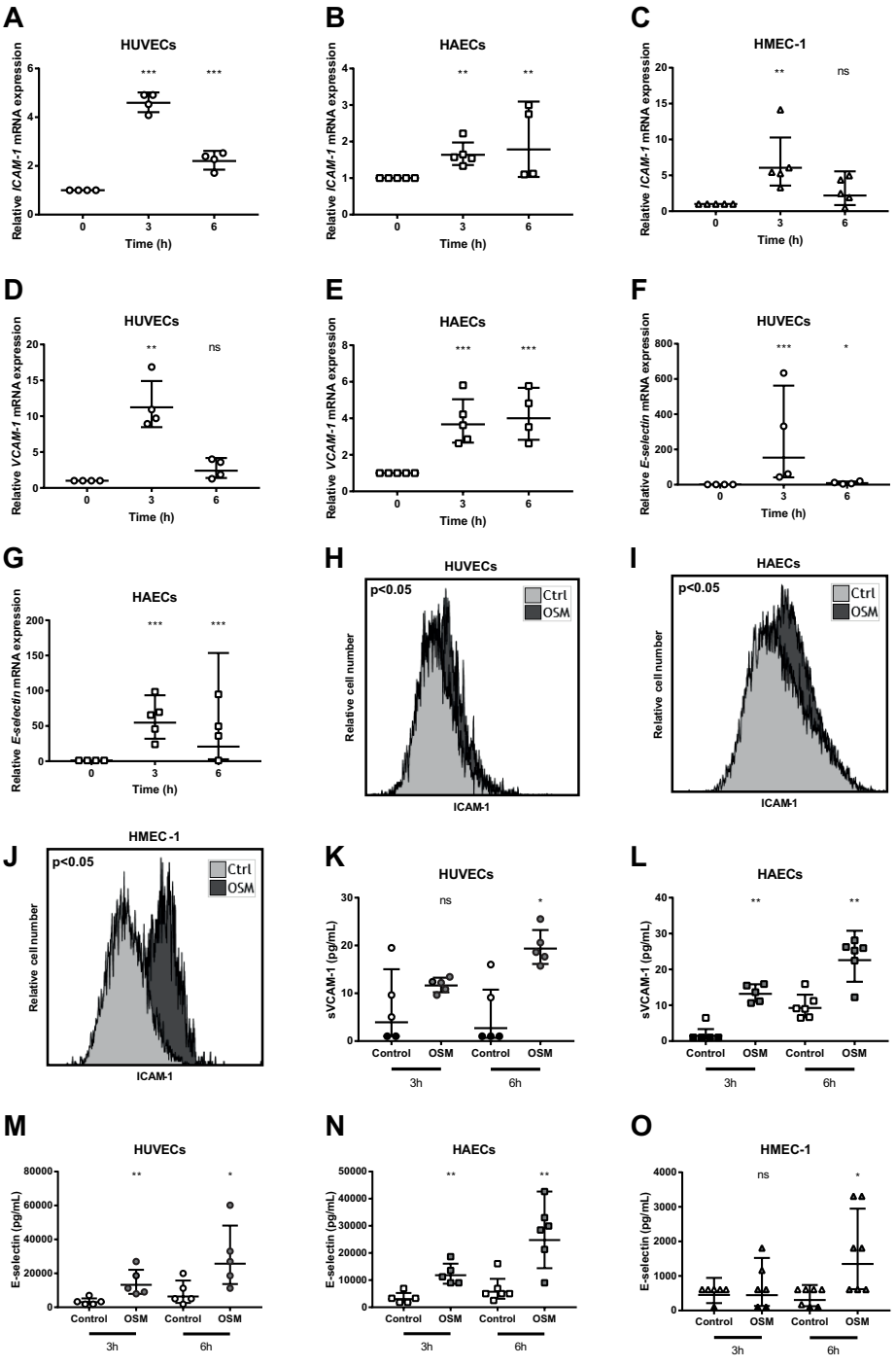


Figure 1. OSM increases cytokine release in different endothelial cells.
HUVECs, HAECs and HMEC-1 cells were incubated with 5 ng/mL OSM for the indicated period of time. All values are relative values figuur to control, which was given an arbitrary value of 1. Values were normalized to *ACTB* and calculated with the ΔΔCt method. A two-way ANOVA with Dunnett's test was performed on the ΔCt values to test for significance (A-F). MCP-1 and IL-6 release was measured in conditioned medium of HUVECs, HAECs and HMEC-1 cells incubated with 5 ng/mL OSM for 3 or 6h. Values too high to measure were arbitrarily set on 100,000 and are indicated with ● or ■. Data sets without missing values were ln transformed and analyzed with an independent t-test for significance while data sets with missing values were analyzed with a Mann Whitney U test (G-L). All data represent geometric mean ± geometric SD. *p<0.05 **p<0.01 ***p<0.001 compared to control (n=4-7).


A
HUVECs
Relative ICAM-1 mRNA expression
Time (h)
B
HAECs
Relative ICAM-1 mRNA expression
Time (h)
C
HMEC-1
Relative ICAM-1 mRNA expression
Time (h)
D
HUVECs
Relative VCAM-1 mRNA expression
Time (h)
E
HAECs
Relative VCAM-1 mRNA expression
Time (h)
F
HUVECs
Relative E-selectin mRNA expression
Time (h)
G
HAECs
Relative E-selectin mRNA expression
Time (h)
H
HUVECs
p<0.05
Ctrl
OSM
Relative cell number
ICAM-1
I
HAECs
p<0.05
Ctrl
OSM
Relative cell number
ICAM-1
J
HMEC-1
p<0.05
Ctrl
OSM
Relative cell number
ICAM-1
K
HUVECs
sVCAM-1 (pg/mL)
Control
OSM
Control
OSM
3h
6h
L
HAECs
sVCAM-1 (pg/mL)
Control
OSM
Control
OSM
3h
6h
M
HUVECs
E-selectin (pg/mL)
Control
OSM
Control
OSM
3h
6h
N
HAECs
E-selectin (pg/mL)
Control
OSM
Control
OSM
3h
6h
O
HMEC-1
E-selectin (pg/mL)
Control
OSM
Control
OSM
3h
6h

◀◀ Figure 2. OSM increases adhesion molecule expression and release in different endothelial cells.
HUVECs, HAECs and HMEC-1 cells were incubated with 5 ng/mL OSM for the indicated period of time. All values are relative values compared to control, which was given an arbitrary value of 1. Values were normalized to *ACTB* and calculated with the ΔΔCt method. A two-way ANOVA with Dunnett's test was performed on the ΔCt values to test for significance (A-C). ICAM-1 membrane expression was determined in HUVECs, HAECs and HMEC-1 cells treated with 5 ng/mL OSM for 18h. A two-way ANOVA was used to test for significance (D-F). Shedding of VCAM-1 and E-selectin was determined in conditioned medium of HUVECs, HAECs and HMEC-1 cells treated with 5 ng/mL OSM for 3 or 6h by measuring soluble VCAM-1 and E-selectin. Soluble VCAM-1 values too low to measure were arbitrarily set on 1 and are indicated with ● or ■. Soluble E-selectin values too low to measure were arbitrarily set on 100 and are indicated with ▲. Data sets without missing values were ln transformed and analyzed with an independent t-test for significance while data sets with missing values were analyzed with a Mann Whitney U test (G-K). All data represent geometric mean ± geometric SD, except for flow cytometry data which shows a representative histogram of control and OSM treated cells (n=3-7). *p<0.05 **p<0.01 ***p<0.001 compared to control, ns = not significant.

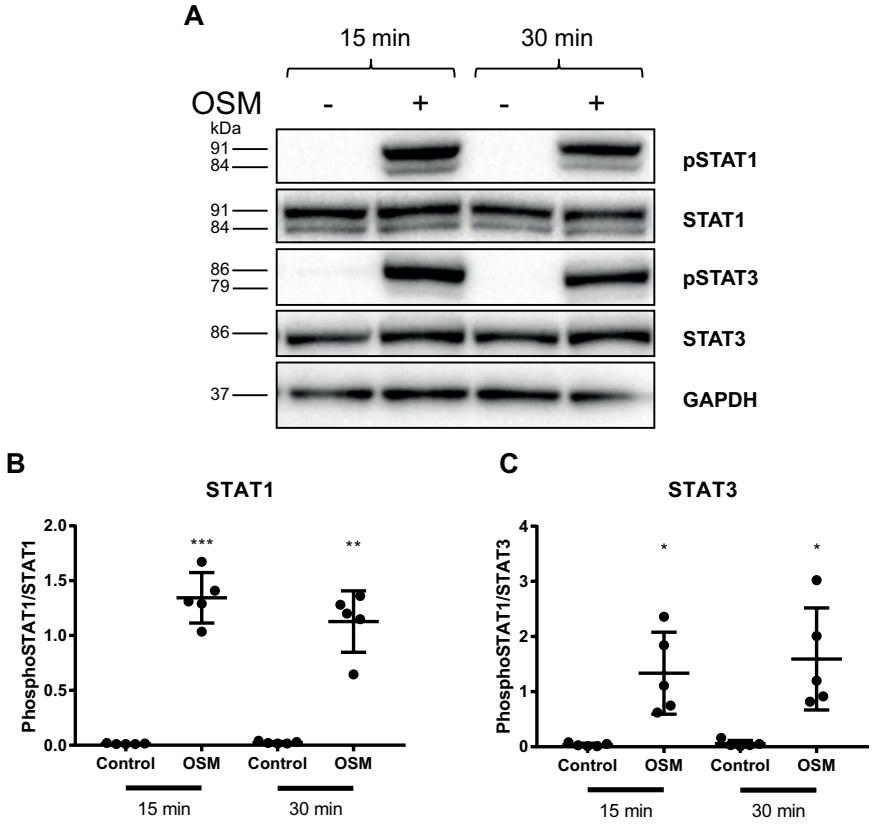


Figure 3. JAK/STAT pathway is involved in OSM induced endothelial activation.
HUVECs were incubated with 5 ng/mL OSM for 15 or 30 min. A representative picture shows STAT1 phosphorylation at Tyr701, STAT1, STAT3 phosphorylation at Tyr705, STAT3 and GAPDH (A). Bar graphs show relative STAT1 and STAT3 phosphorylation (B,C). A two-way ANOVA was performed to test for significance. Data represent mean ± SD (n=5). *p<0.05 **p<0.01 ***p<0.001 compared to control.

OSM induces endothelial activation by simultaneous signaling through the LIFR and OSMR

As OSM can signal through both the OSMR and the LIFR, a siRNA knockdown was performed to investigate which of these receptors is involved in OSM induced endothelial activation. *LIFR* mRNA expression was decreased to 25 ± 6% (mean ± SD), and *OSMR* mRNA expression to 52 ± 15%. Simultaneous knockdown resulted in a decrease of the *LIFR* to 31 ± 8% and of the *OSMR* to 45 ± 11% (Figure 4A,B). Single knockdown of LIFR did significantly decrease MCP-1 ($p=0.019$) and IL-6 secretion ($p=0.005$), but not VCAM-1 or E-selectin shedding. Single knockdown of OSMR did only decrease IL-6 secretion ($p<0.001$), while MCP-1 secretion was significantly increased ($p=0.007$). VCAM-1 and E-selectin shedding were both not significantly changed. Double knockdown did not only decrease IL-6 ($p<0.001$) and MCP-1 ($p<0.001$) secretion, but also VCAM-1 ($p=0.009$) and E-selectin ($p<0.001$) shedding compared to non-targeting siRNA treated cells (Figure 4C,D). A similar effect was observed for STAT1 and STAT3 phosphorylation, which was only reduced by double knockdown ($p<0.05$) compared to control (Figure 4E,F). Altogether, these data indicate that OSM signals through LIFR and OSMR simultaneously in human endothelial cells.

OSM induces an inflammatory response in APOE*3Leiden.CETP mice

To investigate whether OSM activates the endothelium *in vivo* as well, hyperlipidemic APOE*3Leiden.CETP mice were administered OSM for 3 weeks. No clinical signs of deviant behavior and no significant effects on food intake were noted in any treatment group as compared to control. Plasma ALT and AST, measured at end-point as safety markers, showed no aberrant results (S2 Table). Also, no significant difference in body weight, triglyceride, or cholesterol levels were observed compared to control (Figure 5A-C). As endothelial activation goes hand in hand with a pro-inflammatory response, plasma levels of inflammatory markers MCP-1 and E-selectin were measured. Plasma MCP-1 tended to be increased ($p=0.107$) and plasma E-selectin was increased ($p<0.001$) in mice treated with 10 µg/kg/day OSM compared to the control group (Figure 5D-E).

OSM induces endothelial activation in the vasculature of APOE*3Leiden.CETP mice

To further investigate if OSM is able to induce endothelial activation, the aortic root area was examined for relevant markers. ICAM-1 protein expression tended to be elevated from 39 ± 15% (mean ± SD) to 59 ± 22% ($p=0.067$) and an increase in monocyte adhesion to the activated endothelium was observed from 5.7 ± 3.0 to 10.3 ± 4.7 monocytes (mean ± SD, $p<0.05$) in mice treated with 10µg/kg/day OSM (Figure 6). Furthermore, aortic mRNA expression analysis revealed a dose-dependent increase in *Il-6* expression ($p<0.001$) and *Icam-1* expression tended to be increased in the 1µg/kg/day and 10µg/kg/day OSM treated groups ($p=0.101$ and $p=0.133$ respectively) compared to control. *Mcp-1* mRNA expression was not enhanced (Figure 7). These results show that OSM does not only induce endothelial activation *in vitro*, but also *in vivo* in a hyperlipidemic mouse model.

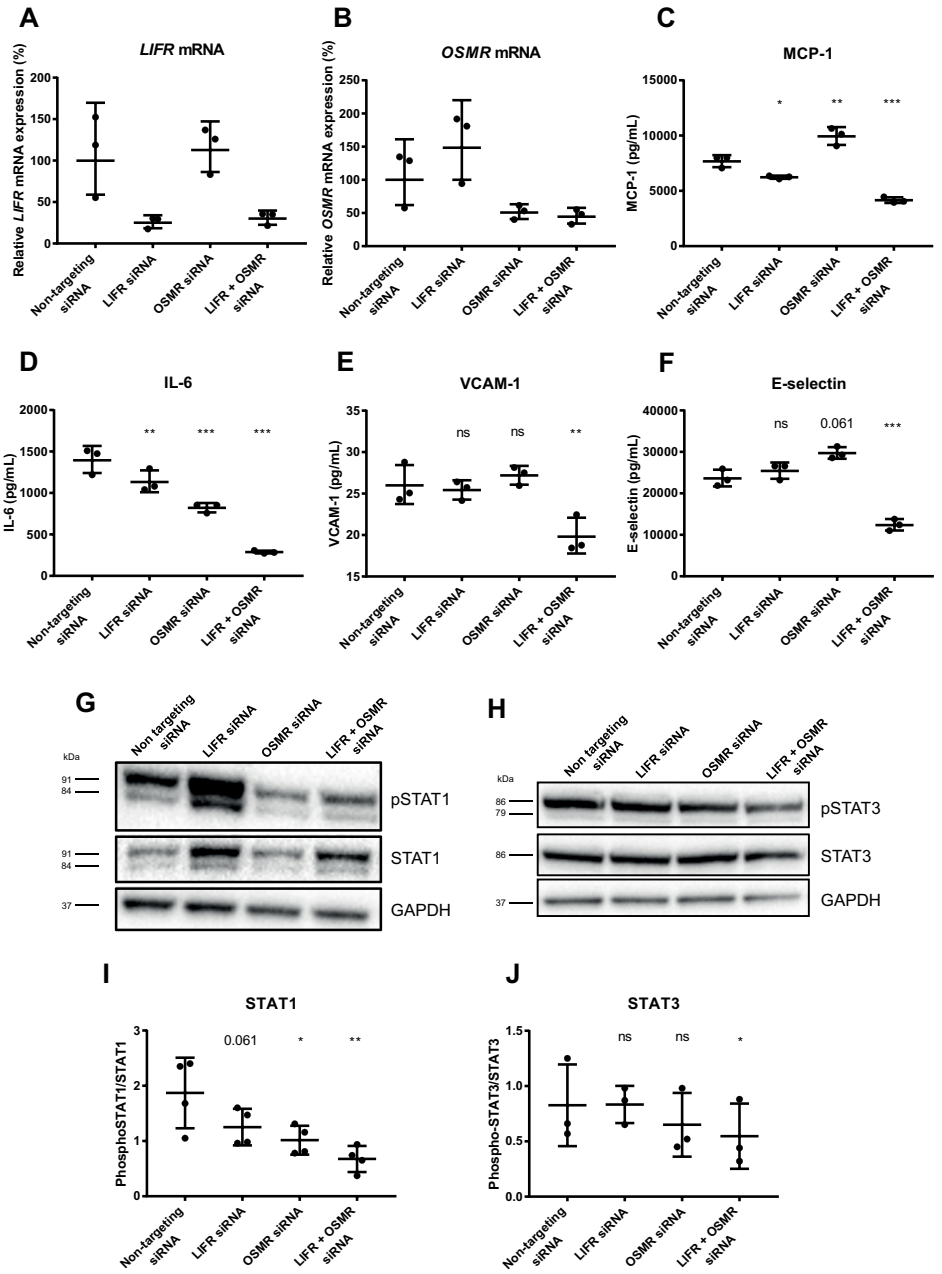


Figure 4. Simultaneous downregulation of LIFR and OSMR decreases IL-6 and MCP-1 release and prevents STAT1 and STAT3 phosphorylation.

LIFR (A) and *OSMR* (B) mRNA expression levels were downregulated by siRNA transfection in HUVECs. 48h post transfection, HUVECs were treated with 5 ng/mL OSM for 6h to determine IL-6 and MCP-1 secretion and VCAM-1 and E-selectin shedding (C-F) or for 15 min to determine STAT1 and STAT3 phosphorylation (G-J). A two-way ANOVA with Dunnett's test was performed to test for significance. All data represent mean ± SD (n=3-4). *p<0.05 **p<0.01 compared to control, ns = not significant.

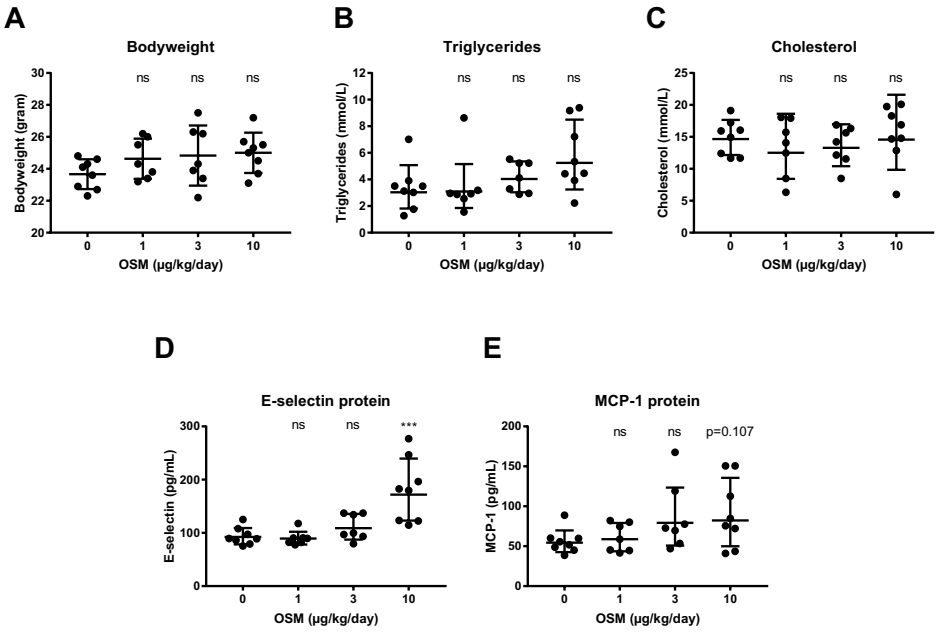


Figure 5. OSM enhances plasma levels of inflammatory markers in APOE*3Leiden.CETP mice treated with OSM.

After 3 weeks of OSM treatment, bodyweight (A), triglyceride (B), cholesterol (C), E-selectin (D) and MCP-1 (E) levels were measured and compared to control mice. A one-way ANOVA with Dunnett's test was performed on ln transformed data, except for the bodyweight, to test for significance. All data represent geometric mean ± geometric SD, except for bodyweight which represents mean ± SD (n=7-8). ***p<0.001 compared to control, ns = not significant.

DISCUSSION

The present study demonstrates that OSM induces endothelial activation in cultured human endothelial cells as well as *in vivo* in APOE*3Leiden.CETP mice. The data show increased release of inflammatory markers and adhesion molecule expression, both features of endothelial activation. Furthermore, OSM increased monocyte adhesion in the aortic root area, as functional marker of endothelial activation.

We studied OSM induced endothelial activation *in vitro* by investigating the effect of OSM in three different types of human endothelial cells. Our data add to and expand on previous data that showed that OSM increases IL-6, IL-8 and MCP-1 secretion, ICAM-1 and VCAM-1 membrane expression and PMN adhesion to endothelial cells *in vitro* [34,42,43]. Consistently, increased VCAM-1 and E-selectin shedding was observed in all three endothelial cell types. ICAM-1 is an important adhesion molecule in monocyte binding as ICAM-1$^{-/-}$ endothelial cells show a strong attenuation in monocyte binding compared to control endothelial cells[44]. Although we did not observe an increase in membrane E-selectin and VCAM-1 expression, OSM did increase soluble E-selectin and VCAM-1. Soluble VCAM-1 was previously shown to serve as a monocyte chemoattractant agent and soluble E-selectin enhances leukocyte

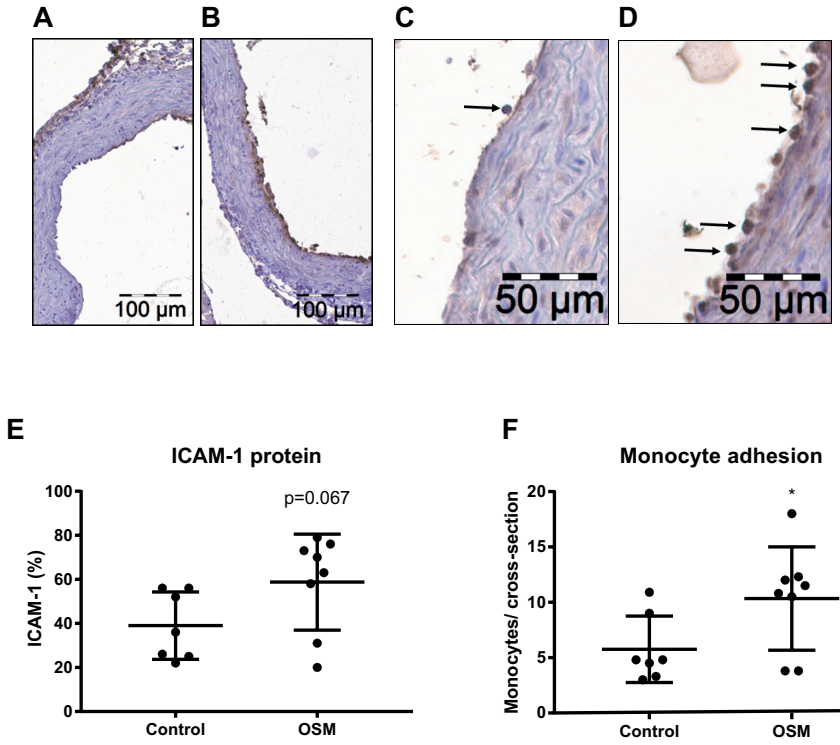


Figure 6. OSM increases ICAM-1 expression and monocyte adherence in the aortic root area in OSM treated APOE*3Leiden.CETP.
Representative pictures showing the endothelial ICAM-1 expression (brown staining) in a control (A) and a 10 µg/kg/day OSM treated (B) mouse and monocyte adherence (arrows) in a control (C) and a 10 µg/kg/day OSM treated (D) mouse. Endothelial ICAM-1 expression was determined as percentage of the endothelial surface in the cross sections (E) and adhering monocytes were counted per cross-section after staining with AIA 31240 (F). Data represent mean ± SD (n = 7-8). An independent t-test was used to test for significance. Data represent mean ± SD. *p<0.05.

migration and binding to endothelial cells[45,46]. Taken together, these observations show that OSM induces different biomarkers of endothelial activation in cultured endothelial cells.
Previous short term *in vivo* studies in healthy wildtype mice with OSM administered for only 6 to 24 hours have shown signs of acute endothelial activation, such as increased angiopoetin 2 expression in cardiac tissue, increased plasma VEGF levels and increased permeability and infiltration of inflammatory cells[33–36]. It is important to note that publicly available datasets show that *Osmr* and *Lifr* mRNA are expressed in aortic endothelial cells from mice as well (data accessible at NCBI GEO database [47,48], accession GSE114805 and [47,48], accession GSE115618).
The aim of the present study was to investigate the effect of chronic OSM exposure on endothelial activation in a hyperlipidemic mouse model, the APOE*3Leiden.CETP mouse. This mouse model features elevated lipid levels, representing humans with hyperlipidemia and mild chronic inflammation who have an increased risk of developing atherosclerosis[31,32,37].

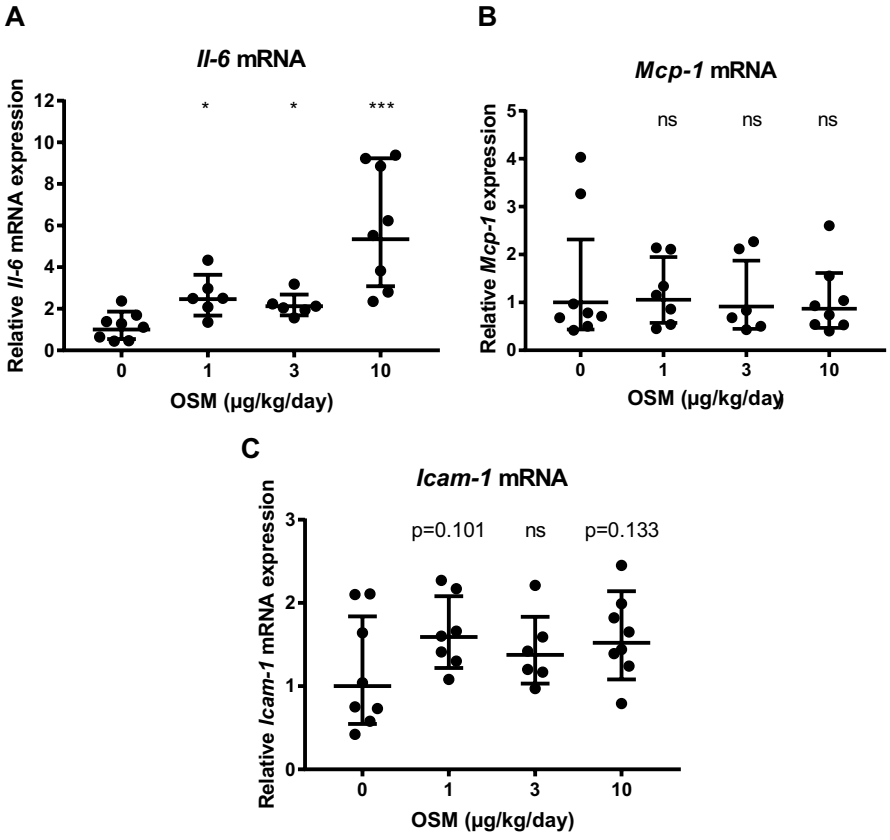


Figure 7. OSM increases *Il-6* mRNA expression in aortic tissue of APOE*3Leiden.CETP mice treated with OSM. After 3 weeks of OSM treatment, mRNA was isolated from the aorta and analyzed by qPCR. *Il-6* (A), *Mcp-1* (B) and *Icam-1* (C) mRNA expression were quantified. All values are relative values compared to the control group, which was given an arbitrary value of 1. Values were normalized to HPRT and calculated with the ΔΔCt method. A one-way ANOVA with Dunnett's test was performed on the ΔCt values to test for significance. Data represent geometric mean ± geometric SD (n=6-8). All values were compared to control. *p<0.05 ***p<0.001 compared to control, ns = not significant.

We found that OSM tended to increase plasma MCP-1 and significantly increased plasma E-selectin, both markers of activated or dysfunctional endothelium[5,49], after 3 weeks of chronic OSM administration. Moreover, mRNA expression of *Il-6* was increased dose-dependently in aortic tissue of OSM treated mice. We also observed a trend towards increased ICAM-1 expression in the aortic root of OSM treated mice and a markedly enhanced monocyte binding as functional marker of activated endothelium, thus demonstrating augmented endothelial activation. ICAM-1 expression and adhesion of monocytes are strongly related, as previous studies show increased monocyte binding upon enhanced ICAM-1 expression and decreased monocyte binding upon reduced ICAM-1 expression[44,50]. Collectively, these findings provide evidence that OSM does not only induce endothelial activation *in vitro,* but also *in vivo* on top of the inflammatory state that is present

in hyperlipidemic mice, resulting in increased monocyte recruitment and adherence.
Even though, endothelial cells are directly activated by OSM *in vitro*, it is important to note that the *in vivo* situation is much more complex and other cell types may have contributed to the observed effects as well. For instance, the increase in plasma MCP-1 and cardiac *Il-6* expression can partly be caused by fibroblasts or smooth muscle cells, as these two cell types also show increased IL-6 and MCP-1 expression upon OSM treatment *in vitro*[51,52]. Furthermore, OSM can promote growth factor and cytokine release in cell types other than endothelial cells, these released growth factors and cytokines can in turn activate the endothelium, thereby inducing indirect endothelial activation[51–53]. An example of such a growth factor is vascular endothelial growth factor (VEGF), which can be upregulated by OSM in multiple cell types[35,54–56] and is known to induce endothelial activation by increasing adhesion molecule expression and leukocyte adhesion[57].
Although our *in vivo* study was not aimed at and was too short to investigate whether chronic OSM exposure aggravates atherosclerosis, our results do give clues that OSM may be involved in the initiation of the atherosclerotic process. Some of the diverse hallmarks of endothelial activation that we observed, have previously been associated with atherosclerosis development in humans[49,58]. Further indications come from reports showing that OSM is present in both murine and human plaques[16], and higher mRNA expression levels of OSM in PBMCs derived from coronary artery disease patients compared to healthy individuals[59]. Moreover, a recent paper showed that prevention of OSM signaling, as opposed to stimulation of OSM signaling in our study, in OSMR-$\beta^{-/-}$ApoE$^{-/-}$ mice resulted in less and smaller atherosclerotic lesions and less macrophages compared to ApoE$^{-/-}$ mice[60].
Other studies have shown that partial inhibition of endothelial activation by knockdown of E-selectin, P-selectin, ICAM-1 or MCP-1 attenuates atherosclerosis development in mice[61,62]. Therefore, lowering of plasma OSM levels or intervention in OSM signaling might be worth investigating as a possible future approach in the treatment of atherosclerosis.
As it is currently unknown which of the OSM receptors is involved in OSM induced endothelial activation, we performed a siRNA knockdown of the LIFR and the OSMR. Single knockdown experiments showed that solely LIFR or OSMR downregulation is not sufficient to prevent OSM induced endothelial activation or JAK/STAT signaling. Only simultaneous knockdown of both receptors was able to dramatically decrease IL-6 and MCP-1 release, VCAM-1 and E-selectin shedding and STAT1 and STAT3 phosphorylation. Hence, it is essential to block both receptors simultaneously or to target OSM when considering intervening in OSM signaling as a possible future therapy. Targeting both receptors or OSM itself could be a relative safe approach since OSM$^{-/-}$ mice are viable and healthy[63].
Taken together, our comprehensive study provides new evidence that OSM induces activation of human endothelial cells from different vascular beds and in APOE*3Leiden. CETP mice chronically treated with OSM. Moreover, we provided data indicating both receptors for OSM as well as OSM itself as potential therapeutic targets in atherosclerosis and other chronic inflammatory diseases in which endothelial activation is involved such as rheumatoid arthritis, abnormal angiogenesis and thrombosis[64–67].

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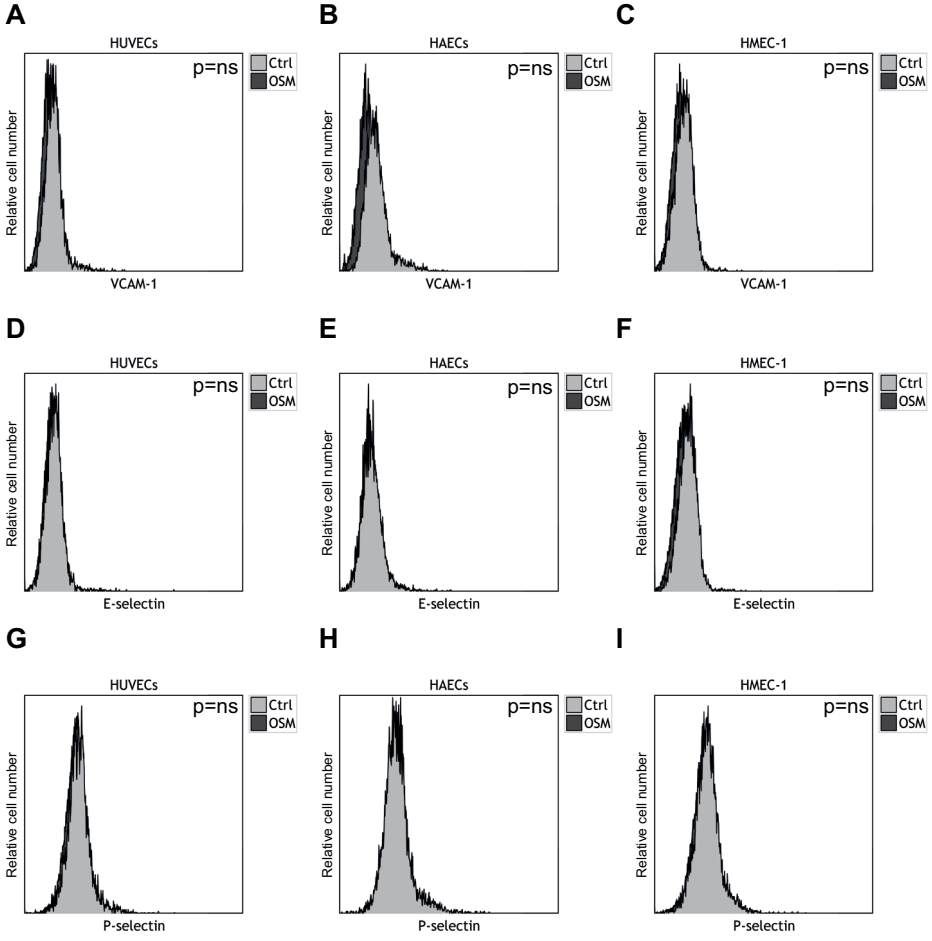
SUPPLEMENTAL DATA

S1 Table. List of antibodies used for flow cytometry, immunohistochemistry and western blot.

Primary antibodies	Clone	Reactivity	Species/ Isotype	Dilution	Company	Cat. No
VCAM-1 conjugated with FITC	STA	Human	Mouse IgG	2.5 μL/ 1,000,000 cells	Thermo Fisher, Waltham, MA	11-1069-42
ICAM-1 conjugated with PerCP-eFluor 710	HA58	Human	Mouse IgG1	2.5 μL/ 1,000,000 cells	Thermo Fisher, Waltham, MA	46-0549-42
P-selectin conjugated with APC	Psel.KO2.3	Human, Mouse	Mouse IgG	2.5 μL/ 1,000,000 cells	Thermo Fisher, Waltham, MA	17-0626-82
E-selectin conjugated with PE	P2H3	Human	Mouse IgG	2.5 μL/ 1,000,000 cells	Thermo Fisher, Waltham, MA	12-0627-42
ICAM-1		Human Rat mouse	Mouse monoclonal IgG_{2a}	1:400	Santa Cruz Biotechnology, Dallas, TX	sc-8439
AIA 31240		Mouse	Rabbit	1:500	Accurate Chemical and Scientific, Westbury, NY	J1857
STAT1		Human, Mouse, Rat, Monkey	Rabbit	1:1,000	Cell signaling, Danvers, MA	9172
phosphoSTAT1 (Tyr701)	58D6	Human, Mouse	Rabbit IgG	1:1,000	Cell signaling, Danvers, MA	9167
STAT3	D3Z2G	Human, Mouse, Rat, Monkey	Rabbit IgG	1:1,000	Cell signaling, Danvers, MA	12640
phosphoSTAT3 (Tyr705)	D3A7	Human, Mouse, Rat, Monkey	Rabbit IgG	1:2,000	Cell signaling, Danvers, MA	9145
GAPDH	14C10	Human, Mouse, Rat, Monkey, Bovine, Pig	Rabbit	1:7,500	Cell signaling, Danvers, MA	2118S
Secondary antibodies		**Reactivity**	**Isotype**	**Dilution**	**Company**	**Cat. No**
Goat Anti-Rabbit antibody conjugated with HRP		Rabbit	Goat	1:2,000	Dako, Glostrup, Denmark	P0448
Goat Anti-Mouse antibody conjugated with HRP		Mouse	Goat	1:2,000	Dako, Glostrup, Denmark	P0447

S2 Table. Average food intake and ALT and AST levels in mice.

	Dose (μg/kg/day)	Food intake (g/mouse/day)	ALT (U/L)	AST (U/L)
Control	-	2.4 ± 0.2	53.1	324
OSM	1	2.7 ± 0.4	52.5	333
OSM	3	2.4 ± 0.3	92.1	669
OSM	10	2.4 ± 0.3	59.4	293



S1 Figure OSM does not affect membrane expression of VCAM-1, E-selectin and P-selectin in endothelial cells.
HUVECs, HAECs and HMEC-1 cells were incubated with 5 ng/mL OSM for 18h. A two-way ANOVA with Dunnett's test was performed on the median to test for significance (n=3). ns = not significant.

CHAPTER 3

Oncostatin M reduces atherosclerosis development in APOE*3Leiden.CETP mice and is associated with increased survival probability in humans

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ABSTRACT

Objective

Previous studies indicate a role for Oncostatin M (OSM) in atherosclerosis and other chronic inflammatory diseases for which inhibitory antibodies are in development. However, to date no intervention studies with OSM have been performed, and its relation to coronary heart disease (CHD) has not been studied.

Approach and Results

Gene expression analysis on human normal arteries (n=10) and late stage/ advanced carotid atherosclerotic arteries (n=127) and *in situ* hybridization on early human plaques (n=9) showed that *OSM,* and its receptors, OSM receptor (*OSMR*) and Leukemia Inhibitory Factor Receptor (*LIFR*) are expressed in normal arteries and atherosclerotic plaques. Chronic OSM administration in APOE*3Leiden.CETP mice (n=15/group) increased plasma E-selectin levels and monocyte adhesion to the activated endothelium independently of cholesterol but reduced the amount of inflammatory Ly-6C^{High} monocytes and atherosclerotic lesion size and severity. Using aptamer-based proteomics profiling assays high circulating OSM levels were shown to correlate with post incident CHD survival probability in the AGES-Reykjavik study (n=5457).

Conclusions

Chronic OSM administration in APOE*3Leiden.CETP mice reduced atherosclerosis development. In line, higher serum OSM levels were correlated with improved post incident CHD survival probability in patients, suggesting a protective cardiovascular effect.

INTRODUCTION

Cytokines have an indisputable role in all stages of atherosclerosis development. In the initial stages of the disease, cytokines induce endothelial activation leading to endothelial adhesion molecule expression and leukocyte recruitment to the activated endothelium. In later stages of the disease, cytokines are involved in smooth muscle cell (SMC) migration, foam cell formation and enhanced MMP activity leading to plaque destabilization[1,2].
Similarly, a role for Oncostatin M (OSM) in atherosclerosis has been suggested[3,4]. This cytokine is secreted by activated macrophages and neutrophils and signals through the Leukemia Inhibitory Factor Receptor (LIFR) and the OSM receptor (OSMR)[5–7]. OSM induces endothelial activation by increasing cytokine release, adhesion molecule expression, and leukocyte adhesion to the activated endothelium in cultured endothelial cells[8–10]. Moreover, OSM reduces vascular integrity of rat blood brain barrier endothelial cells and enhances angiogenesis[11,12]. Next to its effects on the endothelium, OSM enhances SMC proliferation, migration and differentiation[4,12,13].
Additional evidence for this potential role of OSM in atherosclerosis, was provided by Albasanz-Puig *et al.,* who showed that OSM is expressed in both murine and human atherosclerotic plaques[13]. Furthermore, in ApoE$^{-/-}$ mice, OSMR deficiency attenuated atherosclerosis development and increased plaque stability[14].
Using a different approach, we recently demonstrated that short-term OSM administration (for 3 weeks) to APOE*3Leiden.CETP mice increased plasma E-selectin levels, Interleukin (IL)-6 mRNA expression in the aorta and Intercellular Adhesion Molecule 1 (ICAM-1) expression and monocyte adherence to the activated endothelium in the aortic root[10]. Collectively, these findings suggest that OSM may be involved in atherosclerosis development but so far this has never been studied.
The aim of this study is to investigate whether OSM is involved in atherosclerosis development in a humanized mouse model and in man. Therefore, we first investigated if OSM and its receptors are expressed in human normal and atherosclerotic arteries and if circulating OSM levels correlate with markers of endothelial activation in humans. Next, we explored the effect of long-term OSM administration on endothelial activation, atherosclerosis development and lesion composition in APOE*3Leiden.CETP mice, a translational model for human lipoprotein metabolism and atherosclerosis development[15]. Finally, we investigated if circulating OSM levels were associated with survival probability post coronary heart disease (CHD) in humans.

MATERIALS AND METHODS

Microarray on BiKE study material
Late stage/ advanced atherosclerotic plaques were obtained from patients undergoing surgery for high grade (>50%) carotid stenosis and retained within the BiKE study. Normal artery controls were obtained from nine macroscopically disease-free iliac arteries and one aorta from organ donors without history of cardiovascular disease. All samples were

collected with informed consent from patients or organ donor guardians. 127 plaques from BiKE patients and 10 normal arteries were analyzed by Affymetrix HGU133 plus 2.0 GeneChip microarrays. Robust multiarray average normalization was performed and processed gene expression data was transformed in log2-scale. The microarray dataset is available from Gene Expression Omnibus (GSE21545). The BiKE study cohort demographics, details of sample collection, processing, and analyses were previously described[16].

In situ hybridization (ISH) on SOCRATES study material

Early stage atherosclerotic lesions for *in situ* hybridization were obtained from the SOCRATES biobank (Leiden University Medical Center, the Netherlands). Details of this biobank have been described previously[17]. Briefly, this biobank contains aortic wall patches obtained during kidney transplantation with grafts derived from cadaveric donors. Sample collection and handling were performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, the Netherlands, and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (https://www.federa.org/?s=1&m=82&p=0&v=4#827). Chromogenic mRNA-ISH was essentially performed as previously described[18,19] on 9 atherosclerotic lesions from the SOCRATES biobank. For detection of the *OSM*, *OSMR* and *LIFR* mRNAs, ISH was performed in a Ventana Discovery ULTRA instrument (Ventana Medical Systems Inc., AZ, USA) using the ACD RNAscope® 2.5 Red Kit (Advanced Cell Diagnostics, Newark, CA, USA) and the mRNA Discovery ULTRA RED 4.0 procedure. RNAscope® 2.5 VS. Probes for Hs-OSM (#456389), Hs-OSMR-tv1 (#445699) and Hs-LIFR (#441029) were designed by the probe manufacturer (Advanced Cell Diagnostics). FFPE sections (5 µm) were applied to Superfrost Plus (Thermo Fisher Scientific) slides, and all operations including deparaffinization, pretreatment, ISH and counterstaining using hematoxylin were performed in a Ventana Discovery ULTRA instrument. Following the ISH-procedure in the Ventana instrument, slides were washed in lukewarm tap water with detergent until oil from the slides was fully removed. Subsequently, slides were washed in demineralized water, air dried and mounted in EcoMount mounting medium (Advanced Cell Diagnostics) prior to scanning in a bright-field whole-slide scanner (Axio Scan.Z1, Zeiss, Oberkochen Germany) using a 20x objective. The resulting digital images were inspected and regions of interest were selected.

Proteomics on AGES-Reykjavik study material

Association between OSM levels and IL-6, vascular cell adhesion molecule (VCAM)-1, P-selectin, E-selectin, ICAM-1 and Monocyte chemoattractant protein-1 (MCP-1) levels, and between OSM levels and survival were explored in the AGES-Reykjavik cohort (n=5457)[20], a single-center prospective population-based study of deeply phenotyped elderly European Caucasians (aged 66 through 96, mean age 75±6 years) who survived the 50-year-long prospective Reykjavik study. Phenotype description, patient numbers and other details related to the present study have been described previously[21]. The AGES-Reykjavik study was approved by the NBC in Iceland (approval number VSN-00-063), the National Institute on Aging Intramural Institutional Review Board (USA), and the Data Protection Authority in Iceland. We applied a custom version of the Slow Off-rate Modified Aptamer (SOMAmer)

platform targeting proteins known or predicted to be found in the extracellular milieu, including the predicted extracellular domains of single- and certain multi-pass transmembrane proteins, as previously described[21].
For survival analysis post CHD, we used 698 incident CHD cases exhibiting 307 deaths during the survival follow-up period of 12 years. Follow-up time for survival post incident CHD was defined as the time from 28 days after an incident CHD event until death from any cause or end of follow-up time.

Animals and treatments
Sixty-five female in-house bred APOE*3Leiden.CETP transgenic mice (10-15 weeks of age) were used. Mice were housed under standard conditions with a 12h light-dark cycle and free access to food and water. Body weight, food intake and clinical signs of behavior were monitored regularly. Mice received a Western type diet (semi-synthetic containing 15 w/w% cacao butter and 0.15% dietary cholesterol, Altromin, Tiel, the Netherlands). At t=0 weeks, after a run-in period of 3 weeks, mice were matched based on body weight, age, plasma total cholesterol and E-selectin levels in 4 groups: a control group, and three intervention groups, two of which were treated with 10 or 30 µg/kg/day OSM for 16 weeks, and an initial priming group, which received 30 µg/kg/day OSM for the first 5.5 weeks only. All groups consisted of 15 mice except for the control group which had an additional 5 mice to monitor the atherosclerosis development. Five mice were removed from the study based on human end-point criteria and were excluded from all analyses: 2 mice in the 16 week 30 µg/kg/day OSM group and 1 in each of the other 3 groups. At t=0 weeks, an ALZET® Osmotic Pump Type 1004 (Durect, Cupertino, CA) containing either 10 or 30 µg/kg/day murine OSM (R&D systems, Minneapolis, MN) or the vehicle (PBS + 1% mouse serum) was placed subcutaneously in the flank and were replaced at t=5.5 and 11 weeks. Doses were based on our previous research[10]. Prior to surgery, mice received the analgesic Carprofen (5 mg/kg s.c.) and were anesthetized with isoflurane (induction 4%, maintenance 2%). EDTA blood samples were drawn after a 4 hour fast at t=0, 4, 8, 12 and 16 weeks for determination of total cholesterol and inflammatory markers. At t=12 weeks, 4 mice from the control group were euthanized to assess atherosclerosis development for the determination of the end-point of the study. At t=16 weeks, mice were euthanized by gradual CO_2 inhalation. Death was confirmed by exsanguination (via heart puncture) and hearts were isolated. All animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines. Approval was granted by the ethics committee on animal experiments (approval reference number DEC-3683) and the institutional animal welfare body (approval reference number TNO-255).

Plasma parameters
Plasma cholesterol was measured spectrophotometrically with enzymatic assays (Roche Diagnostics). E-selectin and Monocyte Chemoattractant Protein 1 (MCP-1) were measured with ELISA kits from R&D (Minneapolis, MA, USA), and Serum Amyloid A (SAA) with an ELISA kit from Tridelta Development Limited (Maynooth, County Kildare, Ireland). All assays were performed according to the manufacturer's instructions.

Histological assessment of atherosclerosis and plaque composition

Atherosclerotic lesion area and severity were assessed in the aortic root area, as reported previously[22,23]. Briefly, the aortic root was identified by the appearance of aortic valve leaflets, and serial cross-sections of the entire aortic root area (5 µm thick with intervals of 50 µm) were mounted on 3-aminopropyl triethoxysilane-coated slides and stained with haematoxylin-phloxine-saffron (HPS). For each mouse, the lesion area was measured in 4 subsequent sections. Each section consisted of 3 segments (separated by the valves). For determination of atherosclerotic lesion severity, the lesions were classified into five categories according to the American Heart Association (AHA) criteria[24]: type 1 (early fatty streak), type 2 (regular fatty streak), type 3 (mild plaque), type 4 (moderate plaque), and type 5 (severe plaque). The total lesion area was calculated per cross-section. Due to a technical error one mouse of the OSM (30 µg/kg) was excluded from analysis. Lesion severity was calculated as relative amount of type I-V lesions in which the lesion-free segments are included. From this, the relative amounts of lesion-free segments and diseased segments were calculated, and the relative amount of diseased segments was further subdivided into type I–V lesions, where types I-III refer to mild, and types IV-V to severe lesions. Lesion composition of type IV and V lesions was assessed after double immunostaining with anti-α smooth muscle actin (1:400; PROGEN Biotechnik GmbH, Germany) for smooth muscle cells (SMC), and anti-mouse MAC-3 (1:400; BD Pharmingen, the Netherlands) for macrophages. Anti-α smooth muscle actin was labeled with Vina green (Biocare Medical, Pacheco, USA), and MAC-3 with DAB (Vector laboratories, Burlingame, USA). After slides were scanned and analyzed, cover slips were detached overnight in xylene and Sirius Red staining for collagen was performed. The necrotic area was measured in the Sirius Red-stained slides. Lesion stability index, as the ratio of collagen and αSMC area (i.e. stabilization factors) to macrophage and necrotic area (i.e. destabilization factors) was calculated as described previously[22]. Lesion composition was assessed in all type IV-V lesions with a mean of 5.9 ± 3.1 lesions in control, 5.6 ± 2.5 lesions in OSM 10 µg/kg/d, 2.9 ± 2.0 lesions in OSM 30 µg/kg/d temporary and 2.8 ± 2.9 lesions in OSM 30 µg/kg/d. Eight mice were excluded from analysis as there were no type IV-V lesions present (n=1 in control; n=4 in OSM 30 µg/kg/d temporary and n=3 in OSM 30 µg/kg/d). In each segment used for lesion quantification, ICAM-1 expression and the number of monocytes adhering to the endothelium were counted after immunostaining with mouse monoclonal ICAM-1 antibody (1:400; Santa Cruz Biotechnology, Dallas, USA) and AIA 31240 antibody (1:500; Accurate Chemical and Scientific, New York, USA) respectively[25]. NLRP3 expression in the macrophages was quantified after staining with rabbit polyclonal antibody to NLRP3 (1:400; Abcam, Cambridge, UK). All slides were scanned by an Aperio AT2 slide scanner (Leica Biosystems). Atherosclerotic area, monocyte adherence and ICAM-1 expression were measured in Image Scope (version 12-12-2015), and the area that stained positive for αSMA, MAC-3, Sirius Red and NLRP3 in the plaques was quantified automatically in Fiji (version 30-5-2017) using a threshold method.

Flow cytometry

To analyze the different monocyte subsets, 25 µL whole blood was incubated with antibodies against CD11b (APC-eFluor780-conjugated, eBioscience, San Diego, California, USA), Ly-6C

(eFluor450-conjugated, eBioscience, San Diego, California, USA) and Ly-6G (A647-conjugated, Biolegend, San Diego, California, USA) for 30 min at RT. Erythrocytes were lysed with lysis buffer (deionized water with 168 mM ammonium chloride (Merck, Darmstadt, Germany), 9.99 mM potassium bicarbonate (Merck, Darmstadt, Germany) and 0.11 mM Na2EDTA (Sigma-Aldrich, St. Louis, MO, USA)) for 10 min on ice and remaining erythrocytes were lysed with fresh lysis buffer for 5 min on ice. After washing, cells were fixed in 1% paraformaldehyde for 10 min on ice, measured with flow cytometry (Gallios, Beckman Coulter Fullerton, CA, USA) and analyzed with Kaluza Flow Analysis Software Version 2.1 (Beckman Coulter). Monocytes were defined as $CD11b^{+}Ly\text{-}6G^{-}$.

Statistics

BiKE transcriptomic dataset analyses were performed with GraphPad Prism 6 and Bioconductor software using a linear regression model adjusted for age and gender and a two-sided Student's t-test assuming non-equal deviation, with correction for multiple comparisons according to Bonferroni, as previously described[16]. Data is presented as mean ± SD and adjusted $p<0.05$ was considered to indicate statistical significance.

Prior to protein data analyses, we applied a Box-Cox transformation on the proteins to improve normality, symmetry and to maintain all protein variables on a similar scale[21]. For protein to protein correlation we used linear regression analysis. Given consistency in terms of sample handling including time from blood draw to processing, same personnel handling all specimens and the ethnic homogeneity of the Icelandic population we adjusted only for age and sex in all our regression analyses.

Mouse data analyses were performed with GraphPad Prism 7.04 and IBM SPSS v25.0. Data are presented as mean ± SD. Normally (Gaussian) distributed mouse parameters were analyzed with a t-test or one-way ANOVA and not normally distributed mouse parameters with a Kruskal-Wallis test followed by a Mann-Whitney U test if significant. A significant difference between the 16 week 10 and 16 week 30 µg/kg/day groups was considered as a dose-dependent difference. The rejection criteria were adjusted using a Bonferroni-Holm correction. Correlation between plaque size and $Ly\text{-}6C^{High}$ monocytes was tested with a Pearson correlation. A two-tailed p-value of 0.05 was regarded statistically significant in all analyses.

Cox proportional hazards regression was used for post incident CHD and Kaplan-Meier plots were applied to display survival data.

RESULTS

mRNAs coding for *OSM*, *OSMR* and *LIFR* are present in human atherosclerotic plaques

To explore if OSM signaling can be involved in human plaque development, we first investigated if *OSM* mRNA and the mRNAs for the receptors for OSM, *OSMR* and *LIFR*, were present in late-stage human carotid plaques from the BiKE study. Gene expression analysis revealed presence of *OSMR, LIFR* and *OSM* mRNAs at low to moderate levels. mRNA expression of both receptors was significantly downregulated in plaques ($p<0.0001$)

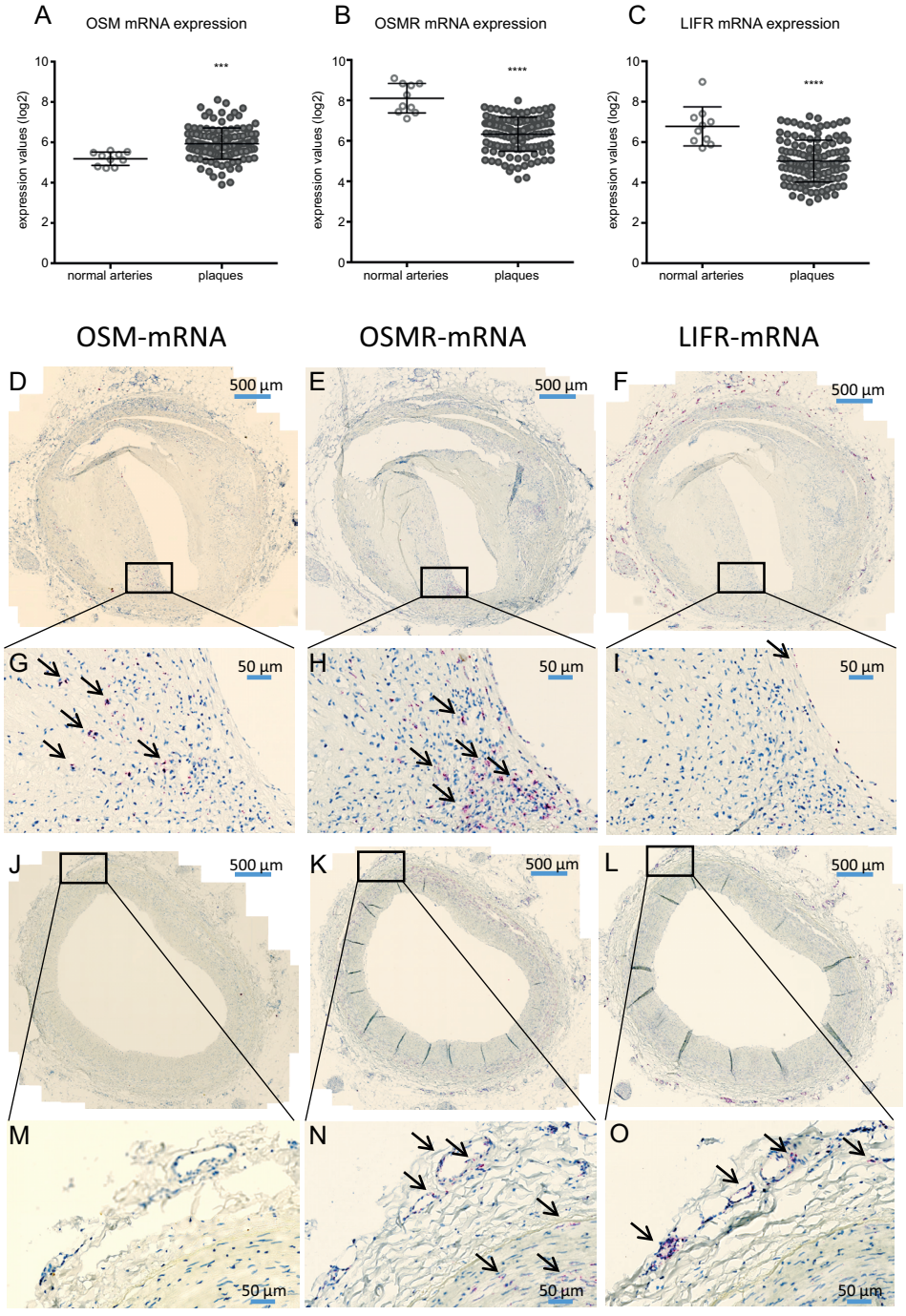


Figure 1. *OSM*, *OSMR* and *LIFR* mRNA expression is present in human atherosclerotic plaques.
mRNA expression was measured in normal arteries and in carotid plaques by microarray analysis (A-C) and ►►

◀◀ ISH was used to visualize *OSM, OSMR* and *LIFR* mRNA expression (red spots and shown by the black arrows) in two different stages of atherosclerosis development, the late fibroatheroma (D-I) and intimal xanthoma (J-O). A two-sided Student's t-test assuming non-equal deviation was used to test for significance between normal and atherosclerotic arteries. ***p<0.001, ****p<0.0001

compared to normal arteries, while *OSM* expression was significantly increased (p=0.003) (Figure 1A-C). *OSM* mRNA expression positively correlated with macrophage markers and negatively with SMC markers (S1 Table). Subsequent *in situ* hybridization confirmed the presence of *OSMR, LIFR* and *OSM* mRNAs in all investigated atherosclerotic plaque stages (Figure 1D-O), which is reflected in S2 Table.

OSM is associated with endothelial activation markers IL-6 and VCAM-1 in humans

We previously found that OSM induces endothelial activation both *in vitro* in human endothelial cells and *in vivo* in APOE*3Leiden.CETP mice[10]. To investigate if OSM can be linked with markers of endothelial activation in a human setting as well, we measured serum levels of OSM and several circulating endothelial activation markers in the AGES-Reykjavik study. OSM levels modestly correlated with IL-6 ($\beta = 0.210$, $p = 5x10^{-56}$) and VCAM-1 ($\beta = 0.130$, $p = 4x10^{-20}$) levels, but inversely with P-Selectin ($\beta = -0.115$, $p = 5x10^{-17}$), E-Selectin ($\beta = -0.092$, $p = 2x10^{-11}$) and ICAM-1 ($\beta = -0.013$, $p = 5x10^{-7}$) levels (Figure 2). No correlation of OSM with MCP-1 was observed.

Chronic exposure to OSM results in a pro-inflammatory vascular phenotype in APOE*3Leiden.CETP mice

The above and our previous data[10] suggest a role for OSM in atherosclerosis development. Therefore, we performed a long-term study in which we administered OSM to APOE*3Leiden.CETP mice for 16 weeks. At t=0, no difference in body weight was observed between the groups, but at t=16, APOE*3Leiden.CETP mice treated with 30 μg/kg/day OSM for 16 weeks had a higher body weight than mice in the control group (p=0.007) and mice treated with 10 μg/kg/day OSM for 16 weeks (p=0.007). No difference in food intake was observed between the different groups. To specifically investigate the effect of OSM on the initiation of atherosclerosis, we added an initial priming group that was treated with OSM only for the first 5.5 weeks of the study. As previous studies had a much shorter duration (ranging from 6 hours to 3 weeks), we first investigated if long-term OSM treatment persistently causes an inflammatory phenotype by measuring E-selectin, MCP-1 and Serum amyloid A (SAA) plasma levels, as markers of vessel wall, general and liver-derived inflammation. Treatment groups receiving either 10 μg/kg/day ($p \leq 0.002$) or 30 μg/kg/day ($p < 0.001$) OSM for 16 weeks showed markedly increased E-selectin levels at all time points and a dose-dependent increase at t=4 ($p < 0.01$) and 8 weeks ($p < 0.01$). The group receiving 5.5 weeks 30 μg/kg/day OSM treatment also showed markedly increased E-selectin levels at t=4 ($p < 0.001$), though after discontinuation of OSM treatment, E-selectin levels dropped and declined to similar levels as the control group. MCP-1 and SAA levels did not differ between the OSM treated groups and control (Figure 3A-C). Also, no statistical difference was observed in ICAM-1 expression at the endothelium in the aortic root area (Figure 3D). In contrast,

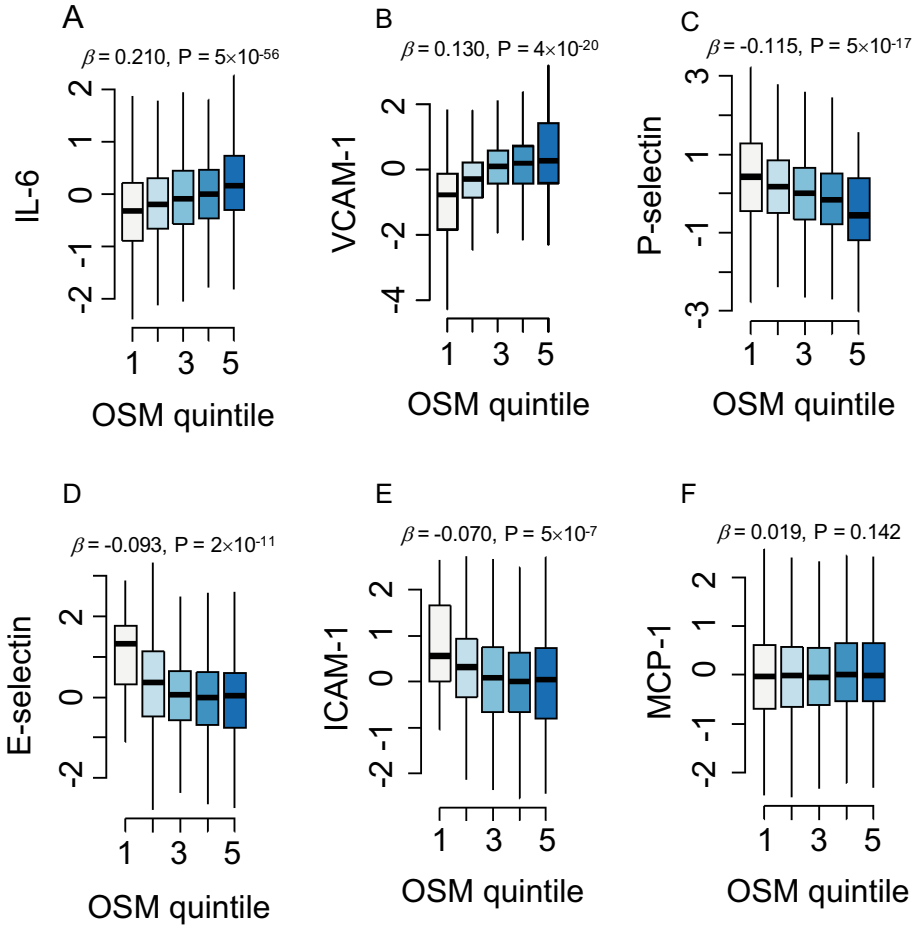


Figure 2. OSM is associated with endothelial activation markers.
Association of serum IL-6 (A), VCAM-1 (B), P-selectin (C), E-selectin (D), ICAM-1 (E) and MCP-1 (F) levels (y-axis) with quintiles of increasing OSM serum levels (x-axis) using specific aptamers measured in 5457 subjects of the AGES cohort. Linear regression analyses were used to test for association.

monocyte adhesion, as functional marker of endothelial activation, in the aortic root area was increased from 4.9 ± 3.3 monocytes per cross-section in the control group to 17.9 ± 10.7 in the 16 weeks 30 µg/kg/day group (p=0.003) (Figure 3E). These results indicate that continuous OSM exposure results in a sustained pro-inflammatory vascular phenotype, even after 16 weeks of treatment.

OSM reduces atherosclerotic lesion area and severity in APOE*3Leiden.CETP mice

Total plasma cholesterol levels, a risk factor for cardiovascular disease, did not differ between any of the groups (S1 Figure). Atherosclerotic lesion size and severity were investigated in the aortic root area of which representative pictures are shown in Figure 4.

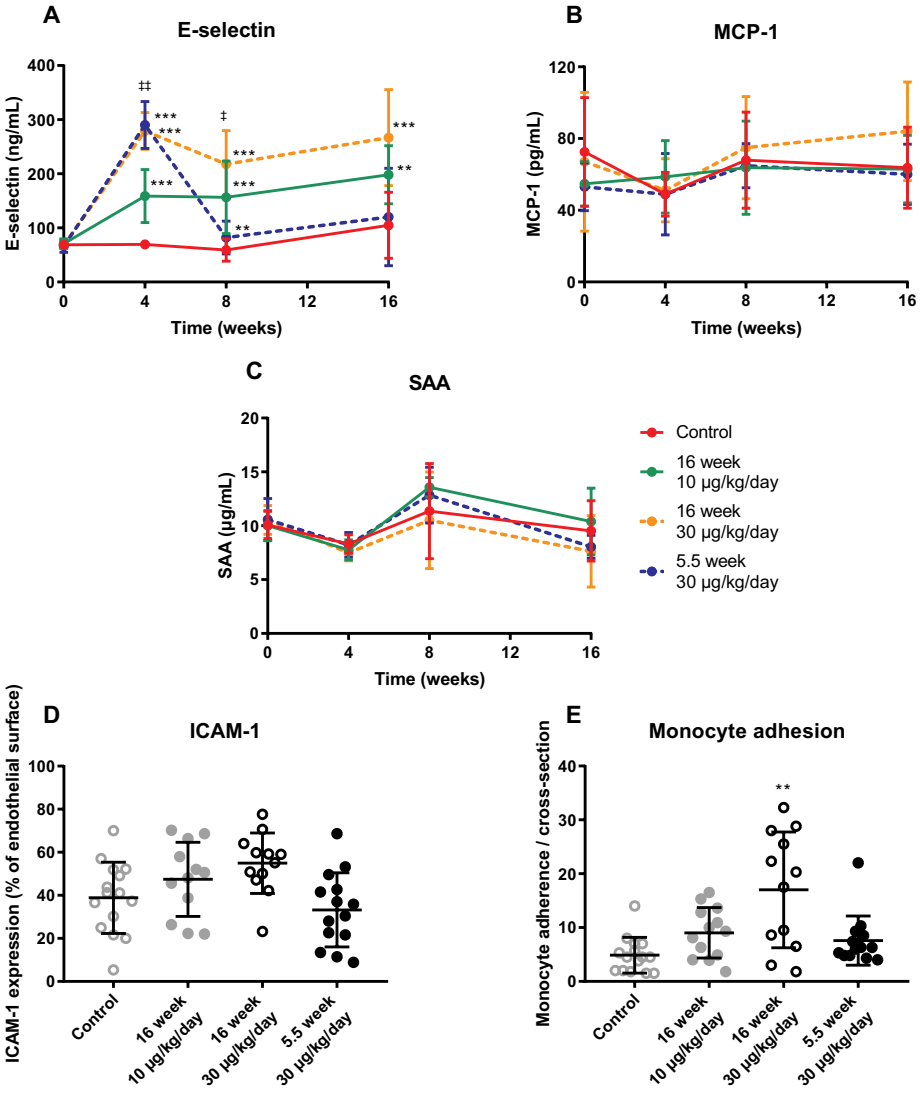


Figure 3. OSM induces a pro-inflammatory vascular phenotype in APOE*3Leiden.CETP mice.
Plasma E-selectin, MCP-1 and SAA (A-C) were measured at multiple time points during the study. Monocyte adhesion (D) and endothelial ICAM-1 expression were assessed per cross-section in the aortic root area (E). Data represent mean ± SD (n=12-20). The Kruskal-Wallis test was used to test for overall significance. If significant, the Mann-Whitney U test was performed to test which treatment groups were significantly different from the control group. Except for monocyte adhesion, which was tested with a t-test. Rejection criteria were adjusted using a Bonferroni-Holm correction. ‡ p<0.05 compared to 10 µg/kg/day; **p<0.01 compared to control; ‡‡ p<0.01 compared to 10 µg/kg/day; ***p<0.001 compared to control.

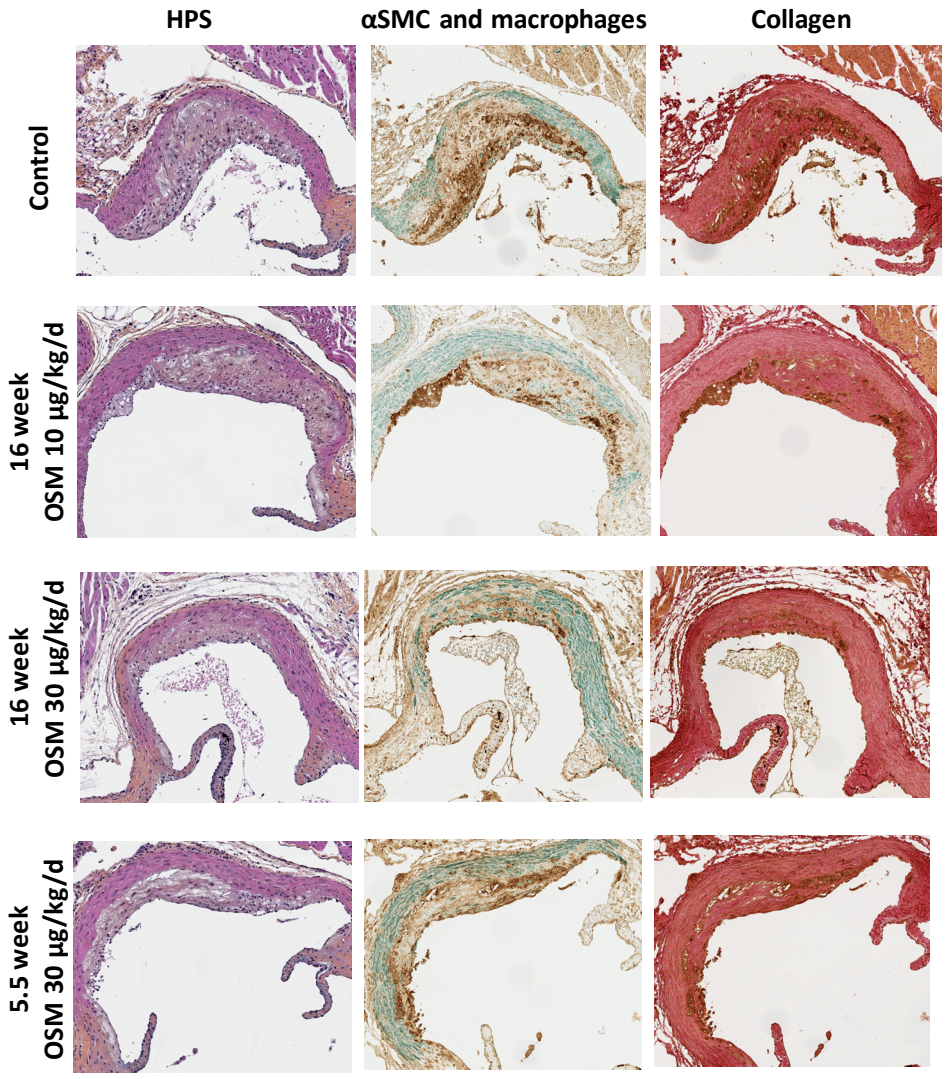


Figure 4. Effect of OSM on plaque composition in APOE*3Leiden.CETP mice.
Representative pictures showing severe lesion types (type IV and V) stained with HPS staining, SMC staining (green), macrophage staining (brown) and collagen staining (red) to determine the effect of OSM on the lesion composition.

The control group had an average lesion size of 119 ± 64 *1000 µm^2. In the 5.5 week 30 µg/kg/day OSM group, plaque size was reduced by 59% (p=0.002) and in the 16 week 30 µg/kg/day OSM group by 58% (p=0.002), while the 16 week 10 µg/kg/day OSM treated group did not differ from the control (Figure 5A). The decrease in plaque area was dose-dependent (p=0.006). In the control group, 62 ± 27% of the lesions were classified as severe lesions, while only 23 ± 22% (p=0.001) and 26 ± 24% (p=0.002) of the lesions were severe in the 16

week 30 µg/kg/day and 5.5 week 30 µg/kg/day OSM treated group, respectively. Again, the 16 week 10 µg/kg/day OSM treatment group did not differ from the control group. In line with plaque area, we observed a dose-dependent decrease in lesion severity (p=0.003) (Figure 5B). Collectively, these results show that early continuous exposure to OSM reduces atherosclerotic lesion size and severity independently from plasma cholesterol in APOE*3Leiden.CETP mice.

OSM has no effect on the stability of severe lesions in APOE*3Leiden.CETP mice

To assess the effect of OSM treatment on plaque stability of the severe lesions, we determined the amount of necrosis and macrophages, as indicators of unstable plaques and the amount of SMCs and collagen, as indicators of stable plaques (Figure 5C) in the severe lesions. Lesions in the control group consisted of 6 ± 3% necrosis, 37 ± 18% macrophages, 5 ± 2% SMCs and 38 ± 10% collagen. The amount of necrosis was decreased to 3 ± 1% in the 5.5 week 30 µg/kg/day OSM group (p=0.012) and to 2 ± 1% in the 16 week 30 µg/kg/day OSM group (p=0.01), while the macrophage content was slightly increased in the 16 week 30 µg/kg/day OSM group (55 ± 10%) (p=0.016) only. The collagen content was decreased in the 5.5 week 30 µg/kg/day OSM group to 28 ± 17% (p=0.012) and to 27 ± 13% in the 16 week 30 µg/kg/day OSM group (p=0.018). No difference was observed in SMC content. The plaque composition of the 16 week 10 µg/kg/day OSM group was similar as in the control group. No differences were observed in the plaque stability ratio between the control and OSM treated groups (Figure 5D). As the amount of macrophages is not necessarily a measure for macrophage activity, we measured the expression of the caspase-1-activating inflammasome protein NLRP3 as marker of macrophage activation[26]. No significant difference was observed in NLRP3 expression in the plaque area (Figure 5E). In conclusion, although OSM does affect lesion composition by slightly increasing the amount of macrophages and decreasing the amount of necrosis and collagen, it does not affect overall plaque stability of the severe lesions.

OSM reduces the inflammatory Ly-6C^{High} monocyte subset

No difference in the percentage of circulating CD11b^{+} cells or CD11b^{+}Ly-6G^{-} cells was observed between the groups (Figure 6A, 6B). As the Ly-6C^{High} monocyte subset is linked to atherosclerosis development[27], we investigated the effect of OSM on the circulating monocyte subtype composition (S2 Figure). In the control group 20.8 ± 6.5% of the monocytes belonged to the Ly-6C^{High} subset and 79.2 ± 6.5% to the Ly-6C$^{Low+Intermediate}$ subset. The amount of Ly-6C^{High} monocytes was decreased to 13.2 ± 3.8% in the 16 week 30 µg/kg/day OSM group (p=0.004) and the amount of Ly-6C$^{Low+Intermediate}$ monocytes increased to 86.8 ± 3.8% (p=0.004) (Figure 6C, D). The Ly-6C^{High} subset showed a positive correlation with lesion size (r=0.303, p=0.029), supporting a role of the Ly-6C^{High} monocytes in the development of atherosclerosis (Figure 6E). Thus, OSM decreases the percentage of Ly-6C^{High} monocytes which may contribute to the smaller atherosclerotic lesion size.

Serum OSM levels are associated with increased post incident CHD in humans

We next explored if variable levels of OSM in the human circulation were associated with

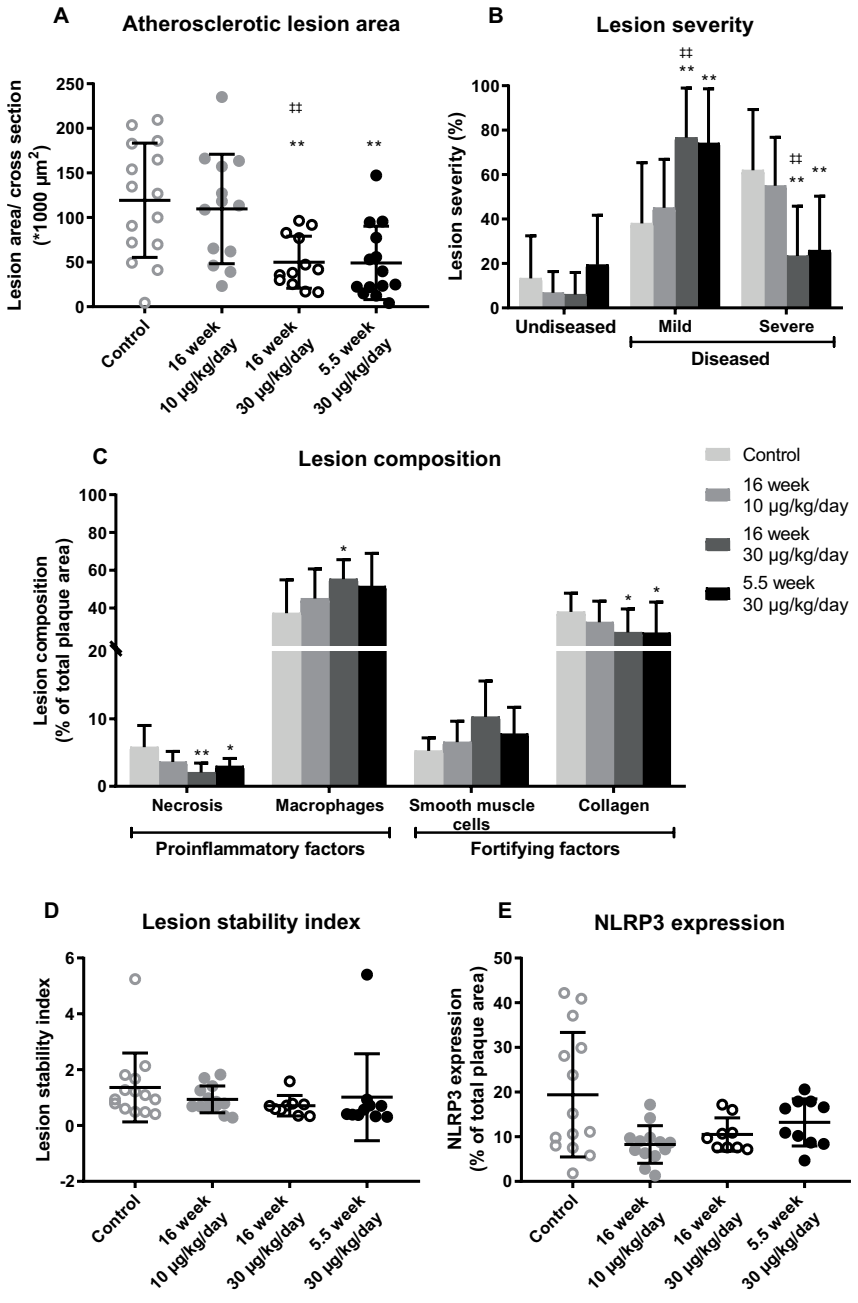


Figure 5. OSM reduces lesion size and severity in APOE*3Leiden.CETP mice.
The atherosclerotic lesion size was determined in the aortic root area (A) and the lesions were classified as mild (type I-III) or severe (IV and V) lesions (B). Furthermore, the amount of necrosis, macrophages, smooth muscle cells and collagen was quantified (C) and the lesion stability index was calculated by dividing the summed proportions of SMCs and collagen, as stabilizing factors, by the summed proportions of necrosis and macrophages, as destabilizing factors (D). Additionally, the amount of NLRP3 expression was examined as ►►

◀◀ percentage of the macrophage area (E). Data represent mean ± SD (n=9-15). The t-test was used for statistical testing of the lesion area. The Kruskal-Wallis test was used to test all other parameters for overall significance. If significant, the Mann-Whitney U test was performed to test which treatment groups were significantly different from the control group. The rejection criteria were adjusted using a Bonferroni-Holm correction *p<0.05 **p<0.01 compared to control; ‡‡ p<0.01 compared to 10 µg/kg/day.

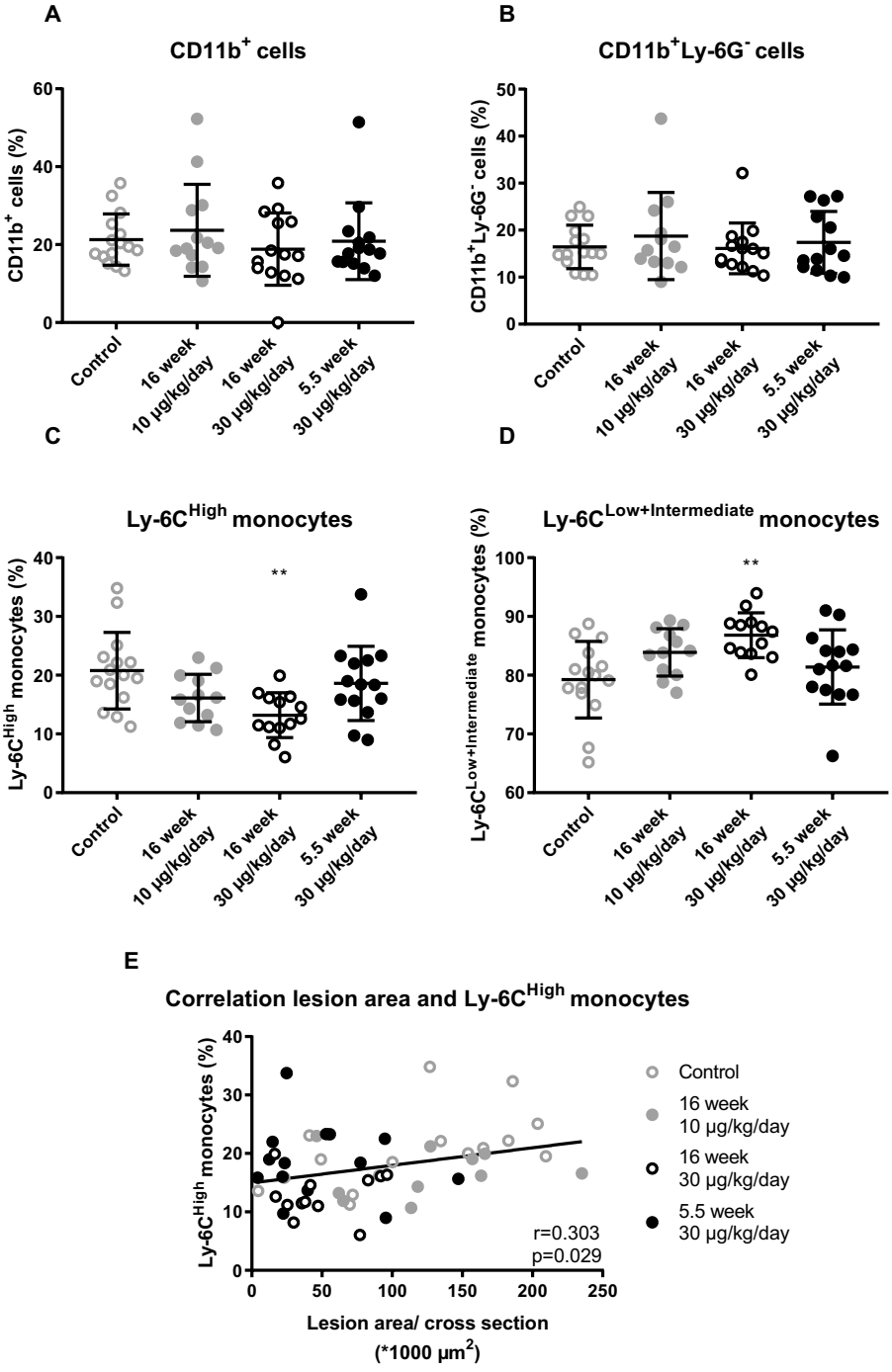
survival probability in the AGES-Reykjavik study. We found that higher serum OSM levels were associated with increased survival probability post incident CHD (HR=0.838, $p=2\times10^{-6}$) (Figure 7A), also using adjusted survival curves for the Cox model[28] (Figure 7B). Thus, elevated levels of OSM predicted reduced mortality in humans.

DISCUSSION

In the present study, we showed that mRNAs coding for *OSM* as well as its receptors*, OSMR* and *LIFR,* were expressed in human normal arteries and carotid atherosclerotic plaques. We demonstrated that serum OSM levels in humans were positively correlated with several but not with other well-known markers of endothelial activation. Chronic OSM administration to APOE*3Leiden.CETP mice reduced atherosclerotic lesion size and severity even after initial priming. In line with these data, increased OSM levels in humans were associated with decreased post incident CHD mortality.

Extending the previous finding by Albasanz-Puig *et al*[13]*,* who showed that OSM is present in both human and murine atherosclerotic plaques, we here demonstrated the presence of *OSMR* and *LIFR* mRNA in human normal and atherosclerotic arteries as well. The relatively higher *OSMR* and *LIFR* expression in normal arteries compared to atherosclerotic arteries may be explained by the high expression of the receptors on endothelial and vascular SMCs[8,29]. These cells make up a relatively large proportion of the normal artery, but less of the atherosclerotic plaque, in which there is influx and proliferation of inflammatory cells, which might dilute *OSMR* and *LIFR* expression. The opposite can be reasoned for the increased *OSM* expression in atherosclerotic arteries, as OSM is mainly produced by activated macrophages and neutrophils[5,6,30]. Moreover, *OSMR* and *LIFR* expression may be downregulated in endothelial and SMCs in plaques compared to endothelial and SMCs in normal arteries. Besides, the chronic inflammatory state during atherosclerosis development drives vascular SMC differentiation, which reduces the expression of SMC specific markers[31] and may therefore also reduce expression of *LIFR* and *OSMR*. This contention is in line with our observation that OSM is negatively correlated with SMC markers and with Kakutani *et al.*, who showed that OSM induces SMC differentiation[4].

The correlation of OSM with IL-6 and VCAM-1 in the AGES-Reykjavik study is in line with previous findings *in vitro*[10]. However, the inverse association of OSM with E-selectin and ICAM-1 contradicts with previous data showing increased levels induced by OSM in human endothelial cells *in vitro*[10] and increased serum E-selectin levels in APOE*3Leiden.CETP mice. The absence of a positive correlation between OSM and ICAM-1, E-selectin and P-selectin may be caused by statin use in the AGES-Reykjavik study (approx. 22%)[21], as statins reduce


A
CD11b+ cells
CD11b+ cells (%)
60
40
20
0
Control
16 week 10 µg/kg/day
16 week 30 µg/kg/day
5.5 week 30 µg/kg/day
B
CD11b+Ly-6G- cells
CD11b+Ly-6G- cells (%)
50
40
30
20
10
0
Control
16 week 10 µg/kg/day
16 week 30 µg/kg/day
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C
Ly-6CHigh monocytes
Ly-6CHigh monocytes (%)
40
30
20
10
0
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Control
16 week 10 µg/kg/day
16 week 30 µg/kg/day
5.5 week 30 µg/kg/day
D
Ly-6CLow+Intermediate monocytes
Ly-6CLow+Intermediate monocytes (%)
100
90
80
70
60
**
Control
16 week 10 µg/kg/day
16 week 30 µg/kg/day
5.5 week 30 µg/kg/day
E
Correlation lesion area and Ly-6CHigh monocytes
Ly-6CHigh monocytes (%)
40
30
20
10
0
r=0.303
p=0.029
0
50
100
150
200
250
Lesion area/ cross section
(*1000 µm2)
Control
16 week 10 µg/kg/day
16 week 30 µg/kg/day
5.5 week 30 µg/kg/day

◀◀ Figure 6. OSM reduces the percentage of circulating Ly-6C^{High} monocytes.
No difference in percentage of CD11b^{+} cells (A) or CD11b^{+}Ly-6G^{-} cells (B) was observed between the groups. But, ApoE*3Leiden.CETP mice treated with OSM have a higher percentage of circulating Ly-6C^{High} monocytes (C) and a lower percentage of circulating Ly-6C$^{Low+Intermediate}$ monocytes (D). The percentage of Ly-6C^{High} monocytes was correlated with an increased lesion size (E). Data represent mean ± SD (n=12-20). One-way ANOVA with Dunnett's correction was used to test for significant differences between treatment groups and control. Pearson correlation was used to test the correlation between lesion size and Ly-6C^{High} monocytes. **p<0.01 compared to control.

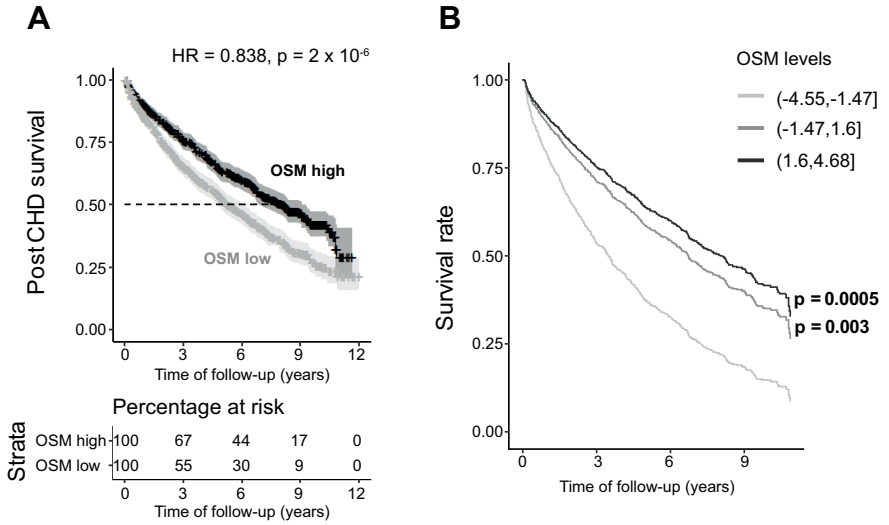

Figure 7. High OSM is associated with reduced post CHD mortality.
Serum OSM levels of CHD patients were significantly associated with CHD related mortality rates when comparing the lower 25% quantile to the upper 75% quantile in OSM levels (hazard ratio (HR)=0.838, p=2×10^{-6}) (A), and in the adjusted survival curves for the Cox model for three groups of OSM protein levels (top vs. bottom HR=0.618, p=0.0005) (B). Cox proportional hazards regression was performed to test for statistical significance and Kaplan-Meier plots were applied to display the survival data.

ICAM-1, E-selectin and P-selectin plasma levels in patients with coronary artery disease[32]. Regardless, mice treated with OSM in the present study did show increased serum E-selectin levels which dropped after discontinuation of OSM treatment, indicating a causal relationship between OSM and E-selectin *in vivo* in mice.

As our present study had a much longer duration than previous intervention studies with OSM in mice[9,10], we first verified if the previously observed short-term inflammatory state[10] is also present after 16 weeks of OSM administration. OSM increased plasma E-selectin levels and monocyte adhesion in the aortic root area, similarly as in our previous study[10], indicating that OSM induces a sustained inflammatory state even after long-term OSM treatment. Although inflammation has been reported to contribute to atherosclerosis development[33], our results show, to our knowledge for the first time, that long-term chronic OSM treatment independently of cholesterol-lowering, results in significantly smaller and less severe atherosclerotic lesions in APOE*3Leiden.CETP mice, clearly indicating that

prolonged exposure to OSM has anti-atherogenic effects. Previously, Zhang *et al.,* using a different approach, showed that OSMR deficient ApoE$^{-/-}$ mice have smaller and more stable plaques than their OSMR expressing littermates[14], suggesting that signaling via the LIFR alone or prevention of IL-31 and OSM signaling through OSMR[34] has a similar beneficial effect.

No difference was observed in the lesion stability index, and although we observed a slight increase in the amount of macrophages as percentage of the total plaque area, the amount of NLRP3 expression was very low and did not differ between any of the groups, indicating that the pro-inflammatory macrophage activity was not affected[26]. In line with this, the percentage of pro-inflammatory Ly-6C^{High} monocytes[35] was decreased and the percentage of non-inflammatory Ly-6C$^{Low+Intermediate}$ monocytes, which actively patrol the luminal site of the endothelium where they remove debris and damaged cells and are associated with reparative processes[35], was increased in OSM treated mice. The decrease in Ly-6C^{High} monocytes plausibly contributes to the attenuated atherosclerosis development.

Although our findings are counter-intuitive with several previously described pro-inflammatory characteristics of OSM[9,36], they are in line with studies addressing the anti-inflammatory properties of OSM. It has been shown that OSM administration suppresses TNFα[37] and IL-1β release *in vitro*[38], whereas TNFα, IL-1β and IFN-γ expression is increased in adipose tissue of OSMR knockout mice[39]. Both cytokines are involved in atherosclerosis progression in mice as TNFα promotes atherosclerosis[40] and IL-1β knockout mice have smaller and less severe atherosclerotic lesions[41]. In humans, anti-inflammatory treatments targeting TNFα or IL-1β are associated with decreased risk of myocardial infarction and overall cardiovascular events[42,43]. Collectively, these and our data indicate that OSM has anti-inflammatory effects as well which may contribute to its anti-atherogenic properties. Moreover, OSM has been reported to induce endothelial proliferation[12,44] and to increase expression of adhesion molecules that bind endothelial progenitor cells[45,46], suggesting that OSM stimulates replacement of leaky, dysfunctional endothelial cells by new and healthy endothelial cells[47] and may therefore attenuate atherogenesis in the initial stages of the disease. This contention is in line with our finding that mice treated with OSM for only 5.5 weeks had a similar lesion size and severity as mice receiving OSM during a 16 week period and suggests that the observed anti-atherogenic effects of OSM have taken place during the initial stages of atherosclerosis development. Furthermore, although the observed increase in SMCs observed in this study was not significant, others have reported that OSM significantly enhances SMC proliferation *in vitro*[13], which is a contributor to a stable plaque phenotype[48]. Finally, the anti-atherogenic effects of OSM that were observed in this study might be caused by OSM-induced alterations in estrus cycling. In human breast cancer cell lines, OSM was shown to suppress oestrogen receptor-α expression[49], which may affect atherosclerosis development as estrogens increase VLDL production[50], and might thereby contribute to an atherogenic phenotype[51]. However, no changes in total cholesterol and increased triglyceride levels were observed in OSM treated mice (S1 Figure), making this possibility unlikely. Since OSM affects many different cell types and the mice in this study received OSM systemically, it is important to note that the observed effects on atherosclerosis development could also be caused by indirect effects of OSM. To conclude, OSM may

contribute to attenuation of plaque development and improvement of plaque severity by: (1) its anti-inflammatory properties, (2) regenerating the endothelial barrier, (3) induction of SMC proliferation, (4) reducing the pro-inflammatory monocyte phenotype and promoting a more regenerative phenotype[48] and (5) indirectly by asserting its effects on other cell types that are involved in atherosclerosis development.

The anti-atherogenic effect of OSM in APOE*3Leiden.CETP mice is consistent with the increased post incident CHD survival probability in humans with higher OSM levels in the AGES-Reykjavik study. Similarly, OSM treatment increased survival in a mouse injury model of acute myocardial infarction[52], emphasizing the regenerative properties of this cytokine[44,53]. As OSM has been suggested to have a progressive effect in chronic inflammatory diseases such as, RA[54] and inflammatory bowel disease[36,55], it has been proposed as a possible pharmaceutical target to suppress inflammation in these diseases[36,54,55] and the effect of anti-OSM treatment in RA has already been investigated in a phase 2 clinical trial[54]. However, considering the anti-atherogenic effects and positive effect of OSM on survival in the present study, we strongly recommend that cardiovascular disease markers and survival are carefully monitored when testing an OSM inhibiting approach. In addition, since this study shows that OSM has beneficial immune modulating effects, the role of OSM in inflammatory diseases possibly needs to be reconsidered.

Taken together, our study provides more insight into the role of OSM in atherosclerosis development. APOE*3Leiden.CETP mice treated with OSM had smaller and less severe plaques associated with a decrease in pro-inflammatory Ly-6C^{High} monocytes. In line with the favorable effect in mice, we found an increased survival probability in humans that have high OSM levels, suggesting an atheroprotective effect for OSM.

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S1 Table. Correlation between OSM and genes of interest in plaques. Pearson correlation analyses were calculated from n=127 human plaque microarrays, p-values are corrected for multiple comparisons according to the Bonferroni method. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Correlation considered weak if r < 0.3 moderate if 0.3 < r < 0.5 and strong if r > 0.5.

	Gene symbol	Pearson r	p-value	Significance level
Cell type markers				
Smooth muscle cells				
Myosin heavy chain 11	*MYH11*	-0.4327	< 0.0001	****
Smoothelin	*SMTN*	-0.4437	< 0.0001	****
Alpha smooth muscle actin	*ACTA2*	-0.3476	< 0.0001	****
Myocardin	*MYOCD*	-0.4119	< 0.0001	****
Transgelin	*TAGLN*	-0.3127	0.0004	***
Endothelial cells				
von Willebrand factor	*VWF*	0.1486	0.0967	ns
Pecam-1 (CD31)	*PECAM1*	0.3009	0.0006	***
Dendritic cells				
Itgax (CD11c)	*ITGAX*	0.4738	< 0.0001	****
Ly75 (CD205)	*LY75*	-0.03098	0.7295	ns
CD80	*CD80*	0.6013	< 0.0001	****
T Lymphocytes				
CD11b	*ITGAM*	0.4048	< 0.0001	****
ITGAL	*ITGAL*	0.5012	< 0.0001	****
CD27	*CD27*	0.107	0.233	ns
CD28	*CD28*	0.2859	0.0012	**
CD3 delta	*CD3D*	0.3678	< 0.0001	****
CD4	*CD4*	0.1078	0.2295	ns
CD8A	*CD8A*	0.2258	0.0107	*
PTPRC (CD45RA)	*PTPRC*	0.3758	< 0.0001	****
CD69	*CD69*	0.4909	< 0.0001	****
ITGAE	*ITGAE*	0.2827	0.0013	**
FABP4	*FABP4*	0.3884	< 0.0001	****
Macrophages				
CD83	*CD83*	0.5474	< 0.0001	****
CD86	*CD86*	0.4934	< 0.0001	****
CD163	*CD163*	0.4434	< 0.0001	****
TNFRSF9	*TNFRSF9*	0.3696	< 0.0001	****
CD40	*CD40*	0.3422	< 0.0001	****
CD36	*CD36*	0.4466	< 0.0001	****
Inflammation / Apoptosis / Calcification markers				
IL-1beta	*IL1B*	0.5657	< 0.0001	****
NFkB	*NFKB1*	0.1764	0.0481	*
MCP-1	*CCL2*	0.5311	< 0.0001	****
Caspase-3	*CASP3*	0.2726	0.002	**
Caspase-7	*CASP7*	0.05738	0.5233	ns
Caspase-9	*CASP9*	0.2318	0.009	**
BCL2	*BCL2*	0.2761	0.0018	**
RANTES	*CCL5*	0.3821	< 0.0001	****
BMP4	*BMP4*	-0.1434	0.1091	ns

S1 Table continued

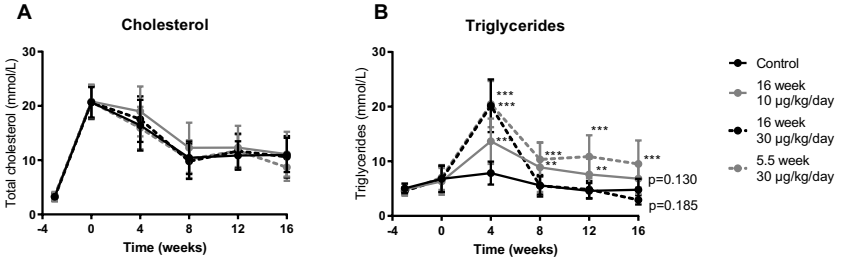
	Gene symbol	Pearson r	p-value	Significance level
Extracellular matrix/ degradation				
MMP9	*MMP9*	0.4202	< 0.0001	****
TIMP1	*TIMP1*	0.3891	< 0.0001	****
Growth factors				
TGFB1	*TGFB1*	0.4113	< 0.0001	****
TGFA	*TGFA*	0.328	0.0002	***
IGF1	*IGF1*	0.256	0.0038	**
PDGFA	*PDGFA*	-0.02346	0.7943	ns
PDGFB	*PDGFB*	0.2417	0.0064	**
PDGFC	*PDGFC*	-0.2382	0.0072	**
PDGFD	*PDGFD*	-0.2889	0.001	**
Chemokines and receptors				
Interferon gamma	*IFNG*	0.2032	0.0225	*
IL2	*IL2*	0.2446	0.0058	**
IL4	*IL4*	0.03414	0.7043	ns
IL5	*IL5*	0.1947	0.0289	*
IL6	*IL6*	0.5659	< 0.0001	****
IL9	*IL9*	0.05453	0.5442	ns
IL10	*IL10*	0.4213	< 0.0001	****

S2 Table. Quantification of ISH signal in various atherosclerotic plaque stages. The amount of ISH signal was scored in various atherosclerotic plaque stages. A general score and a single cell score was given. 0 = No signal, 1 = Few cells expressing mRNA, 2 = Low expression, 3 = Moderate expression and 4 = High expression.

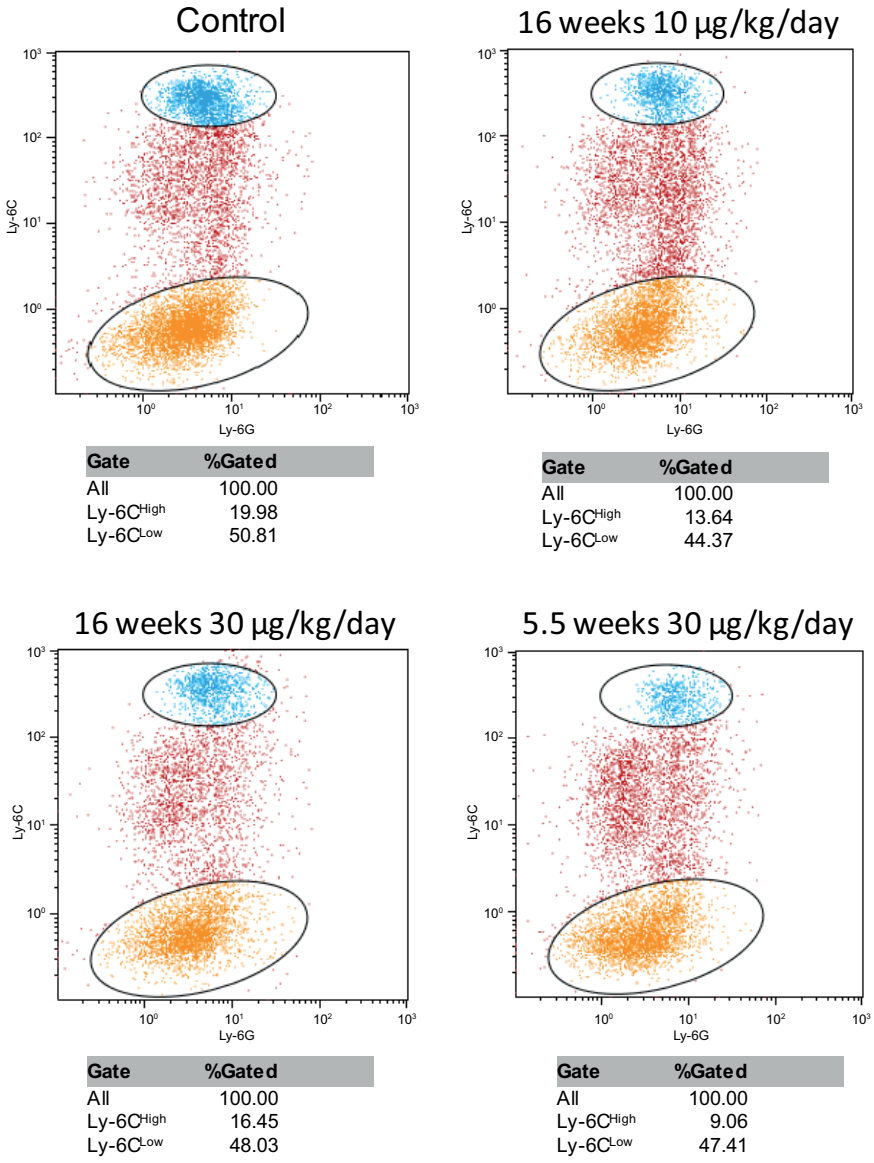
		mRNA expression		
		OSM	**OSMR**	**LIFR**
Adaptive Intimal Thickening	Neo-intima	1	2	2
	Media	1	3	2
	Adventitia	1	3	3
Intimal Xanthoma	Neo-intima	1	2	2
	Media	0	2	3
	Adventitia	0	2	3
Pathological Intimal Thickening	Neo-intima	1	2	2
	Media	1	3	2
	Adventitia	1	3	3
Early Fibroatheroma	Neo-intima	2	2	2
	Media	1	2	2
	Adventitia	1	3	3
Late Fibroatheroma	Neo-intima	2	2	2
	Media	2	2	2
	Adventitia	1	2	4
Fibrous Calcified Plaque	Neo-intima	2	2	2
	Media	1	3	2
	Adventitia	1	3	2

S3 Table. Effect of OSM on body weight and food intake.
At t=0, no difference in body weight was observed between the groups. At t=16, APOE*3Leiden.CETP mice treated with 30 µg/kg/day OSM for 16 weeks had a higher body weight than mice in the control group and mice treated with 10 µg/kg/day OSM for 16 weeks. No difference in food intake was observed between the different groups. Body weight at t=0 was normally distributed and therefore analyzed with a One-way ANOVA, while body weight at t=16 was not normally distributed and therefore analyzed with the Kruskal-Wallis test with subsequent Mann-Whitney U tests to test which groups were significantly different from the control group and to test if there was a dose-dependent effect. Food intake was measured with the Kruskal-Wallis test as too little data points were available to evaluate the distribution of the data. The rejection criteria were adjusted using a Bonferroni-Holm correction. **p<0.01 compared to control; ‡‡ p<0.01 compared to 10 µg/kg/day.

Treatment group	Bodyweight t=0 (g)	Bodyweight t=16 (g)	Food intake (g/mouse/day)
Control	21.1 ± 1.2	23.5 ± 1.9	3.0 ± 0.1
16 week 10 µg/kg/day	21.1 ± 1.8	23.3 ± 1.5	3.0 ± 0.1
16 week 30 µg/kg/day	21.1 ± 1.5	24.8 ± 1.2** ‡‡	3.2 ± 0.1
5.5 week 10 µg/kg/day	21.1 ± 1.5	22.9 ± 1.4	3.1 ± 0.1
p-value	0.995	0.007	0.169



S1 Figure. OSM does not affect total plasma cholesterol levels and increases triglyceride levels in APOE*3Leiden.CETP mice.
Total plasma cholesterol (A) and triglyceride (B) levels were measured at multiple time points during the study. Data represent mean ± SD (n=13-20). The Kruskal-Wallis test was used to test for overall significance. If significant, the Mann-Whitney U test was performed to test which treatment groups were significantly different from the control group.



Gate	%Gated
All	100.00
Ly-6C^{High}	19.98
Ly-6C^{Low}	50.81

Gate	%Gated
All	100.00
Ly-6C^{High}	13.64
Ly-6C^{Low}	44.37

Gate	%Gated
All	100.00
Ly-6C^{High}	16.45
Ly-6C^{Low}	48.03

Gate	%Gated
All	100.00
Ly-6C^{High}	9.06
Ly-6C^{Low}	47.41

S2 Figure. Representative pictures of the distribution of the Ly-6C monocyte subsets.
Based on the Ly-6C expression, monocytes were distributed into 3 monocyte subsets, the Ly-6C^{Low}, Ly-6C^{Intermediate} and Ly-6C^{High} monocyte subset.

CHAPTER 4

Common variants in OSMR contribute to carotid plaque vulnerability

In preparation

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ABSTRACT

Background

Oncostatin M (OSM) signaling is implicated in atherosclerosis, however the mechanism remains unclear. We investigated the impact of common genetic variants in *OSM* and its receptors, *OSMR* and *LIFR,* on overall plaque vulnerability (based on macrophage, collagen, smooth muscle cell and fat content) and on seven individual atherosclerotic plaque phenotypes (calcification, collagen, atheroma size, macrophages, smooth muscle cells, vessel density and intraplaque hemorrhage).

Methods and results

We queried Genotype-Tissue Expression (GTEx) data and selected one variant, rs13168867 (C allele), that associated with decreased *OSMR* expression and one variant, rs10491509 (A allele), that associated with increased *LIFR* expression in arterial tissue. No variant was associated to significantly altered *OSM* expression.

We associated these two variants with plaque characteristics from 1,443 genotyped carotid endarterectomy patients in the Athero-Express Biobank Study. The rs13168867 variant in *OSMR* was significantly associated with an increased overall plaque vulnerability ($\beta = 0.118 \pm$ s.e. $= 0.040$, $p = 3.00 \times 10^{-3}$, C allele). With respect to different plaque phenotypes, this variant showed strongest associations with intraplaque fat ($\beta = 0.248 \pm$ s.e. $= 0.088$, $p = 4.66 \times 10^{-3}$, C allele) and collagen content ($\beta = -0.259 \pm$ s.e. $= 0.095$, $p = 6.22 \times 10^{-3}$, C allele). No associations were found for rs10491509 in the *LIFR* locus.

Conclusion

Our study suggests that genetically decreased arterial *OSMR* expression, possibly resulting in decreased OSM signaling, contributes to increased carotid plaque vulnerability.

INTRODUCTION

Oncostatin M (OSM) is an inflammatory cytokine[1] that is released by activated monocytes[2], macrophages[2], T-lymphocytes[3] and neutrophils[4], and mediates its effects through binding to either the glycoprotein (gp) 130/ oncostatin M receptor (OSMR) heterodimer or the gp130/ leukemia inhibitory factor receptor (LIFR) heterodimer[5–7]. Binding of OSM to either of the receptor heterodimers can activate multiple pathways, including the janus kinase (JAK)/ signal transduction and activator of transcription (STAT), the mitogen-activated protein kinase (MAPK), and the Phosphoinositide 3-kinase (PI3K)/AKT pathway[6]. It is suggested that the ratio of the two receptor types expressed on the cell membrane is a potential regulatory mechanism for the multiple and sometimes opposing effects that are exerted by OSM[8]. The cytokine is associated to multiple inflammatory diseases, including chronic periodontitis[5,9], rheumatoid arthritis[10] and inflammatory bowel disease[11].

There are multiple indications, that OSM is involved in atherosclerosis. OSM is present in both murine and human atherosclerotic plaques[12] and $OSMR^{-/-}ApoE^{-/-}$ mice show reduced plaque size and improved plaque stability compared to their *OSMR* expressing littermates[13], indicating that OSM drives atherosclerosis development. To our knowledge, no studies have been performed to investigate the involvement of LIFR in OSM driven atherosclerosis development. However, we previously showed that OSM signals through both receptors simultaneously to induce activation in human endothelial cells, suggesting that also LIFR is involved in atherosclerosis development[14].

Little is known about the effect of OSM on plaque composition. Since OSM affects multiple cell types and processes, it is difficult to predict how OSM contributes to atherosclerotic plaque formation. As OSM promotes angiogenesis[15], endothelial activation[14], vessel permeability[16] and osteoblastic differentiation[17], it hypothetically results in a higher intraplaque microvessel density and intraplaque hemorrhages and plaque calcification, thereby contributing to the formation of a vulnerable plaque[18,19]. On the other hand, OSM also promotes fibroblast proliferation[20], collagen formation[20], smooth muscle cell proliferation[12] and M2 macrophage polarization[21], hypothetically resulting in enhanced fibrosis and attenuates inflammation, thereby contributing to plaque stabilization[22–24].

We aimed to investigate these theorized opposing effects of OSM signaling on the atherosclerotic plaque using data from the Athero-Express Biobank Study, which comprises a large collection of human plaque specimens obtained through carotid endarterectomy[25]. Common genetic variation in gene expression is key to disease susceptibility, and *cis*-acting genetic variants, single-nucleotide polymorphisms (SNPs), have been mapped to expression quantitative trait loci (eQTLs)[26]. Likewise, eQTLs modulate transcriptional regulation of *OSM*, *OSMR*, and *LIFR* in arterial tissues. We hypothesized that eQTLs for these genes, can be used as proxies of gene expression to examine the effect on overall plaque vulnerability[27] and individual plaque characteristics, including collagen, lipid, macrophage and smooth muscle cell content, calcification, and intraplaque microvessel density and hemorrhage.

MATERIALS AND METHODS

Sample collection

The Athero-Express Biobank Study (https://www.atheroexpress.nl) contains plaque material of patients that underwent carotid endarterectomy (CEA) or femoral endarterectomy at two Dutch tertiary referral centers[25]. Details of the study design were described before. Briefly, blood and plaque material were obtained during endarterectomy and stored at -80˚C. Only CEA patients were included in the present study. All patients provided informed consent and the study was approved by the medical ethics committee.

Athero-Express genotyping, quality control, and imputation

Details of genotyping have been previously described[28]. In short, DNA was extracted from EDTA blood or (when no blood was available) plaque samples of 1,858 consecutive patients from the Athero-Express Biobank Study and genotyped in 2 batches. For the Athero-Express Genomics Study 1 (AEGS1) 836 patients, included between 2002 and 2007, were genotyped using the Affymetrix Genome-Wide Human SNP Array 5.0 (SNP5) chip (Affymetrix Inc., Santa Clara, CA, USA). For the Athero-Express Genomics Study 2 (AEGS2) 1,022 patients, included between 2002 and 2013, were genotyped using the Affymetrix Axiom® GW CEU 1 Array (AxM). Both studies were carried out according to OECD standards. After genotype calling, we adhered to community standard quality control and assurance (QCA) procedures of the genotype data from AEGS1 and AEGS2. Samples with low average genotype calling and sex discrepancies (compared to the clinical data available) were excluded. The data was further filtered on 1) individual (sample) call rate > 97%, 2) SNP call rate > 97%, 3) minor allele frequencies (MAF) > 3%, 4) average heterozygosity rate ± 3.0 s.d., 5) relatedness (pi-hat > 0.20), 6) Hardy–Weinberg Equilibrium (HWE $p < 1.0\times10^{-6}$), and 7) population stratification (based on HapMap 2, release 22, b36) by excluding samples deviating more than 6 standard deviations from the average in 5 iterations during principal component analysis and by visual inspection as previously described[28]. After QCA 657 samples and 403,789 SNPs in AEGS1, and 869 samples and 535,983 SNPs in AEGS2 remained. Before phasing using SHAPEIT2[29], data was lifted to genome build b37 using the liftOver tool from UCSC (https://genome.ucsc.edu/cgi-bin/hgLiftOver). Finally, data was imputed with 1000G phase 3[30] and GoNL 5[31] as a reference.

Variant selection

We queried data from the Genotype-Tissue Expression (GTEx) Portal (https://gtexportal.org)[26] for variants that alter *OSM* expression in the blood, and *OSMR* or *LIFR* expression in arterial tissue. We selected common variants with a MAF >3%, which yielded 2 variants in total. We harmonized the effect alleles and effect sizes from these eQTLs to the Athero-Express Biobank Study data.

Plaque phenotyping

The (immuno)histochemical analyses of plaque phenotypes have been described previously[25,28,32]. Briefly, the culprit lesion was identified directly after dissection, fixed in 4%

formaldehyde and embedded in paraffin. The tissue was cut in 5µm sections on a cryotome for (immuno)histochemical analysis by pathology experts. Calcification (hematoxylin & eosin, H&E) and collagen content (picrosirius red) were semi-quantitatively scored and defined as no/minor or moderate/heavy. Atheroma size (H&E and picrosirius red) was defined as <10% or ≥10% fat content. The amount of macrophages (CD68) and smooth muscle cells (ACTA2) were quantitatively scored and classified as percentage of plaque area. The presence of intraplaque hemorrhage (H&E and fibrin) was defined as absent or present, and vessel density was classified as the number of intraplaque vessels (CD34)/ hotspot.

Plaque vulnerability

Assessment of overall plaque vulnerability was performed as previously described[27]. In short, the amount of macrophages and smooth muscle cells were also semi-quantitatively defined as no/minor or moderate/heavy. Each plaque characteristic that defines a stable plaque (i.e. no/minor macrophages, moderate/heavy collagen, moderate/heavy smooth muscle cells and <10% fat) was given a score of 0, while each plaque characteristic that defines a vulnerable plaque (i.e. moderate/heavy macrophages, no/minor collagen, no/minor smooth muscle cells and ≥10% fat) was given a score of 1. The score of each plaque characteristic was summed resulting in a final plaque score ranging from 0 (most stable plaque) to 4 (most vulnerable plaque). Intraobserver and interobserver variability were examined previously and showed good concordance (κ 0.6-0.9)[33].

Statistical analyses

Quantitatively scored characteristics (macrophages, smooth muscle cells, and the vessel density) were Box-Cox transformed[34] to obtain a normal distribution. Association of the common variants with continuous parameters were statistically tested with linear regression and the categorical parameters with logistic regression. Data was corrected for age, sex, genotyping chip, and genetic ancestry using principal components 1 through 4. Correction for multiple testing resulted in a corrected p-value of $p = 0.05/((7 \text{ plaque phenotypes} + \text{plaque vulnerability}) \times 2 \text{ common variants}) = 3.13 \times 10^{-3}$. The power of the study was estimated at ±75% based on a sample size of 1,443, a minor allele frequency (MAF) of 0.409 and a relative risk of 1.28 (http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/, S1 Figure).

RESULTS

Baseline characteristics

A total of 1,443 patients that underwent carotid endarterectomy were genotyped and included in this study. The genotyped groups (AEGS1 and AEGS2) are not overlapping. As we previously showed that the baseline characteristics of both groups are comparable[28], the groups were combined for overall plaque vulnerability and phenotype analyses. Baseline characteristics of the combined groups are shown in Table 1.

Table 1. Baseline characteristics of genotyped CEA patients from the Athero-Express Biobank Study. Cerebrovascular disease history is defined by ischemic stroke prior to surgery. Coronary artery disease history includes coronary artery disease, myocardial infarction, percutaneous coronary intervention, and coronary artery bypass graft. Peripheral disease history includes diagnosed peripheral arterial occlusive disease, femoral artery interventions, and ankle-brachial index <70. Type 2 diabetes mellitus includes all individuals with diagnosed type 2 diabetes mellitus and those on appropriate medication. Hypertension includes all individuals with self-reported hypertension. Current smokers include all individuals smoking up to 6 months until the surgery date. BMI, kg/m^2. eGFR rate was based on the Modification of Diet in Renal Disease formula, mL/min/1.73m^2. Anti-hypertensives include all anti-hypertension medication. Anti-thrombotics include clopidogrel, dipyridamole, acenocoumarin, ascal, and anti-platelet drugs. Missingness shows the percentage of the patients of which we lack information on the specific patient characteristic.

Patient characteristics	AEGS1+AEGS2 n=1,443	Missingness (%)
Sex, male, n (%)	976 (64.0)	5.7
Age in years, mean (SD)	68.84 (9.33)	5.7
History		
Cerebrovascular disease, n (%)	478 (33.2)	5.7
Coronary artery disease, n (%)	430 (29.9)	5.8
Peripheral artery disease, n (%)	297 (20.7)	5.8
Risk factors		
Type 2 diabetes mellitus, n (%)	332 (23.1)	5.7
Hypertension, n (%)	1017 (73.0)	8.7
Current smoker, n (%)	492 (34.9)	7.5
BMI, median [IQR]	26.0 [24.0-28.4]	11.5
eGFR, median [IQR]	72.3 [58.7-85.4]	8.1
Total cholesterol in mmol/L, median [IQR]	4.38 [3.60-5.25]	22.8
LDL in mmol/L, median [IQR]	2.40 [1.81-3.13]	27.8
HDL in mmol/L, median [IQR]	1.06 [0.87-1.30]	25.0
Triglycerides in mmol/L, median [IQR]	1.50 [1.08-2.04]	24.6
Medication		
Antihypertensives, n (%)	1110 (77.2)	5.8
Lipid lowering drugs, n (%)	1112 (77.4)	5.8
Antithrombotics, n (%)	1272 (88.6)	6.0
Symptoms		
Asymptomatics, n (%)	195 (13.6)	6.0
Ocular, n (%)	221 (15.4)	6.0
TIA, n (%)	634 (44.2)	6.0
Stroke, n (%)	384 (26.8)	6.0

Common variants altering *OSM*, *OSMR* and *LIFR* expression

OSM is secreted by neutrophils[4], monocytes[2], macrophages[2] and T-cells[3], and acts through binding to OSMR and LIFR[5–7] in the arterial wall[13,35]. Thus we queried data from the Genotype-Tissue Expression project (GTEx)[26] for SNPs that alter *OSM* expression in whole blood and *LIFR* and *OSMR* expression in arterial tissue. There were no significant eQTLs for *OSM*, but there were two eQTLs that associated with either altered *OSMR* (rs13168867) or *LIFR* (rs10491509) expression in arterial tissue. The C allele of rs13168867 showed the strongest association with decreased *OSMR* expression in the tibial artery (Figure 1A), and the A allele of rs10491509 showed the strongest association with increased *LIFR* expression in the aortic artery (Figure 1B). Cross-tissue meta-analysis showed that these variants have > 0.9 m-values

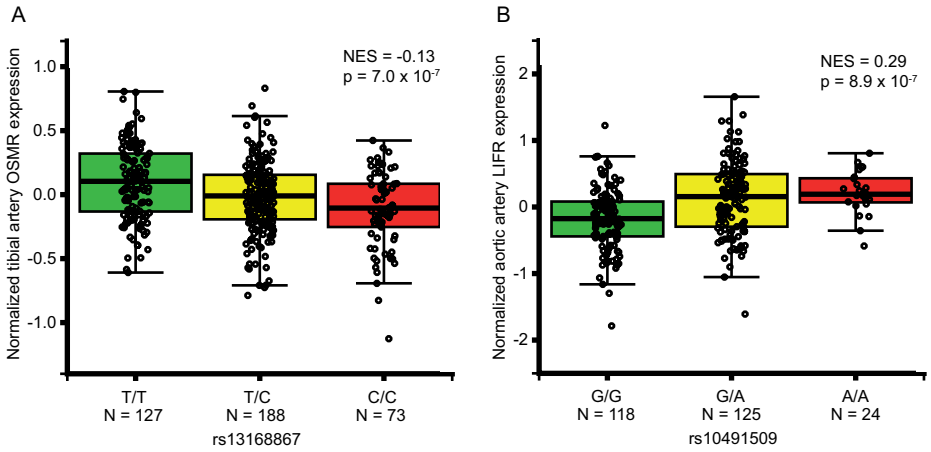

Figure 1. Association of OSMR and LIFR variants in arterial tissues.
Per variant, the normalized expression of *OSMR* (A) and *LIFR* (B) is given in arterial tissue. Data from GTEx Portal (www.gtexportal.org). NES = Normalized effect size. In aortic arterial tissue, rs13168867 had a NES of -0.123 and in tibial arterial tissue, rs10491509 had a NES of 0.0881 (S2 and S3 Figure).

in both tibial and aortic artery tissue, indicating a high probability that they are single *cis*-eQTLs in both tissues (S2 and S3 Figure).

Genetic association with plaque vulnerability

To determine the effect of OSM signaling on the overall plaque vulnerability, we correlated the rs13168867 and rs10491509 genotypes to the overall plaque vulnerability, which was given a score ranging from 0 (least vulnerable plaque) to 4 (most vulnerable plaque). The effect allele of variant rs13168867 in the *OSMR* locus was significantly correlated with an increased overall plaque vulnerability (β = 0.118 ± s.e. = 0.040 (C allele), $p = 3.00 \times 10^{-3}$, Table 2), which is visualized in Figure 2. No association was observed with rs10491509 and overall plaque vulnerability.

Genetic association with plaque phenotypes

To determine the effect of OSM signaling on the plaque phenotype, we assessed the association between rs13168867 and rs10491509 and seven plaque phenotypes in the Athero-Express Biobank Study. The strongest associations were observed between the effect allele of variant rs13168867 in the *OSMR* locus and intraplaque fat (β = 0.248 ± s.e. = 0.088 (C allele), $p = 4.66 \times 10^{-3}$), and collagen content (β = -0.259 ± s.e. = 0.095 (C allele), $p = 6.22 \times 10^{-3}$, Table 3). No associations were observed between rs10491509 and any of the plaque phenotypes.

Table 2. OSMR and LIFR variants and their association with overall plaque vulnerability. For each variant, the association with overall plaque vulnerability is given. NEF: the normalized effect size on expression (from GTEx Portal, www.gtexportal.org)[26]); Alleles: the effect allele and the other allele, respectively; EAF: effect allele frequency; Info: estimated imputation score; β: effect size; s.e.: standard error; *P*: p-value of association.

Gene	Variant	NEF	Alleles	EAF	Info	β (s.e.)	*P*
OSMR	rs13168867	-0.13	C/T	0.393	0.999	0.118 (0.040)	**3.00×10^{-3}**
LIFR	rs10491509	0.29	A/G	0.351	0.989	0.001 (0.043)	0.981

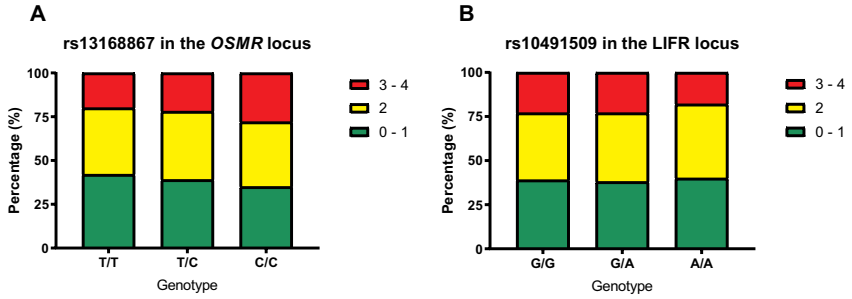

Figure 2. Association of *OSMR* and *LIFR* variants with overall plaque vulnerability.
The plaques were given a score ranging from 0 (least vulnerable plaque) to 4 (most vulnerable plaque) and divided in three groups (Group 1 contains plaque vulnerability scores 0 and 1, group 2 contains the plaque vulnerability score 2 and group 3 contains the plaque vulnerability scores 3 and 4). The bars represent the proportion of each group per genotype for rs13168867 in the *OSMR* locus (A) and rs10491509 in the *LIFR* locus (B). A bar chart showing the proportion of each individual score is shown in Supplemental Figure 4.

DISCUSSION

We investigated whether common variants associated to arterial gene expression, eQTLs, near *OSM, OSMR* and *LIFR* affect overall plaque vulnerability and phenotype. We showed that one *cis*-acting eQTL (rs13168867), associated with reduced *OSMR* expression arterial tissue, is associated with increased plaque vulnerability. This suggests that a decrease in *OSMR* expression and therefore possibly a decrease in OSM signaling, increases the chance on a vulnerable plaque. To gain further insight into the role of genetically decreased *OSMR* expression on plaque vulnerability, we examined the effect of rs13168867 on individual plaque characteristics in more detail. The strongest associations were found for rs13168867 with increased intraplaque fat and decreased collagen content, suggesting that reduced OSM signaling results in a larger lipid core and less fibrosis - in line with a more vulnerable plaque phenotype. None of the other plaque characteristics were associated with rs13168867.

The increase in intraplaque fat content that is associated with genetically decreased arterial *OSMR* expression can be related to the effect of OSM signaling on endothelial cells. OSM enhances ICAM-1 expression, but not VCAM-1 expression on endothelial cells[36,37], suggesting that OSM enhances recruitment of the non-classical monocyte subset[38,39]has a protective

Table 3. *OSMR* and *LIFR* variants and their association with plaque phenotypes. For each variant, the association with plaque phenotypes is given. NEF: the normalized effect size on expression; Alleles: the effect allele and the other allele, respectively; EAF: effect allele frequency; Info: estimated imputation score; β: effect size; s.e.: standard error; *P*: p-value of association. Calcification and collagen were classified as no/minor vs moderate/heavy, fat content as 10% vs >10% fat of plaque area, intraplaque hemorrhage was classified as absent vs present. Smooth muscle cells and macrophages were classified as Box-Cox transformed percentage of plaque area and vessel density as Box-Cox transformed number of vessels/ hotspot.

Gene	Variant	NEF	Alleles	EAF	Info	Phenotype	β (s.e.)	*P*
OSMR	rs13168867	-0.13	C/T	0.393	0.999	Calcification	0.036 (0.077)	0.637
						Collagen	-0.259 (0.095)	6.22×10^{-3}
						Fat content	0.248 (0.088)	4.66×10^{-3}
						Intraplaque hemorrhage	-0.014 (0.080)	0.862
						Smooth muscle cells	0.001 (0.011)	0.913
						Vessel density	-0.000 (0.004)	0.976
						Macrophages	0.004 (0.015)	0.809
LIFR	rs10491509	0.29	A/G	0.351	0.989	Calcification	0.046 (0.082)	0.577
						Collagen	0.134 (0.104)	0.194
						Fat content	0.086 (0.094)	0.363
						Intraplaque hemorrhage	0.071 (0.086)	0.414
						Smooth muscle cells	-0.003 (0.012)	0.840
						Vessel density	0.002 (0.004)	0.577
						Macrophages	0.015 (0.016)	0.354

effect on the endothelium[38,39] Furthermore, this monocyte subset is biased to turn into the M2 macrophage subset[40], which is associated with plaque regression[41–43]. OSM could even accelerate this process as OSM induces M2 macrophage polarization [21]. Yet, this hypothesis remains to be investigated and future studies should explore whether genetically reduced OSM signaling indeed increases intraplaque lipid content by reduced recruitment of non-classical monocytes and impaired M2 macrophage polarization.

The decrease in collagen content associated to genetically decreased OSM signaling could be attributed to the increase in fibroblast proliferation and enhancement of collagen formation that is induced by OSM[20]. Moreover, OSM enhances liver fibrosis in mice[44] and is upregulated in patients with pulmonary fibrosis[45]. Future studies are required to investigate if decreased OSM signaling indeed contributes to a decreased collagen content in plaques by reduced fibroblast proliferation and collagen formation.

No significant associations were found for rs10491509 in the *LIFR* locus and overall plaque vulnerability or any of the investigated plaque phenotypes. This suggests that an increase in *LIFR* expression does not affect OSM signaling or that OSMR is the dominant receptor in OSM signaling regarding the OSM related effects on atherosclerotic plaque vulnerability and phenotypes.

Recent developments in single-cell expression analyses might extend on the present study by investigating which cell types, that are present in the plaque, most abundantly express *OSM, OSMR* and *LIFR.* Furthermore, it would be interesting to investigate if the *OSMR/LIFR* expression ratio correlates with plaque vulnerability and if this ratio might be a predictor of plaque vulnerability.

Interestingly, a previous study showed that OSMR deficient mice have more stable plaques[13], which runs counter to our findings. This controversy could be explained by the differences in human and murine OSM signaling. In humans OSM binds to OSMR and LIFR with the same affinity, while murine OSM has a much stronger affinity for OSMR[46] In point of fact, while homologous, the sequence similarity of human OSM, OSMR, LIFR with murine is moderate at best (64.75%, 70.79%, 78.51%, respectively based on data from GeneCards[47]) and might directly impact receptor affinity[48].

Based on our data we can conclude that the variant rs13168867 in the *OSMR* locus is associated with increased plaque vulnerability. Given the multiple testing burden for individual plaque characteristics, it remains unclear through which precise biological mechanisms OSM signaling exerts its effects on plaque morphology, although our data point towards lipid metabolism and extracellular matrix remodeling. Compared to genome-wide association studies that include thousands of individuals, the Athero-Express Biobank Study is relatively small (n = 1,443), and given its design finite in size. However it is well suited to examine the effect of common disease associated genetic variation on plaque morphology and characteristics. Indeed, we estimated the power at ±75% given a MAF = 0.40 (approximately the frequency of rs13168867) and relative risk = 1.28[49].

CONCLUSION

We associated one eQTL in the *OSMR* locus, which associates with decreased arterial *OSMR* gene expression, with increased human plaque vulnerability in the Athero-Express Biobank Study. Further analyses of plaque phenotypes showed the strongest associations of this eQTL with increased intraplaque fat and decreased collagen content. No associations were found between an eQTL in the *LIFR* locus and either plaque vulnerability or any of the investigated plaque characteristics. In contrast to earlier mouse studies, our observations in human derived samples suggest that genetically decreased *OSMR* arterial expression contributes to increased carotid plaque vulnerability. Further, our study underscores the need to study prospective therapeutic targets derived in experimental models through human genetics.

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Disclosures

DvK is employee of Quorics B.V. and DT is employee of SkylineDx B.V and Quorics B.V. Quorics B.V. and SkylineDx B.V. had no part whatsoever in the conception, design, or execution of this study nor the preparation and contents of this manuscript.

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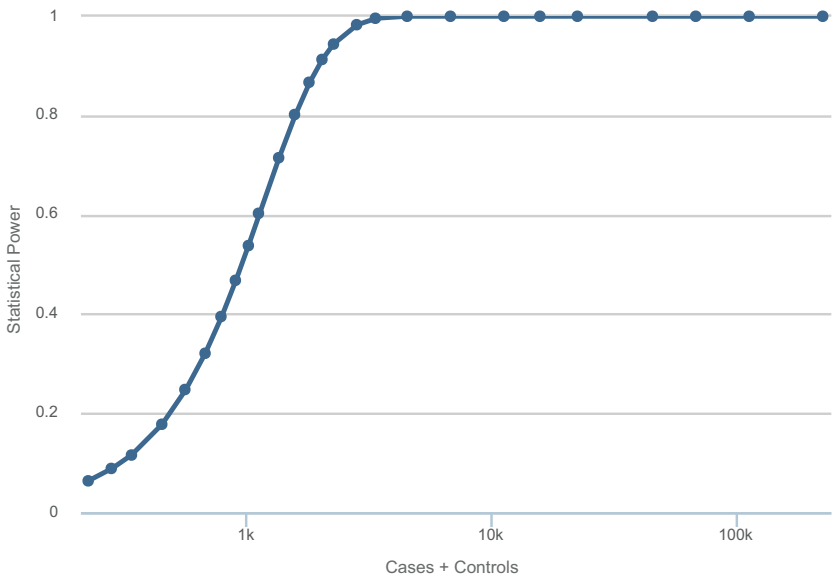
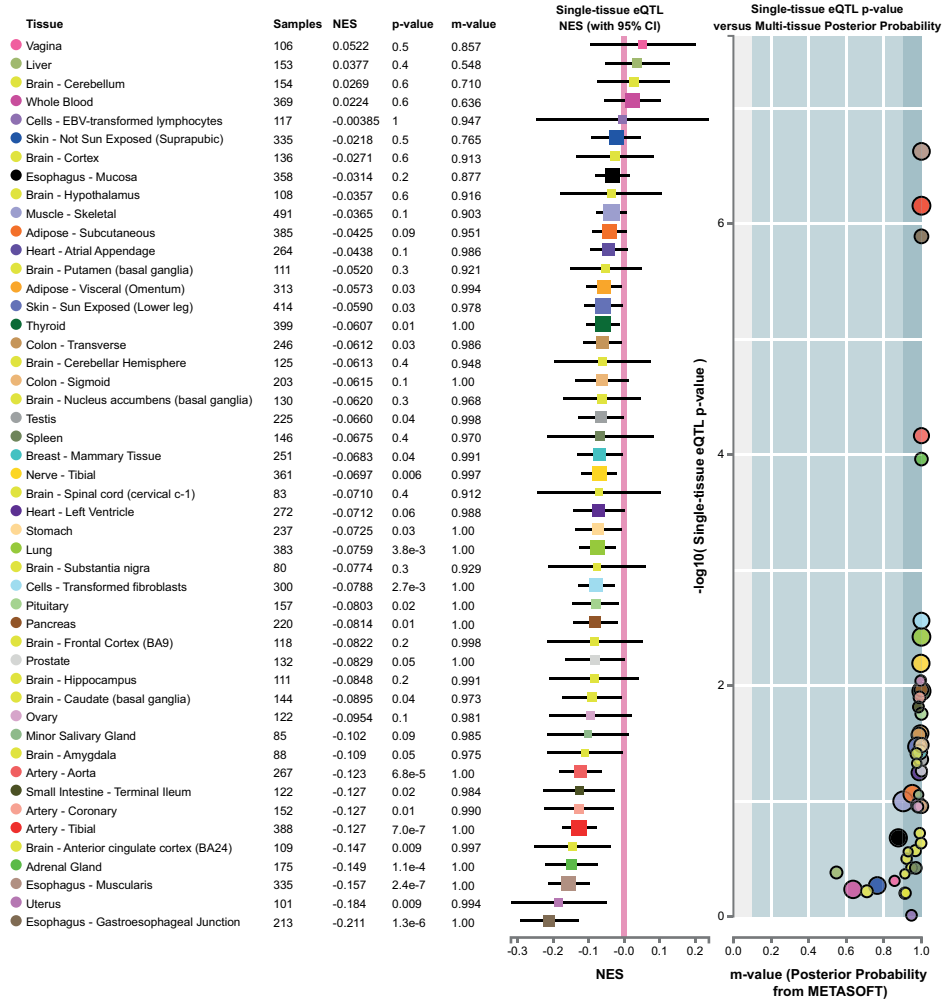
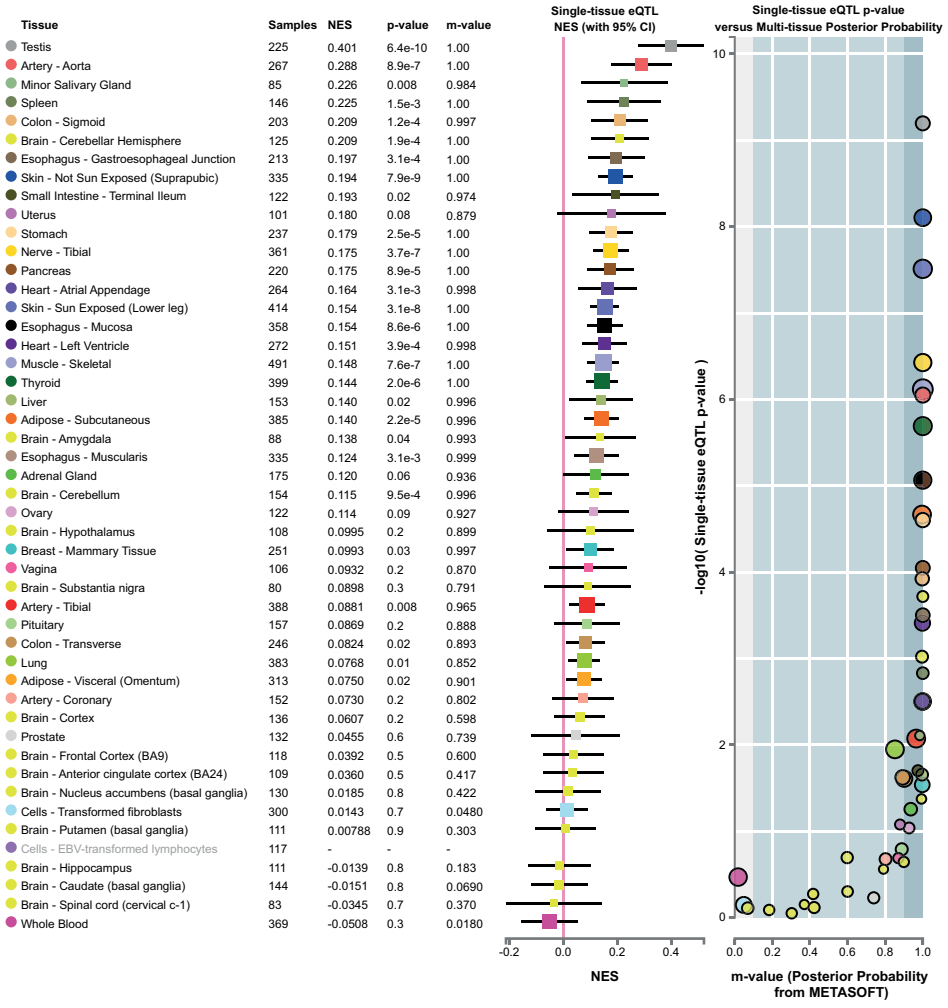
SUPPLEMENTAL MATERIAL



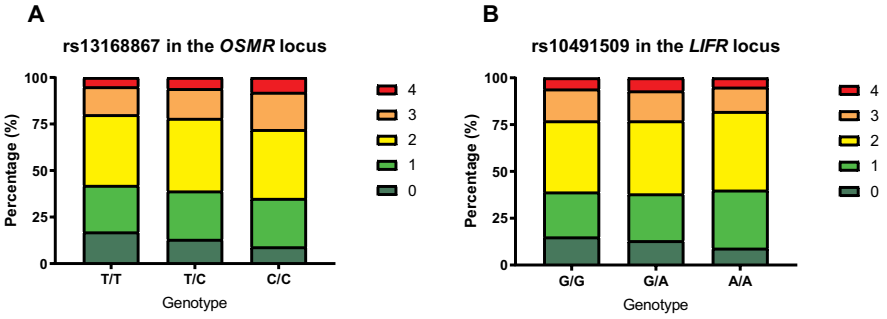
S1 Figure. Statistical power calculation on the Athero-Express Biobank.
The graph shows that based on a p-value of 3.13×10^{-3}, a MAF of 0.409 and a relative risk of 1.28 the 1,443 cases and controls included in this specific study give an estimated statistical power of ±75% (http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/index.html).



S2 Figure. eQTL analysis of rs13168867 with OSMR expression in multiple tissues from the GTEx Project. The forest plot shows the correlation of rs13168867 with *OSMR* expression per tissue and the posterior probability from METASOFT shows the posterior probability that the specific eQTL exists in each tissue (a large m-value indicates that the variant is predicted to be an eQTL for *OSMR* in that specific tissue). Data is obtained from GTEx Portal[1,2].



S3 Figure. eQTL analysis of rs10491509 with *LIFR* expression in multiple tissues from the GTEx Project. The forest plot shows the correlation of rs10491509 with *LIFR* expression per tissue and the posterior probability from METASOFT shows the posterior probability that the specific eQTL exists in each tissue (a large m-value indicates that the variant is predicted to be an eQTL for *LIFR* in that specific tissue). Data is obtained from GTEx Portal[1,2].



S4 Figure: Association of *OSMR* and *LIFR* variants with overall plaque vulnerability.
The plaques were given a vulnerability score ranging from 0 (least vulnerable plaque) to 4 (most vulnerable plaque). The bars represent the proportion of each plaque score per genotype for rs13168867 in the *OSMR* locus (A) and rs10491509 in the *LIFR* locus (B).

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PART TWO

PHOSPHOLIPID PHOSPHATASE 3

CHAPTER 5

Integrated human evaluation of the lysophosphatidic acid pathway as a novel therapeutic target in atherosclerosis

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ABSTRACT

Variants in the *PLPP3* gene encoding for lipid phosphate phosphohydrolase 3 have been associated with susceptibility to atherosclerosis independently of classical risk factors. PLPP3 inactivates lysophosphatidic acid (LPA), a pro-inflammatory, pro-thrombotic product of phospholipase activity. Here, we performed the first exploratory analysis of PLPP3, LPA and LPA receptors (LPARs 1-6) in human atherosclerosis. PLPP3 transcript and protein were repressed comparing plaques *vs.* normal arteries and plaques from symptomatic *vs.* asymptomatic patients, and negatively associated with risk of adverse cardiovascular events. PLPP3 localized to macrophages, smooth muscle and endothelial cells (ECs) in plaques. LPAR 2, 5, and especially 6 showed increased expression in plaques, with LPAR6 localized in ECs and positively correlated to PLPP3. Utilising *in situ* mass spectrometry imaging, LPA and its precursors were found in the plaque fibrous cap, co-localizing with PLPP3 and LPAR6. *In vitro,* PLPP3 silencing in ECs under LPA stimulation resulted in increased expression of adhesion molecules and cytokines. LPAR6 silencing inhibited LPA-induced cell activation, but not when PLPP3 was silenced simultaneously. Our results show that repression of PLPP3 plays a key role in atherosclerosis by promoting EC activation. Altogether, PLPP3 pathway represents a suitable target for investigations into novel therapeutic approaches to ameliorate atherosclerosis.

INTRODUCTION

Therapies that can reduce the deposition of apolipoprotein-B-containing lipoproteins in lesion-susceptible arterial segments, can slow down atherosclerotic cardiovascular disease (ACVD) progress[1]. Efficient therapies for suppression of the inflammatory response, which accompanies lipoprotein deposition and contributes to plaque development and ACVD, may add further cardiovascular benefit, as shown recently with the CANTOS clinical trial[2].
Several genome-wide association studies (GWAS) including a meta-analysis, have identified *PLPP3,* the gene encoding lipid phosphate phosphatase 3, as a candidate locus causally related to ACVD risk[3,4]. Furthermore, *PLPP3* single nucleotide variant (SNP) rs17114036 was included in a genetic risk score (GRS) study showing that individuals with high GRS appear to have a larger benefit in cardiovascular absolute risk reduction from statin treatment than those with low GRS[5], thus suggesting that *PLPP3* genetic data may be applied to enhance clinical benefit with already established therapy for ACVD. We identified PLPP3 and the associated lysophosphatidic acid (LPA) axis as possible anti-atherosclerotic drug candidates after applying a target selection workflow within the CARTARDIS Consortium (www.cartardis.eu), aimed at identifying targets suitable for pharmaceutical drug development. In our approach, candidate targets were first assembled from published data related to human genetics of CVD and subsequently prioritized using stringent target discovery filters including: genetic correlation to ACVD clinical phenotypes; novelty; feasibility to validate (*in vitro, in vivo*); and relation to known ACVD mechanisms[6].
PLPP3 enzyme is an integral membrane protein that dephosphorylates and inactivates various lipid-phosphate mediators such as LPA and sphingosine-1-phosphate, thus blocking the downstream signals of these bioactive lipids[7]. Expression quantitative trait locus (eQTL) analyses linked the major ACVD risk-associated allele with lower expression of PLPP3 in human endothelial cells (ECs) but not in other tissues[8,9]. These results suggest that ACVD risk-associated SNPs may increase the disease susceptibility by regulating PLPP3 expression in an endothelial-specific manner. Elegant mouse *in vitro* and *in vivo* studies have shown that PLPP3 expression in the arterial wall is modulated by hemodynamic forces and that it is involved in arterial wall pathology by modulating vascular cell functions[9-11]. Mechanistic studies with isolated arterial cells showed that lower expression of PLPP3 sensitizes the response of ECs to LPA[9,12].
Several studies have shown that LPA accumulates in human atheroma where it can exert prothrombotic effects[13,14]. LPA is an amphipatic phospholipid that can interact with cell membranes by its detergent properties but also by its association to lysophosphatidic acid specific receptors (LPARs 1 to 6)[15]. Experimental evidence supports a role for LPA and its G-protein-coupled receptors in promoting pro-atherogenic, pro-inflammatory and pro-thrombotic processes[16,17]. LPA in lesions can be generated by enzymatic hydrolysis of lysophospholipids by extracellular lysophospholipase D, autotaxin, and by local deposition of modified apoB-lipoprotein carriers of LPA and its phospholipid precursors[7,18,19]. For example, PC (34:1) and PC (34.2) are two phosphatidylcholine (PC) species connected to the lysophosphatidylcholine (LPC), which are the degradation products from PC hydrolysis by phospholipases and precursors for LPA production. LPA molecules can be inactivated by

the enzymatic action of PLPP3 which removes the phosphate group generating the corresponding alcohols that are not agonists for LPA receptors[20]. Thus, PLPP3 expression levels in the arterial wall may have a relevant function in disease progression by affecting local LPARs-LPA mediated signalling.

To explore the putative association of the PLPP3-LPAR(s) pathway with ACVD and its potential for drug development, we performed a comprehensive analysis on genetic, transcriptomic and proteomic level in human carotid plaques and normal arteries. In the same tissues, we also evaluated their possible co-localization with LPA and its phospholipid precursors. *In vitro* silencing experiments were conducted to characterize the role of PLPP3 and LPA receptors on endothelial activation. Our results from investigation of this human genetic association and the pathway biology as a marker of atherosclerosis, provide insight for selection and identification of potential new therapeutic approaches to prevent ACVD and its complications.

RESULTS

PLPP3 is repressed in human carotid atherosclerotic lesions

We first evaluated the genetic association of a previously reported rs17114036 variant with PLPP3 levels in human plaques by expression quantitative locus (eQTL) analyses. Two suitable proxies were identified (rs6588634 and rs9970807, both R^2>0.8 and D'=1) that confirmed a marginal association with PLPP3 mRNA levels in plaques from n=127 patients that were available for these analyses (p=0.07, Figure 1A). Next, gene expression analysis of totally n=177 lesions showed a significant downregulation of PLPP3 transcript in carotid plaques (CP) compared to macroscopically normal arteries (NA) in two non-overlapping microarray datasets (n=127 CP vs. n=33 NA from the larger discovery dataset and n=50 CP plaque vs. n=5 NA from the smaller validation dataset, Figure 1B) with mean log2 difference ± SD = -0.6311 ± 0.1731 and mean log2 difference ± SD = -1.012 ± 0.3323, respectively. Notably, PLPP3 mRNA was also significantly downregulated in CPs from symptomatic patients compared with asymptomatic ones (mean difference ± SD = -0.2803 ± 0.1031). Importantly, this data was confirmed on the protein level by mass spectrometry analyses comparing plaques with adjacent control arterial tissue (n=18 matched samples) and plaques from symptomatic vs. asymptomatic patients (n=9 in each group, Figure 1C), strongly suggesting a correlation between lower PLPP3 levels and a more severe clinical phenotype. Moreover, patients with below median expression levels of PLPP3 in their plaques at surgery, conferred a significantly higher risk of future adverse cardio- and cerebro-vascular events during the follow-up period after CEA (p=0.0072, Figure 1D).

To begin delineating the cellular processes associated with PLPP3 expression, correlation analyses were performed in plaques from the BiKE discovery dataset between PLPP3 and various cellular markers (Table S1). These analyses showed positive correlation (Pearson *r* >0.3, *p*<0.0001) between PLPP3 expression and typical SMC markers; endothelial marker CD31; T-Lymphocyte marker CD45RA; pro-inflammatory markers NFkB and BMP4; extracellular matrix degradation via SULF1; growth factors TGFB1, IGF1, PDGFB, PDGFD; and chemokine CCR2. Weak positive correlations were found also with some macrophage markers.

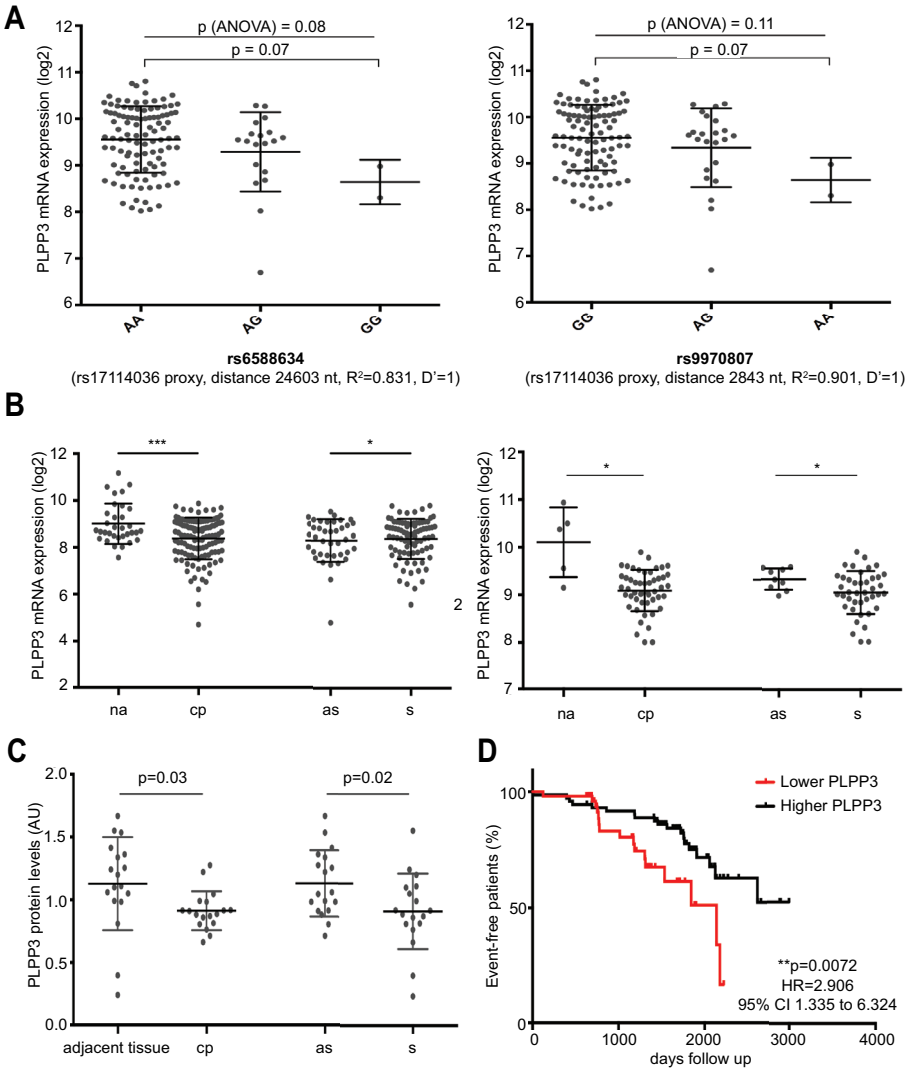
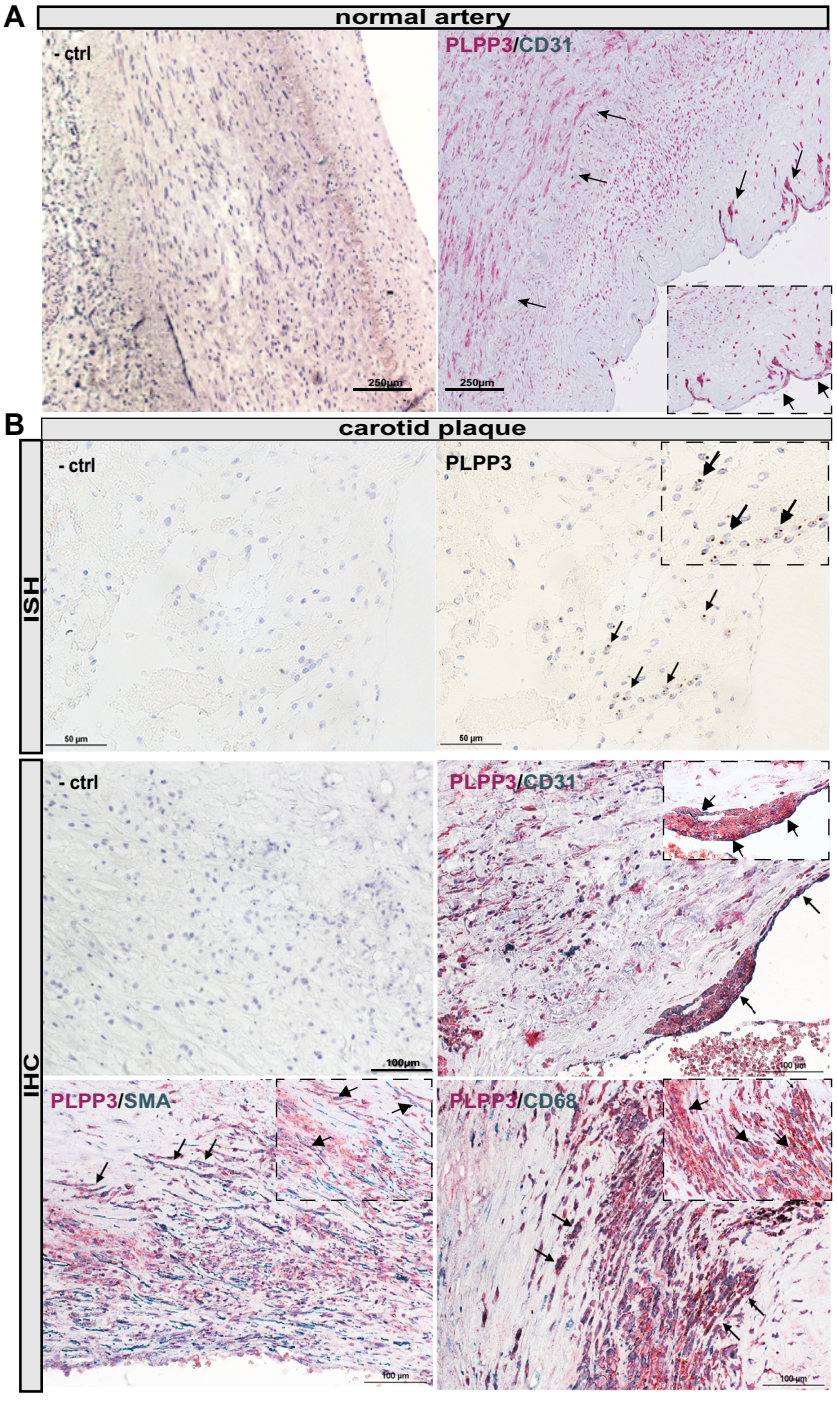


Figure 1. Genetic association and expression of PLPP3 in advanced human atherosclerotic plaques.
A) Genetic variant rs17114036 was found to be tentatively associated with PLPP3 mRNA expression in carotid plaques by expression quantitative train locus (eQTL) analysis of two suitable proxies in n=127 patients. Plots show median ± standard deviation (SD). **B)** Two microarray datasets (discovery set on the left and validation set on the right) showing the PLPP3 mRNA expression in carotid plaques (cp) compared to normal arteries (na), along with the comparison between plaques from symptomatic (s) and asymptomatic (as) patients. Discovery set contained n=33 na and n=127 cp, and validation set n=5 na and n=50 cp. Values are expressed as log2 mean ± SD. *P<0.05, ****P<0.0001, ns - not significant. **C)** Evaluation of PLPP3 protein levels by mass spectrometry comparing plaques (CP) with adjacent control arterial tissue (n=18 matched samples), and plaques from s *vs.* as patients (n=9 per group). Values are expressed in arbitrary units (AU) as mean ± SD. **D)** Survival curves illustrating MACCE-free survival of patients during the follow-up period after surgery, based on PLPP3 mRNA expression in BiKE plaques above (black) and below (red) the median values (X-axis, days event-free survival). Each mark along the lines indicates an event. Total number of events in the cohort was n=58. MACCE - major adverse cerebro- and cardio-vascular event.


A
normal artery
- ctrl
PLPP3/CD31
250µm
250µm
B
carotid plaque
ISH
- ctrl
PLPP3
50 µm
50 µm
IHC
- ctrl
PLPP3/CD31
100µm
100 µm
PLPP3/SMA
PLPP3/CD68
100 µm
100 µm

◀◀ **Figure 2. Localization of PLPP3 in normal arteries and carotid plaques.**
Immunohistochemical staining shows the distribution of PLPP3 (red signal) in normal human artery, in medial smooth muscle cells and CD31+ (green signal) luminal endothelial cells (A). *In situ* hybridization detection of PLPP3 mRNA transcript in human lesions (top raw). Arrows indicate the RNA probe signals (also shown in the zoomed inset). Immunohistochemical stainings showing the distribution of PLPP3 in human plaques. Double staining shows the colocalisation of PLPP3 (red signal) with endothelial cell marker (CD31, green signal), smooth alpha actin (SMA, green signal) and macrophage cell marker (CD68, green signal). Arrows indicate double positive cells, insets show higher magnification (B). Images taken with the 40x objective.

To evaluate these bioinformatic results, we further examined PLPP3 mRNA and protein expression levels and localization in various cell types in normal arteries and plaques *in situ*. Overall, PLPP3 protein signal was strong in normal arteries, particularly in CD31+ endothelial cells but was also found in medial SMCs (Figure 2A). PLPP3 transcript and protein (Figure 2B) expression was also detected in CPs (n=10 samples examined), particularly localized in the fibrous cap and in the areas lining the necrotic core. PLPP3 was absent from peripheral, subintimal plaque areas. Double immuno-staining with specific cell markers showed PLPP3 protein localization in various cell types, such as SMCs (SMA^+), endothelial cells ($CD31^+$) and macrophages ($CD68^+$, Figure 2B), confirming the correlation analyses. These results confirmed overall lower levels of PLPP3 in advanced human plaques, but indicated a wide expression pattern linked to several major plaque cell types.

LPAR6 is the most abundantly expressed LPA receptor in human plaques, localized to endothelial cells and positively correlated with PLPP3

Expression of LPAR1-6 transcripts was then analysed in microarrays from human CPs compared to NA (discovery dataset) (Figure 3). LPAR1 was the only one downregulated in CP *vs.* NA comparison (mean log2 difference ± SD = -1.836 ± 0.1106) while LPAR4 did not show any significant difference and was detected at low levels, similarly as LPAR3. LPARs 2, 5 and 6 were all significantly upregulated in this comparison and showed moderate to high expression levels in plaques, particularly LPAR6 (mean log 2 difference ± SD LPAR2 = 1.037 ± 0.0876; LPAR5 = 1.604 ± 0.1306; LPAR6 = 1.032 ± 0.1268).

Expression correlations of LPARs with markers of cell types and processes in plaques, showed association between LPAR1 and typical SMC markers, whereas LPAR2 was negatively correlated with SMCs and showed moderate positive correlation with inflammatory cells (T lymphocytes and macrophages). Correlation analyses for LPAR3 and LPAR4 were inconclusive, likely due to low expression levels of these receptors in the tissue. LPAR5 and LPAR6 correlated to endothelial- and inflammatory cell markers (dendritic cells, T lymphocytes and macrophages) (Table S2).

These analyses were followed by ISH and IHC of the LPARs in consecutive sections of human lesions, which generally confirmed the differential expression data and correlation pattern of the receptors (S1 Figure). LPAR1 was detected at low levels while LPARs 3 and 4, in line with the low expression levels from gene arrays, could not be detected in plaques (data not shown). LPAR2 localized exclusively in inflammatory cells as determined by double staining with CD3, CD8, CD68 and CD163 (S2 Figure). LPAR6 showed the most abundant expression of all receptors, both by ISH (Figure 4A) and IHC (Figure 4B), with localization in $CD34^+$ ECs and in the fibrous cap smooth muscle-like cells. Together our analyses on transcript and

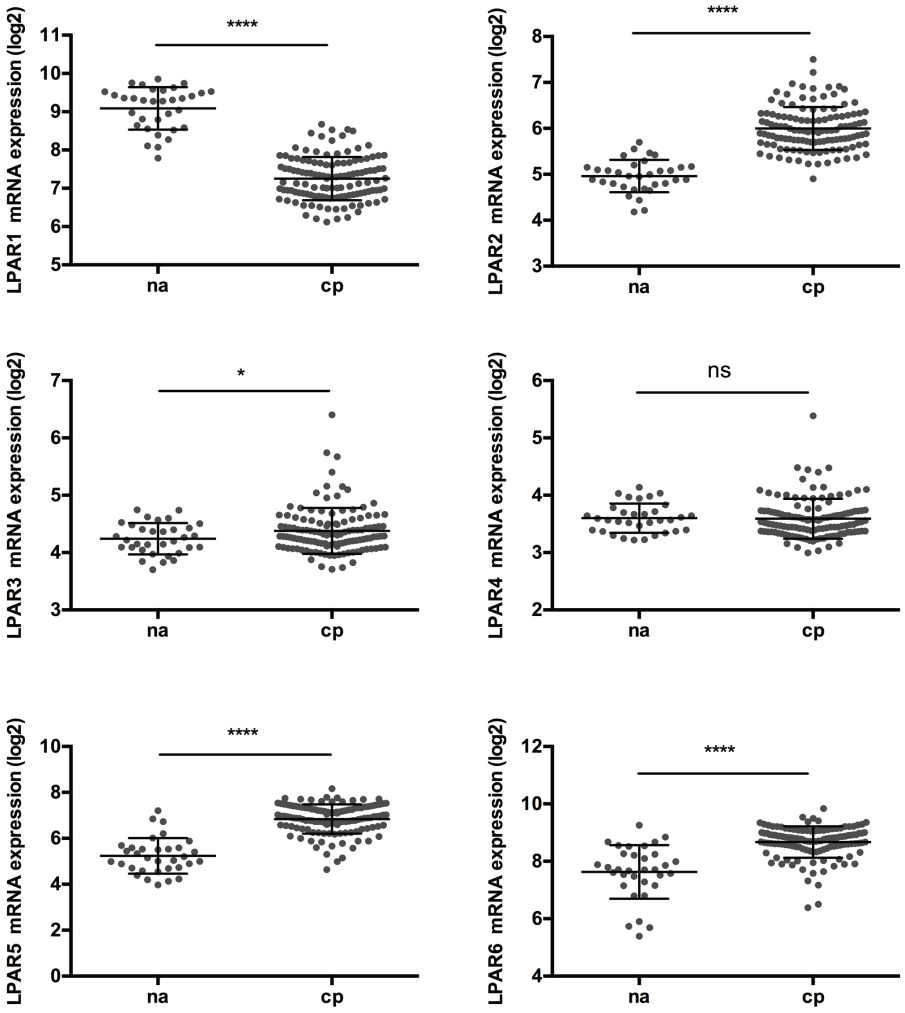


Figure 3. LPARs expression in comparison between normal arteries and plaques.
mRNA levels of LPA-receptors (LPARs 1-6) in normal arteries (n=33) and in human plaques (n=127) were interrogated in the BiKE discovery microarray dataset. Values are expressed as log2 mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

protein level showed the differential expression of LPARs in human plaques and revealed a strong enrichment of primarily LPAR6, but also LPAR2 and LPAR5 to a lesser extent.
Next, the relationship between PLPP3 and LPARs in plaques was assessed by expression correlations from microarrays (Table S3). These analyses indicated a significantly positive association of PLPP3 with LPAR6 and negative with LPAR2 (Figure 5A). Non-significant correlations were found between PLPP3 and other LPARs. A combined ISH/IFL staining was thus performed for PLPP3 and LPAR6 in human early fibroatheroma lesions, including

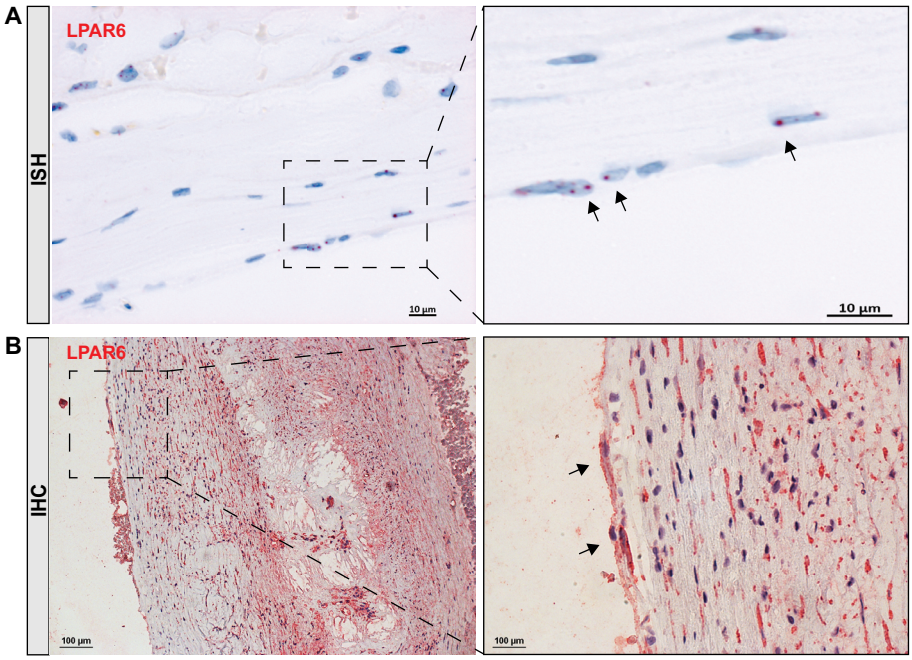


Figure 4. Localisation of LPAR6 in plaques.
In situ hybridization detection of LPAR6 mRNA transcript in human lesions. Arrows indicate the RNA probe signals (red) (A). Immunohistochemistry staining of LPAR6 in plaque (red signal) depicts its strong overall expression and localization in cells lining the luminal wall of the fibrous cap (arrows) as well as in the underlying cells with elongated nuclei. Nuclei (purple) are stained with hematoxylin (B). Images were taken with 20x, 40x and 63x objectives.

markers of ECs and SMCs. These experiments convincingly showed that both PLPP3 and LPAR6 mRNA localize in $CD34^+$ ECs on the luminal side of the fibrous cap, and localization in SMCs could also be confirmed (Figure 5B, C).

PLPP3, LPAR6 and LPA pathway-related lipids co-localize in plaques

To examine the spatial relationship between PLPP3, LPAR6 and lipid species that constitute PLPP3 substrates in human plaques, a combination of immune- and lipid-imaging *in situ* was employed (n=4 CP tested). First, ten lipid species between 400 and 800 Da were detected in plaques (Table S4). Replicates of MALDI-MSI in detection mode at 30 µm showed the presence of cholesteryl ester (CE) stearate (18:0); oleate (18:1) and linoleate (18:2), localized particularly in the necrotic core and upper border of the fibrous cap. Phosphatidylcholine (PC), the main phospholipid type, was detected in the necrotic core and in the middle area of the fibrous cap. LPC stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) were mainly detected in the fibrous cap and shoulder region lining the necrotic core (S3 Figure).
PLPP3 is an enzyme that de-phosphorylates lysophosphatidic acid (LPA) into monoacylglycerol (MAG), therefore we next sought to examine the spatial relationship among PLPP3, LPAR6

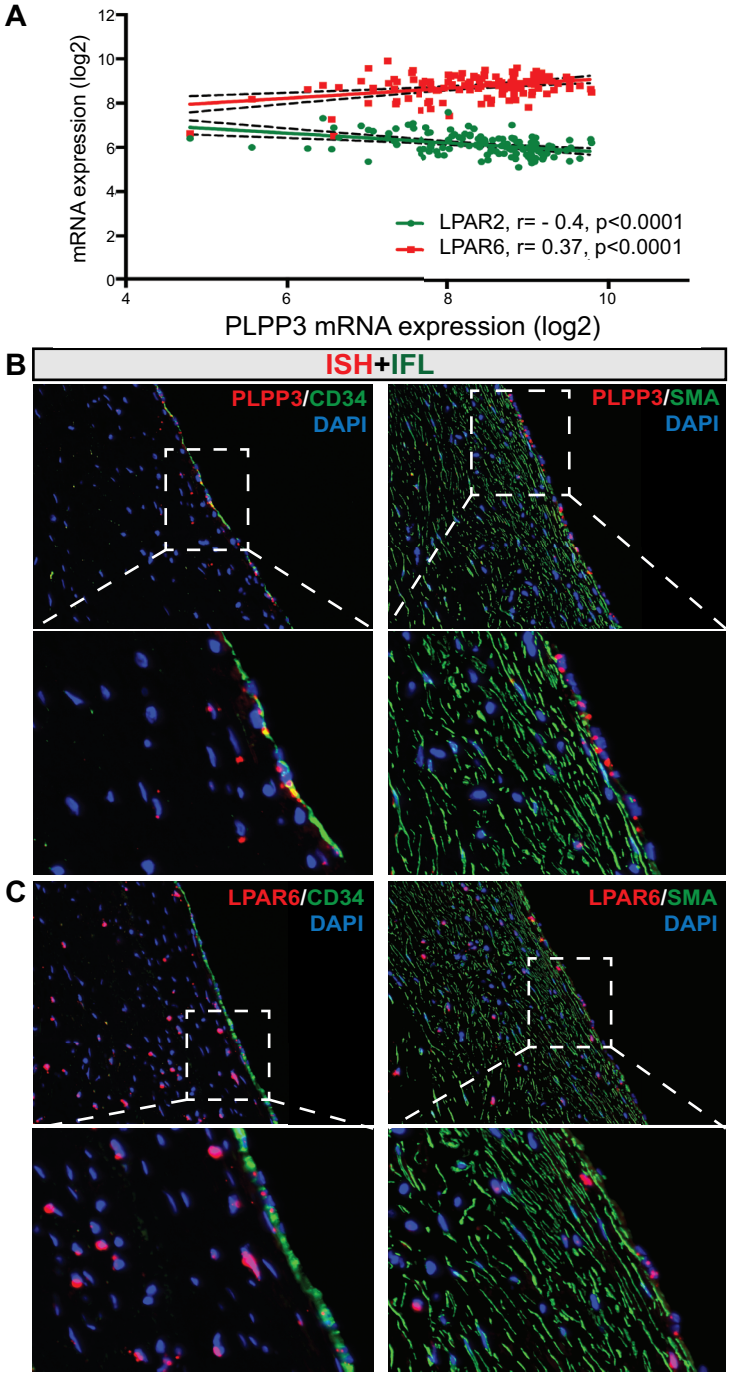


Figure 5. Association between PLPP3 and LPAR6 in human lesions.
Correlation plot showing significantly positive association of PLPP3 with LPAR6 mRNA expression levels in ►►

◀◀ plaques, while inverse with LPAR2 (A). Pearson correlation calculated based on the discovery dataset (n=127 samples). Localisation of PLPP3 and LPAR6 mRNA in $CD34^+$ endothelial and SMA^+ smooth muscle cells in the fibrous cap, shown by combined *in situ* hybridization (red signal) and immunofluorescence (green signal) in human early fibroatheroma lesions (B). Nuclei are stained with DAPI. Images were taken with 20x objective. IFL-immunofluorescence.

and LPA in plaques. We could show that positive PLPP3 immune detection (Figure 6A) co-localized with high intensity signal for LPA, a PLPP3 substrate, but not with PA signal, an LPA precursor (Figure 6B and overlay in 6C). Similarly, co-localization was observed between LPAR6 and LPA, an LPAR ligand, but not with PA. Moreover, LPA and PA appeared to be mutually anti-localized based on the low spatial correlation factor of r = 0.2.

Silencing of LPAR6 diminishes LPA induced endothelial activation in vitro, but not after PLPP3 silencing

The possible effect of a reduced PLPP3 expression in ECs, which would resemble that observed in human plaques, was assessed using siRNA-mediated gene silencing. Silencing of PLPP3 in HUVECs (by 47% as compared with a non-targeting scramble siRNA) resulted in a significant increase in mRNA expression of pro-inflammatory cytokines MCP-1 and IL-6 as well as the adhesion molecules VCAM-1 and ICAM-1 (Figure 7A). Importantly, similar effect of PLPP3 silencing was previously reported in human aortic endothelial cells[9,12]. Together, these results supported the anti-inflammatory function of PLPP3 in ECs.

To further explore this finding, we next stimulated the HUVECs with LPA, which lead to increased expression of cytokines IL-6 and MCP-1 as well as ICAM-1 and VCAM-1 (Figure 7B). These effects were comparable to when PLPP3 was silenced, which again suggested that lower PLPP3 levels may cause endothelial activation through enhanced LPA signalling. We further hypothesized that the effect of LPA would be mediated *via* LPAR6, shown to be the most prominent LPAR expressed in ECs in human plaques from our cohort. Interestingly, silencing of LPAR6 in HUVECs (by 57% on mRNA level and confirmed also on protein level, S4 Figure) inhibited LPA-induced endothelial activation, observed by the reduced expression of ICAM-1, VCAM-1, IL-6 and MCP-1 (Figure 7B).

Surprisingly, simultaneous silencing of both LPAR6 and PLPP3 did not result in any diminishing effects on endothelial activation compared to those observed after silencing PLPP3 alone (Figure 7C). In addition, expression of LPAR1 was enhanced after silencing of LPAR6 and LPA stimulation, but not when in combination with the silencing of PLPP3. The mRNA expression of other LPARs (LPAR2, 4 and 6) was not significantly affected by the knockdown of PLPP3 and LPAR3 and LPAR5 were undetectable in ECs (data not shown). LPAR2 was only upregulated after stimulation with LPA and this effect could be inhibited by silencing of LPAR6.

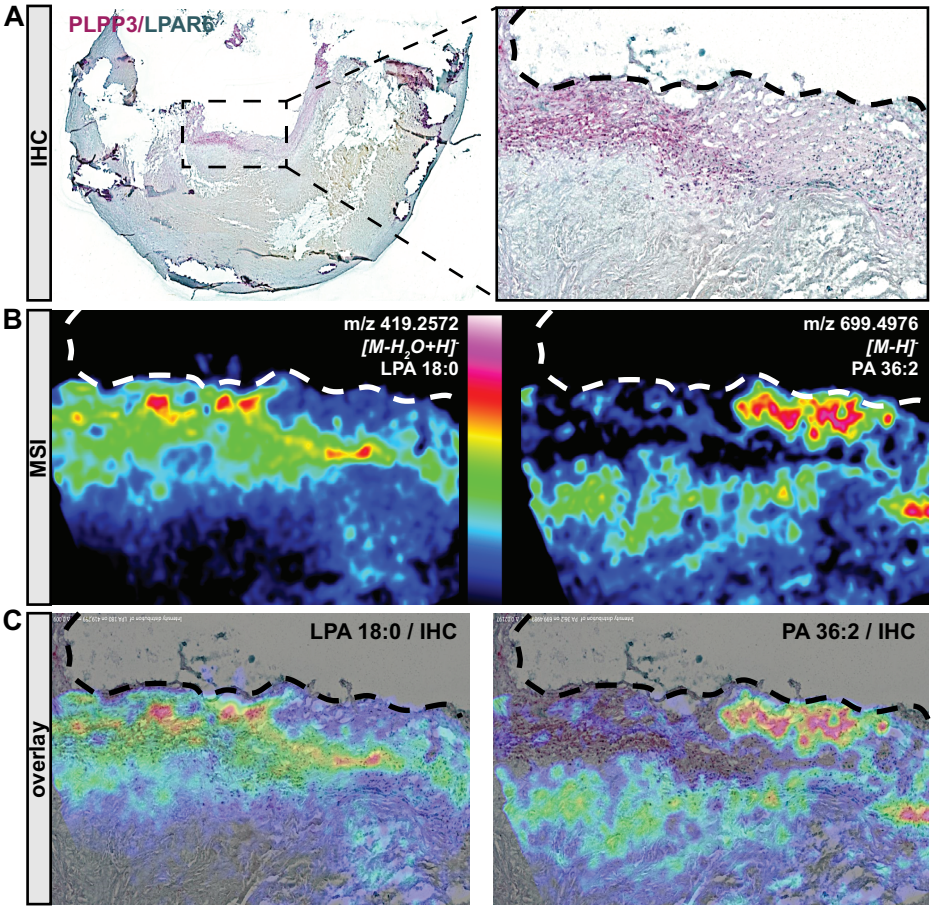


Figure 6. Relationship of PLPP3, LPAR6 and LPA pathway-related lipids in atherosclerotic plaques.
Double immunostaining of PLPP3 (red) and LPAR6 (green) in atherosclerotic plaques, showing their co-localisation and accumulation of positive signal in the fibrous cap and shoulder region (A). Distribution of lysophosphatidic acid (LPA 18:0, to the left) and PA 36:2 (to the right) on consecutive plaque sections by mass spectrometry imaging (MSI). Red signal indicates the highest amount of detected lipid (colour scale reported in the middle) (B). Overlay of the PLPP3 and LPAR6 staining by IHC with MSI images for LPA (left panel) or PA (right panel), showing an increase of LPA and a decrease of PA in PLPP3/LPAR6 double positive regions (C). Images taken with 2x objective, representative of n=4 independent experiments.

DISCUSSION

To the best of our knowledge, this is the first study to systematically characterize the expression of the PLPP3 pathway, including all six LPA-receptors as well as substrates LPA and its precursors PA and LPC, in advanced human atherosclerotic plaques. A reduced PLPP3 mRNA and protein expression was demonstrated in human plaques compared with normal disease-free artery specimens, suggesting more permissive conditions for signalling

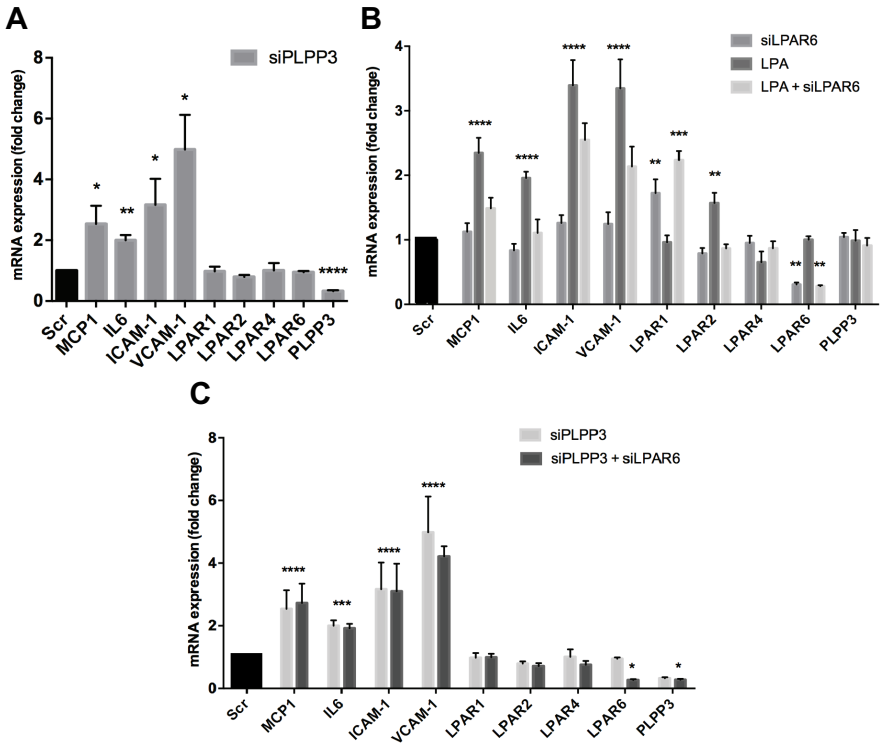


Figure 7. Effect of PLPP3 and LPAR6 silencing on endothelial cell activation *in vitro*.
Silencing of PLPP3 in cultured endothelial cells lead to induced mRNA expression of the inflammatory cytokines MCP1 and IL-6 and adhesion factors VCAM-1 and ICAM-1 compared to control cells (scramble). No significant change in expression of LPARs 1-6 could be observed upon PLPP3 silencing (A). Inhibition of LPAR6 alone had no prominent effect on MCP1, IL-6, VCAM-1 or ICAM-1, but the upregulation of these genes caused in response to LPA stimulation (10 μM, 2h treatment) could be significantly attenuated by simultaneous silencing of LPAR6. PLPP3 expression was not affected by LPA stimulation nor the LPAR6 silencing (B). Combined silencing of both LPAR6 and PLPP3 did not result in any diminishing effects on endothelial activation compared to those already observed after silencing PLPP3 alone. Note also that neither inhibition of PLPP3 nor LPA stimulation had any effect on the LPAR1 expression, but its induction was observed when LPAR6 was silenced. The expression of LPAR2 was strongly enhanced after LPA stimulation alone, but not in combination with inhibition of LPAR6 or PLPP3. Expression of LPAR4 was not affected in any of these experiments (C). All experiments were repeated 4 times, n=4 samples per group in all analyses. Significance indicated above bars calculated in comparison to scramble/control cells (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).

through LPA and its receptors in late-stage human atherosclerosis. This assumption was further supported by indications of an even lower levels of PLPP3 in plaques from symptomatic patients than in those of asymptomatic ones, and an inverse relationship between plaque PLPP3 levels and future adverse cardio- and cerebro-vascular events. Together with our eQTL studies confirming the genetic link between *PLPP3* and carotid atherosclerosis, these findings suggest that suppression of PLPP3 is associated with more severe disease and confirm a potential athero-protective role for the enzyme, as previously proposed[9].

This study is the first to definitively localize LPA and PA molecular species *in situ* in the fibrous cap of human plaques using MS imaging and overlay with PLPP3 and LPAR6 by immunodetection. Our analyses showed that PLPP3 and LPAR6 protein in the fibrous cap co-localize with LPA, the substrate of PLPP3 and the ligand for LPAR6, but not with PA that is a potential precursor of LPA generated by A-type phospholipase activity. Lipoprotein associated phospholipase A2 (Lp-PLA_2), a platelet-activating factor (PAF) acetyl-hydrolase or group VII phospholipase A secreted enzyme which catalyzes the degradation of PAF producing LPA and acetate, could be a source of LPA. However, in a large-scale human genetic study, none of a series of Lp-PLA_2-lowering alleles, including loss-of-function mutation leading to full Lp-PLA_2 deficiency, were related to coronary heart disease risk, suggesting that Lp-PLA_2 is unlikely to be a causal risk factor for ACVD[21]. This conclusion is also supported by the lack of cardiovascular benefit of the anti-PAFAH/Lp-PLA_2 drug darapladib in large phase three clinical trials[22]. Thus, we suggest that other extra- and intracellular enzymatic pathways may be involved in local production of LPA independent of Lp-PLA_2 activity[23]. We identified saturated LPC (LPC18:0) as the main LPC species present in the advanced atherosclerotic lesions, confirming the previous findings that showed the presence and the enzymatic activity of secretory phospholipase A2 in human lesions[24]. LPC associated with plasma lipoproteins accumulating in lesions, can be another source of local LPC species[25]. LPA has been reported by others to be significantly higher in coronary than in peripheral systemic arterial blood[14]. Thus, together with our results, these findings support the hypothesis that higher concentrations of LPA might be associated with atherogenesis and its clinical consequence ACVD.

LPA is a potent lipid mediator with broad cellular effects that are relevant to atherothrombosis[13,26,27]. PLPP3 deactivates LPA and it has been suggested that the enhancement of its expression and activity might serve for pharmacological therapeutic purposes, however considering that its control mechanisms are yet not understood, this could be a challenging task. An alternative approach to reduce LPA-mediated pro-inflammatory activation could be to target LPA-mediated signalling through modulation of its different receptors (LPARs). However, this requires a detailed investigation of the expression and cellular distribution of LPA receptors and other components of the pathway in human atherosclerosis, and our results should be considered as an initial step in this area.

Our investigation suggests that the repression of PLPP3 in atherosclerotic arterial tissue could contribute to the elevated local accumulation of LPA and increase its biological effects in lesions. It should be noted that PLPP3 fulfils also non-catalytic functions such as its interaction with integrins that can promote endothelial cell-to-cell adhesion [28]. Although PLPP3 expression levels are lower in plaques, we showed that the residual protein localizes to ECs, SMCs and CD68+ cells. However, further studies will be needed to establish whether differential expression of PLPP3 in normal and diseased tissue is translated into differences in the hydrolytic activity required for deactivation of LPA. Similarly, we do not know if increased expression of the enzyme affects its non-enzymatic roles in endothelium and foam-cells[29].

When it comes to the LPA receptors, our results indicated that LPAR2, LPAR5 and especially LPAR6 were the most prominent receptors expressed in lesions. LPAR2 and LPAR6 were the only receptors also showing significant correlation with PLPP3 expression at the mRNA

level. We found that LPAR6 expression was mostly associated with luminal ECs and with SMCs, but less frequently with inflammatory cells. Interestingly, LPAR6 has previously been involved in LPA-induced actin stress formation in ECs[30]. LPAR2 was expressed in a specific subset of inflammatory cells, thus it is possibly limited to certain type of plaques that express less PLPP3 combined with increased leukocyte content. These results are in line with previous data indicating that LPA can stimulate T-cell migration and secretion of matrix metalloproteinases when overexpressing LPAR2[31]. Together, this suggests that LPAR2 might contribute to LPA-mediated atheroma progression and may be a promising LPA receptor for pharmacological intervention to reduce atherogenic inflammation. With respect to the other LPARs, we found that the expression of LPARs 3 and 4 was either absent or too low to be detected in both in plaques and specifically in ECs, indicating that these receptors may be of limited importance in the regulation of LPA activation in atherosclerotic lesions.
Our *in vitro* experiments were targeted to ECs as a plausible cell type to facilitate the future therapeutic application of the PLPP3 pathway. We show that silencing of PLPP3 did not affect the expression levels of LPARs 1, 2, 4 and 6 in ECs, although there was a significantly positive correlation between PLPP3 and LPAR6 in the whole plaque tissue. This suggests a mechanism by which the loss of PLPP3 expression, and the potential subsequent increase in LPA, does not lead to a compensatory increase in the expression of LPARs in ECs. Knockdown of LPAR6 appeared to inhibit the upregulation of LPAR2 after LPA stimulation, which indicates that the LPA-LPAR6 interaction may be involved in endothelial activation, but also in the regulation of LPAR2. Furthermore, the inhibition of LPAR6 induced the expression of LPAR1, suggesting a compensatory mechanism between these two receptors in ECs. Based on our *in vitro* data, it is possible to speculate that, although silencing of PLPP3 and LPA stimulation induce similar phenotypes in ECs, they may not share the same LPA-LPAR6 signalling. Our results also indicate a complexity and flexibility in signalling patterns among LPARs, that can be dictated by repression of PLPP3 in combination with some yet unknown factors in the atherosclerotic tissue environment, considering that silencing of LPAR6 did not have a diminishing effect on the endothelial activation already observed after silencing of PLPP3 alone. Further experiments are needed to better understand the interaction between PLPP3, LPA and the different LPARs.

Limitations

Although BiKE cohort is one of the world's largest when it comes to the molecular profiles of human carotid atherosclerotic plaques, an even larger number of subjects may be necessary to validate our findings in independent biobanks and generalize the observations to other vascular beds. Replication is warranted in heterogeneous cohorts that will permit adjustment for traditional cardiovascular risk factors and evaluation within subgroups of interest such as defined by age, gender, symptoms and comorbidities. This is also important in the context of genetic investigations of PLPP3 and LPARs. Consensus is lacking in the field regarding the selection of appropriate control tissues, and it is worth noting that arteries of various embryonic origins have been used for this purpose as well as adjacent macroscopically intact parts of lesions. In addition, more detailed *in vitro* studies were precluded due to the lack of suitable PLPP3 antibodies that would allow assessment of the

protein levels. Finally, molecular mechanisms behind the interactions among PLPP3, LPA and LPARs that could modulate atherosclerosis, need to be explored beyond *in vitro* findings, using atherosclerotic animal models.

CONCLUSIONS

We found that PLPP3 is repressed in the advanced stages of human atherosclerosis compared with normal arteries. The enzyme co-localizes in lesions mainly with LPA receptors 5 and 6. Furthermore, expression of LPA receptors is high in the atheroma and LPA co-localizes within plaque regions where the cells expressing PLPP3 and LPAR6 are also found. Collectively, our findings indicate that LPA-mediated inflammatory cascades are relevant in the pathophysiology of human atherosclerosis and that the PLPP3 enzyme and LPARs can modulate the potential atherogenicity of LPA signalling.

Clinical Impact

Altogether, our results support the hypothesis that PLPP3 serves to suppress inflammatory pathways in atherosclerotic plaques and that diminished PLPP3 activity may carry higher risk for developing complications of atherosclerosis. In human lesions, the LPA receptors LPAR2, LPAR5 and LPAR6 are expressed in ECs and regulate the response to LPA-induced EC activation in advanced plaques. Low PLPP3 *e*xpression levels in plaques appear to increase the local accumulation of the specific LPA molecular species (18:0). Our proposal is that this phospholipid should be investigated as the plasma biomarker for risk associated with low activity of LPP3 and consequently of LPA-related receptor activation. Moreover, our findings open the possibilities for investigations into the anti-atherosclerotic effects that may be achieved by therapies aimed to (a) increase the levels of PLPP3, thus reducing the levels of pro-inflammatory products, or (b) modulate the activity of the LPA membrane receptor(s). The first option would require the development of therapeutic approaches for increasing the *PLPP3* gene product expression or the PLPP3 enzyme activity (i.e. localized gene therapy, modified RNA or recombinant protein in stable liposomes)[32]. However, increasing PLPP3 expression is hazardous as this enzyme hydrolyses also S1P which is critical for lymphocyte egress and blood vessel integrity. A high affinity S1P-receptor agonist is currently in clinical development as an immunomodulator for transplantation and autoimmunity[33]. Alternatively, modulation of LPA receptor signalling, a target of known druggable class[15], would imply the development of more frequently used therapeutic strategies, such as small molecules or biologicals.

METHODS

Human material

Atherosclerotic plaques were obtained from patients undergoing surgery for high grade (>50% NASCET)[34] carotid stenosis at the Department of Vascular Surgery, Karolinska

University Hospital, Stockholm, Sweden and clinical data were recorded on admission. Symptomatic patients (S) were defined based on the following symptoms: transitory ischemic attack (TIA), minor stroke (MS) and *amaurosis fugax* (AF). Patients without qualifying symptoms within 6 months prior to surgery were categorized as asymptomatic (AS) and indication for carotid endoarterectomy (CEA) based on results from the Asymptomatic Carotid Surgery Trial (ACST). A mandatory examination of the carotid arteries (by duplex US and/or CT angiography) was performed prior to surgery. Patients with severe disability after major stroke were excluded since the remaining cost-benefit of stroke-prevention is limited and does not outweigh the risk of surgery. In the study group, patients with atrial fibrillation were also excluded in order to minimize analysis of carotid lesions in patients with symptoms from cardioembolic- rather than atheroembolic origin.

As previously described[35], human carotid endarterectomy samples (carotid plaques, CP) and blood were collected at surgery and retained within the **Bi**obank of **K**arolinska **E**ndarterectomies (**BiKE**). Normal artery controls (NA) were obtained from nine macroscopically disease-free iliac arteries and one aorta from organ donors without history of cardiovascular disease. The BiKE study is approved by the Ethical Committee of Northern Stockholm with following ethical permits: EPN DNr 95- 276/277; 02-146; 02-147, 2005/83-31; 2009/512-31/2; 2009/295-31/2; 2011/950-32; 2012/619-32 and 213/2137-32. The project is performed under the Swedish biobank regulations and prospective sampling is approved with informed consent procedure (DNr 2009/512-31/2). BiKE is registered at Socialstyrelsen (The National Board of Health and Welfare) and Biobank of Karolinska and approved by the Swedish Data Inspection Agency (approval date/number 2002-09-30 DNr 916-2002). The BiKE database was merged with the Swedish Hospital Discharge Register and the Swedish Cause of Death Register for follow-up of major adverse cardiovascular, cerebrovascular and vascular events (MACCEs). All samples were collected with informed consent from patients or organ donor guardians.

This study involved 3 non-overlapping subsets of BIKE patients, where 2 sets of plaques were analysed by Affymetrix microarrays (larger set with n=127 plaques of which 87 were from S + 40 from AS patients and smaller set with n=50 plaques where 40 from S + 10 from AS patients). DNA genotyping by Illumina chips was carried out on patients from the 'larger' subset (n=127), and used for eQTL analyses. The third subset of n=18 BiKE plaques (n=9 from S + 9 from AS patients, matched for gender, age and statin medication) were analysed using LC-MS/MS as previously described[35]. For proteomic analyses, a central portion of the plaque corresponding to the maximum stenosis was separated from the respective downstream peripheral end (adjacent tissue) of the plaque and used in comparisons. The BiKE study cohort demographics, details of sample collection, processing and analyses were as previously described[36].

Additional control vascular tissues (n=23) and early fibroateroma lesions were obtained from the SOCRATES biobank (Leiden University Medical Center, the Netherlands). Details of this biobank have been described previously [37]. Briefly, this biobank contains aortic wall patches obtained during kidney transplantation with grafts derived from cadaveric donors. All patches were from grafts that were eligible for transplantation (i.e. all donors met the criteria set by The Eurotransplant Foundation). Sample collection and handling was

performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, the Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org/?s=1&m=82&p=0&v=4#827).
Altogether n=33 normal arteries of different embryonic origins were used as controls in this study. No additional demographic information is available for these samples obtained from organ donors.

***In situ* hybridization (ISH)**
Chromogenic ISH for the detection of the LPAR mRNAs was performed in a Ventana Discovery ULTRA instrument (Ventana Medical Systems Inc., AZ, USA) using the ACD RNAscope® 2.5 Red Kit (Advanced Cell Diagnostics, Newark, CA, USA) and the mRNA Discovery ULTRA RED 4.0 procedure. RNAscope® 2.5 VS Probes for Hs-LPAR1 (#483889), Hs-LPAR2 (#428809), Hs-LPAR3 (#428819), Hs-LPAR4 (#458859), Hs-LPAR5 (#456369), and Hs-LPAR6 (#409359) were designed by the probe manufacturer (Advanced Cell Diagnostics). FFPE sections (5 µm) were applied to Superfrost Plus (Thermo Fisher Scientific) slides, and all operations including baking, deparaffinization, conditioning, pretreatment, ISH and counterstaining using hematoxylin were performed in a Ventana Discovery ULTRA instrument. Following the ISH-procedure in the Ventana instrument, slides were washed in lukewarm tap water with detergent until oil from the slides was fully removed. Slides were finally washed in demineralized water and allowed to air dry before mounting in EcoMount mounting medium (Advanced Cell Diagnostics). Slides were subsequently inspected in bright-field microscopy using an AxioImager.Z1 microscope (Zeiss, Oberkochen Germany) and digital images of selected regions of interest were acquired using 40x and 63x objectives.

Double staining by ISH combined with immunofluorescence (IFL)
Non-decalcified FFPE sections (5 µm) from early fibroatheroma lesion were used for co-staining of mRNA and protein markers. Firstly, as described in the previous section, ISH analyses were performed for LPAR6 and PLPP3 mRNA using the Ventana Discovery ULTRA instrument (Ventana Medical Systems Inc., AZ, USA), the ACD RNAscope® 2.5 Red Kit (Advanced Cell Diagnostics, Newark, CA, USA) and the mRNA Discovery ULTRA RED 4.0 procedure. Hs-LPAR6 (#409359) and Hs-PLPP3 (#456371) for detection of mRNA were designed by the probe manufacturer (Advanced Cell Diagnostics). Secondly, following ISH and washes as previously described, two slides each from LPAR6 and PPAP2B mRNA detection were stained for CD34 and SMA, respectively. Briefly, slides were washed for 5 min in 1xPBS, followed by blocking in 0.1 M Tris-HCl, 0.15 M NaCl, 10% fetal bovine serum and 0.01% Tween-20 for 15 min. Anti-CD34 primary antibody (MAb mouse anti human CD34 Class II clone QBEnd10, DAKO, Glostrup, Denmark) diluted 1:100 and anti-SMA primary antibody (rabbit MAb anti human SMA, EPR5368, Abcam, Cambridge, UK) diluted 1:2000, respectively, were added and incubated 30 min at room temperature (RT). Slides were washed three times in 1xPBS, and then fluorophore labelled secondary antibodies were added. For detection of CD34, Alexa Flour 488 Goat anti Mouse IgG(H+L) (Jackson ImmunoResearch, West Grove, PA, USA) (1:50) was added for 30 min at RT. For detection of SMA, Alexa Flour 488 Goat anti Rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA,

USA) (1:50) was added for 30 min at RT. Slides were washed 2 times in 1xPBS, and mounted with DAPI for epifluorescence microscopy inspection and imaging.

Lipid Mass spectrometry Imaging (MSI) *in situ*

Snap frozen human carotid plaque tissues were sectioned (12 µm thickness) using cryostat CM3050S (Leica, Germany) and thaw mounted on SuperFrost glass slide to enable multiple staining modalities or on ITO conductive glass slide for mass spectrometry imaging experiments and then dried in vacuum chamber[38]. Optimal Cutting Temperature (OCT) compound was purchased from Fischer Scientific (Illkirch, France). For MALDI imaging experiment, two MALDI matrices were used to enable the detection of lipids species the 2,5-Dihydroxybenzoic acid (2,5-DHB) and the 9-aminoacridine (9AA, Sigma-Aldrich, saint-quentin Fallavier, France) for positive and negative detection mode respectively. 2,5-DHB powder (150 mg) was used and vaporized on tissue sample using a home-built sublimation apparatus (150°C, 8 min, 2.10-3 mbar). 9AA solution was prepared at 10 mg/mL in methanol (MeOH)/Water (7:3). The 9AA matrix solution was sprayed onto tissue sections using the SunCollect automatic sprayer (SunChrom, Friedrichsdorf, Germany). MS images were obtained using a SolariX MALDI-FTICR 7.0T (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser used at a repetition rate of 1 kHz. Mass spectra were acquired in the 100–1200 *m/z* range in positive and negative detection mode depending on MALDI matrix. The mass spectrum was obtained by mass spectra average of 300 consecutive laser shots on the same location with a time domain of 1MWord, subsequent single zero filling and sine wave apodization. An ICR noise threshold was fixed at 0.97 for imaging acquisition. An image raster size of 30 µm was selected for human plaque tissues analysis. FTMSControl 3.0 and FlexImaging 4.2 software packages (Bruker Daltonics, Bremen, Germany) were used to control the mass and set imaging parameters. The visualization and statistical analysis of imaging data were performed using *MultiImaging* 1.1 software (ImaBiotech, Lille, France). To combine and compare tissue staining with the lipid distribution, MALDI imaging acquisition was performed on adjacent carotid tissue sections and scanned using a 40x magnification objective on an Olympus IX18 microscope (Olympus, Germany). Scans were then integrated in the *MultiImaging* software for a co-registration with imaging data to accurately correlate the distribution of molecular species with human plaque histological regions. Assignment of lipid species was performed by searching the accurate *m/z* values against the Lipidmap database using a mass tolerance of 3 ppm[39].

Bionformatics and statistical analyses

RNA extracted from endarterectomy (carotid plaque, CP) and control normal artery (NA) specimens was analyzed by Affymetrix microarrays. Robust multiarray average (RMA) normalization and correction for batch effect was performed and processed gene expression data was returned in log2-scale. The microarray dataset is available from Gene Expression Omnibus (GSE21545). Target gene and protein analyses were performed with GraphPad Prism 6 using a two-sided Student's t-test assuming non-equal deviation. Pearson correlations were calculated to determine the association between mRNA expression levels from microarrays, with Bonferroni correction for multiple comparisons. Survival analysis

was done using the Cox regression model [40] with event-free survival as the response variable and log2-transformed gene expression levels as the explanatory variable. The covariates age and gender were tested and had no effect on results. Results from qPCR were evaluated by multiple t-tests assuming non-equal deviation. In comparisons with more than two groups ANOVA was used as appropriate. In all analyses p-value <0.05 was considered to indicate statistical significance.

Additional methods are described in the Supplemental Information file.

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Author contributions
SA, LPM, GH, DvK, DT, KH, AS, BSN, VE, RAB, ML performed experiments and analysed data. GPB, JS, PE and JHNL contributed human material, datasets and method development. All authors were involved in designing the study, joint discussions, writing of the manuscript and comments. AJG, UH and EH-C supervised the study.

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SUPPLEMENTAL MATERIAL

METHODS

Proteomic analysis of plaques

Atherosclerotic plaques from n=18 BiKE patients (n=9 symptomatic + 9 asymptomatic; matched for male gender, age and statin medication) were analysed using LC-MS/MS as previously described[1]. Briefly, protein samples were digested by trypsin and the resulting tryptic peptides were TMT-labeled and pooled. Pooled samples were cleaned by Strong Cation exchange columns (Phenomenex) and subjected to LC-MS/MS analysis. The sample pools were separated on a 4-hour gradient using an UPLC-system (Dionex UltiMate™ 3000) coupled to a Q- Exactive mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). The fragment spectra from the mass spectrometer were matched to a database consisting of theoretical fragment spectra from all human proteins and filtered at a 1% False Discovery Rate on the peptide level to obtain protein identities (Uniprot). Quantitative information was acquired using the TMT reporter ion intensities.

Antibodies

For immunohistochemical studies, the following primary antibodies with the relative concentrations were used: PPAP2B 1:50, LPAR1 1:500 (both from Novus Biologicals, Littleton, CO, USA), LPAR2 1:250, LPAR5, LPAR6 1:400 (all from LSBio, Seattle, WA, USA), smooth muscle α- actin 1:1000 (SMA, DAKO Sweden AB, Stockholm, SE), anti-CD68 1:50 (Novocastra, Bromma, SE) and anti-CD31 1:200 (Abcam, Cambridge, UK).

Immunohistochemical (IHC) stainings

IHC reagents were from Biocare Medical (Concord, CA). Tissues were treated as previously described[2]. In brief, tissues were fixed for 48 hours in 4% Zn-formaldehyde at room temperature and paraffin-embedded. Isotype rabbit and mouse IgG were used as negative controls. 5 µm tissue sections were deparaffinized in Tissue Clear and rehydrated in graded ethanol. For antigen retrieval, slides were heated in DIVA buffer (pH 6.0) for 20 min with peak at 121°C. Blocking was performed with Background Sniper for 20 min and primary antibodies were diluted in Da Vinci Green solution, and incubated at room temperature for 1 hour. The single stainings were detected by a probe-polymer system for rabbit, followed by Warp Red chromogen. For double staining, a double-stain probe- polymer system containing alkaline phosphatase and horseradish peroxidase was applied, followed by detection with Warp Red and Vina Green. All slides were counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA) and mounted in Pertex (Histolab, Gothenburg, Sweden). Images were scanned by an automated ScanScope slidescanner (Hamamatsu, Kista, Sweden) or acquired by a Nikon OPTIPHOT-2 microscope equipped with a digital camera and processed with NIS-Elements software. Magnifications are indicated in the figure legends.

***In vitro* experiments**

Primary human umbilical vein endothelial cells (HUVECs, Lonza, the Netherlands) were cultured in EBM2 medium supplemented with bullet kit (EGM-2, Lonza) under normoxic conditions (21% O2). Passages 5 to 7 were used throughout the study. Knockdown of LPAR6 and PPAP2B was achieved by transfection with a mix of 4 specific siRNA sequences directed against the human mRNA (SMARTpool siGENOME, GE Dharmacon, Lafayette, CO) in 70% subconfluent HUVEC cultures. Cells were incubated for 1 hour in a small volume of EGM- 2 medium supplemented with DharmaFECT 1 (GE Dharmacon, Lafayette, CO) according to manufacturer's instructions. After 1-2 hours cells were supplemented with extra EGM-2 medium to complement medium volumes. As controls, HUVECs were transfected with a mix of 4 scrambled, non-targeting siRNAs (siSham Smartpool; GE Dharmacon, Lafayette, CO). 48h after siRNA transfection HUVECs were treated with 10 μM Lysophosphatidic acid (Santa Cruz, Dallas, TX). 2 hours after LPA treatment cells were harvested and RNA was isolated with the NucleoSpin RNA kit according to the manufacturer's protocol (Macherey-Nage, Düren, Germany). Isolated RNA (500 ng) was reverse transcribed into cDNA (iSCript Adv cDNA kit for RT-qPCR, Bio-Rad, Hercules, CA) and analyzed by real-time fluorescence assessment of SYBR® Green signal in the iCycler iQ Detection system (Bio- Rad). Primers were designed for the human genes of interest. mRNA levels were analyzed and corrected for the housekeeping gene beta-actin. Reported values were normalized to sham that was arbitrarily assigned an average value of 1[3].

Table S1. Expression correlation analyses between PPAP2B and genes of interest in plaques.
Pearson correlation analyses were calculated from n=127 human plaque microarrays, p-values are corrected for multiple comparisons according to the Bonferroni method. Correlation considered weak if r < 0.3, moderate if 0.3 < r < 0.5 and strong if r > 0.5. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

	Gene Symbol	Pearson r	p-value	Significance level
Cell type markers				
Smooth muscle cells				
Myosin heavy chain 11	*MYH11*	0.2759	0.0018	**
Smoothelin	*SMTN*	0.2057	0.0209	*
Alpha smooth muscle actin	*ACTA2*	0.3752	< 0.0001	****
Myocardin	*MYOCD*	0.3399	< 0.0001	****
Transgelin	*TAGLN*	0.4809	< 0.0001	****
Endothelial cells				
von Willebrand factor	*VWF*	0.01506	0.8671	ns
PECAM-1 (CD31)	*PECAM1*	0.5245	< 0.0001	****
Dendritic cells				
ITGAX (CD11c)	*ITGAX*	-0.1212	0.1746	ns
LY75 (CD205)	*LY75*	0.04661	0.6028	ns
CD80	*CD80*	-0.1459	0.1018	ns
T Lymphocytes				
CD11b	*ITGAM*	-0.1982	0.0261	*
ITGAL	*ITGAL*	-0.1949	0.0287	*
CD27	*CD27*	-0.07268	0.4187	ns
CD28	*CD28*	0.2105	0.018	*
CD3 delta	*CD3D*	-0.02751	0.7598	ns
CD4	*CD4*	0.1938	0.0297	*
CD8A	*CD8A*	-0.07039	0.4335	ns
PTPRC (CD45RA)	*PTPRC*	0.3796	< 0.0001	****
CD69	*CD69*	-0.06686	0.4552	ns
ITGAE	*ITGAE*	0.08025	0.3698	ns
FABP4	*FABP4*	0.01908	0.8314	ns
Macrophages				
CD83	*CD83*	-0.02925	0.7451	ns
CD86	*CD86*	0.3196	0.0003	***
CD163	*CD163*	0.2817	0.0014	**
TNFRSF9	*TNFRSF9*	-0.1674	0.0611	ns
CD40	*CD40*	-0.1155	0.1977	ns
CD36	*CD36*	0.1858	0.0365	*
Inflammation/ Apoptosis/ Calcification markers				
IL-1beta	*IL1B*	0.05357	0.5513	ns
NFkB	*NFKB1*	0.3644	< 0.0001	****
TNF-alpha	*TNFA*	-0.2183	0.0141	*
MCP-1	*CCL2*	0.3241	0.0002	***
Caspase-3	*CASP3*	0.1682	0.0598	ns
Caspase-7	*CASP7*	0.08284	0.3564	ns
Caspase-9	*CASP9*	0.1407	0.116	ns
BCL2	*BCL2*	0.1983	0.026	*
RANTES	*CCL5*	-0.2318	0.009	**
BMP4	*BMP4*		< 0.0001	****

S1 Table continued

	Gene Symbol	Pearson r	p-value	Significance level
Extracellular matrix/ degradation				
MMP9	*MMP9*	0.03487	0.6982	ns
TIMP1	*TIMP1*	0.3178	0.0003	***
Sulfatase 1	*SULF1*	0.6295	< 0.0001	****
Sulfatase 2	*SULF2*	0.1564	0.0803	ns
Growth factors				
TGFB1	*TGFB1*	0.3565	< 0.0001	****
TGFA	*TGFA*	-0.1275	0.155	ns
IGF1	*IGF1*	0.4746	< 0.0001	****
PDGFA	*PDGFA*	-0.3214	0.0002	***
PDGFB	*PDGFB*	-0.2063	0.0205	*
PDGFC	*PDGFC*	0.3403	< 0.0001	****
PDGFD	*PDGFD*	0.4509	< 0.0001	****
Chemokines and receptors				
CCR2	*CCR2*	0.425	< 0.0001	****
CCR5	*CCR5*	0.0959	0.2835	ns
Interleukin 10	*IL10*	-0.2681	0.0024	**
Interferon gamma	*INFG*	-0.2756	0.0018	**
IL2	*IL2*	-0.1016	0.2578	ns
IL6	*IL6*	0.01629	0.8557	ns
IL4	*IL4*	-0.2411	0.0065	**
IL5	*IL5*	-0.01216	0.8925	ns
IL9	*IL9*	-0.172	0.0542	ns

Table S2. Expression correlation analyses between LPARs and various markers in plaques.
Pearson correlation analyses were calculated from n=127 human plaque microarrays, p-values are corrected for multiple comparisons according to the Bonferroni method. Correlation considered weak if r < 0.3, moderate if 0.3 < r < 0.5 and strong if r > 0.5. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

		LPAR1			LPAR2		
	Gene Symbol	Pearson r	p-value	Signi-ficance level	Pearson r	p-value	Signi-ficance level
Cell type markers							
Smooth muscle cells							
Myosin heavy chain 11	*MYH11*	0.6706	< 0.0001	****	-0.4431	< 0.0001	****
Smoothelin	*SMTN*	0.4657	< 0.0001	****	-0.4536	< 0.0001	****
Alpha smooth muscle actin	*ACTA2*	0.6343	< 0.0001	****	-0.4332	< 0.0001	****
Myocardin	*MYOCD*	0.6585	< 0.0001	****	-0.5676	< 0.0001	****
Transgelin	*TAGLN*	0.5662	< 0.0001	****	-0.5853	< 0.0001	****
Endothelial cells							
von Willebrand factor	*VWF*	-0.3296	0.0002	***	0.4895	< 0.0001	****
PECAM-1 (CD31)	*PECAM1*	-0.1619	0.0701	ns	0.2939	0.0008	***

S2 Table continued

Dendritic cells							
ITGAX (CD11c)	*ITGAX*	-0.5713	< 0.0001	****	0.5664	< 0.0001	****
LY75 (CD205)	*LY75*	-0.002261	0.9799	ns	0.4875	< 0.0001	****
CD80	*CD80*	-0.3983	< 0.0001	****	0.4718	< 0.0001	****
T Lymphocytes							
CD11b	*ITGAM*	-0.5282	< 0.0001	****	0.4891	< 0.0001	****
ITGAL	*ITGAL*	-0.4416	< 0.0001	****	0.6066	< 0.0001	****
CD27	*CD27*	-0.419	< 0.0001	****	0.3069	0.0005	***
CD28	*CD28*	-0.2708	0.0022	**	0.4252	< 0.0001	****
CD3 delta	*CD3D*	-0.3652	< 0.0001	****	0.1419	0.113	ns
CD4	*CD4*	-0.5254	< 0.0001	****	0.08309	0.355	ns
CD8A	*CD8A*	-0.3322	0.0001	***	0.2653	0.0027	**
PTPRC (CD45RA)	*PTPRC*	-0.2482	0.0049	**	0.3816	< 0.0001	****
CD69	*CD69*	-0.07406	0.408	ns	0.4384	< 0.0001	****
ITGAE	*ITGAE*	-0.1859	0.0364	*	0.5556	< 0.0001	****
FABP4	*FABP4*	-0.3392	< 0.0001	****	0.4484	< 0.0001	****
Macrophages							
CD83	*CD83*	-0.4417	< 0.0001	****	0.465	< 0.0001	****
CD86	*CD86*	-0.4989	< 0.0001	****	0.5162	< 0.0001	****
RANK	*TNFRSF11A*	-0.4012	< 0.0001	****	0.6283	< 0.0001	****
CD163	*CD163*	-0.4111	< 0.0001	****	0.5445	< 0.0001	****
TNFRSF9	*TNFRSF9*	-0.621	< 0.0001	****	0.3327	0.0001	***
CD40	*CD40*	-0.5126	< 0.0001	****	0.4241	< 0.0001	****
CD36	*CD36*	-0.3921	< 0.0001	****	0.5142	< 0.0001	****
Inflammation/ Apoptosis/ Calcification markers							
IL-1beta	*IL1B*	-0.2719	0.0021	**	0.242	0.0063	**
NFkB	*NFKB1*	-0.1226	0.1714	ns	0.1519	0.0896	ns
TNF-alpha	*TNFA*	-0.484	< 0.0001	****	0.3666	< 0.0001	****
MCP-1	*CCL2*	-0.1699	0.0571	ns	0.01676	0.8522	ns
Caspase-3	*CASP3*	-0.05183	0.5644	ns	0.5511	< 0.0001	****
Caspase-7	*CASP7*	-0.005042	0.9553	ns	0.3474	< 0.0001	****
Caspase-9	*CASP9*	-0.02516	0.7797	ns	0.2577	0.0036	**
BCL2	*BCL2*	0.4797	< 0.0001	****	-0.4165	< 0.0001	****
RANTES	*CCL5*	-0.4499	< 0.0001	****	0.44	< 0.0001	****
BMP4	*BMP4*	0.04733	0.5987	ns	0.4274	< 0.0001	****
Extracellular matrix/ degradation							
MMP9	*MMP9*	-0.4874	< 0.0001	****	0.4547	< 0.0001	****
TIMP1	*TIMP1*	-0.3947	< 0.0001	****	0.334	0.0001	***
Sulfatase 1	*SULF1*	-0.325	0.0002	***	0.5368	< 0.0001	****
Sulfatase 2	*SULF2*	0.3379	0.0001	***	-0.3382	0.0001	***
Growth factors							
TGFB1	*TGFB1*	-0.2904	0.001	***	-0.4719	< 0.0001	****
TGFA	*TGFA*	-0.3053	0.0005	***	0.5867	< 0.0001	****
IGF1	*IGF1*	-0.1903	0.0328	*	0.4513	< 0.0001	****
PDGFA	*PDGFA*	0.5479	< 0.0001	****	0.02336	0.7951	ns
PDGFB	*PDGFB*	-0.408	< 0.0001	****	0.1198	0.1813	ns
PDGFC	*PDGFC*	0.5948	< 0.0001	****	-0.1977	0.0265	*
PDGFD	*PDGFD*	0.6034	< 0.0001	****	-0.4602	< 0.0001	****

S2 Table continued

		LPAR1			LPAR2		
Chemokines and receptors							
CCR2	*CCR2*	-0.07643	0.3931	ns	0.3701	< 0.0001	****
CCR5	*CCR5*	-0.4306	< 0.0001	****	0.4636	< 0.0001	****
Interleukin 10	*IL10*	-0.3689	< 0.0001	****	0.5818	< 0.0001	****
Interferon gamma	*INFG*	-0.3075	0.0005	***	-0.03486	0.6984	ns
IL2	*IL2*	-0.1837	0.0395	*	-0.2099	0.0183	*
IL6	*IL6*	-0.03277	0.7145	ns	0.2466	0.0052	**
IL4	*IL4*	-0.1667	0.0621	ns	-0.2958	0.0008	***
IL5	*IL5*	-0.007118	0.9369	ns	-0.4802	< 0.0001	****
IL9	*IL9*	-0.1642	0.0662	ns	-0.206	0.0206	*

		LPAR3			LPAR4		
	Gene Symbol	**Pearson r**	**p-value**	**Signi-ficance level**	**Pearson r**	**p-value**	**Signi-ficance level**
Cell type markers							
Smooth muscle cells							
Myosin heavy chain 11	*MYH11*	-0.2946	0.0008	***	0.1502	0.0933	ns
Smoothelin	*SMTN*	0.4251	< 0.0001	****	-0.3744	< 0.0001	****
Alpha smooth muscle actin	*ACTA2*	-0.2514	0.0045	**	0.1165	0.1939	ns
Myocardin	*MYOCD*	-0.09252	0.3028	ns	0.03997	0.6568	ns
Transgelin	*TAGLN*	0.03132	0.7278	ns	-0.1038	0.2472	ns
Endothelial cells							
von Willebrand factor	*VWF*	-0.3106	0.0004	***	0.1736	0.0519	ns
PECAM-1 (CD31)	*PECAM1*	-0.1621	0.0698	ns	0.1127	0.2089	ns
Dendritic cells							
ITGAX (CD11c)	*ITGAX*	0.3511	< 0.0001	****	-0.07009	0.4336	ns
LY75 (CD205)	*LY75*	-0.4494	< 0.0001	****	0.06596	0.4613	ns
CD80	*CD80*	-0.2953	0.0007	***	0.1664	0.0614	ns
T Lymphocytes							
CD11b	*ITGAM*	0.4518	< 0.0001	****	-0.4083	< 0.0001	****
ITGAL	*ITGAL*	-0.1041	0.2458	ns	-0.01843	0.8377	ns
CD27	*CD27*	0.03483	0.6987	ns	-0.2123	0.017	*
CD28	*CD28*	-0.254	0.0041	**	-0.2748	0.0018	**
CD3 delta	*CD3D*	0.1412	0.1148	ns	-0.1471	0.1001	ns
CD4	*CD4*	0.2873	0.0011	**	-0.4718	< 0.0001	****
CD8A	*CD8A*	0.05853	0.515	ns	-0.09318	0.2994	ns
PTPRC (CD45RA)	*PTPRC*	-0.2953	0.0007	***	0.3897	< 0.0001	****
CD69	*CD69*	-0.4398	< 0.0001	****	0.02757	0.7583	ns
ITGAE	*ITGAE*	-0.2002	0.024	*	0.03967	0.6579	ns
FABP4	*FABP4*	-0.02759	0.7582	ns	-0.07478	0.4034	ns
Macrophages							
CD83	*CD83*	0.06016	0.5034	ns	-0.09816	0.2742	ns
CD86	*CD86*	-0.005174	0.9541	ns	-0.09719	0.279	ns
RANK	*TNFRSF11A*	0.02527	0.7788	ns	-0.1704	0.0564	ns

S2 Table continued

CD163	*CD163*	-0.268	0.0024	**	0.02635	0.7696	ns
TNFRSF9	*TNFRSF9*	0.3201	0.0003	***	-0.2192	0.0137	*
CD40	*CD40*	0.3923	< 0.0001	****	0.4597	< 0.0001	****
CD36	*CD36*	-0.1493	0.0939	ns	-0.1578	0.0765	ns
Inflammation/Apoptosis/ Calcification markers							
IL-1beta	*IL1B*	-0.01481	0.8692	ns	-0.1345	0.1333	ns
NFkB	*NFKB1*	0.007307	0.9353	ns	-0.1105	0.2179	ns
TNF-alpha	*TNFA*	0.1344	0.1334	ns	-0.1546	0.0839	ns
MCP-1	*CCL2*	-0.001336	0.9882	ns	-0.1377	0.1243	ns
Caspase-3	*CASP3*	-0.4667	< 0.0001	****	0.2803	0.0015	**
Caspase-7	*CASP7*	-0.446	< 0.0001	****	0.106	0.2374	ns
Caspase-9	*CASP9*	-0.3026	0.0006	***	0.3252	0.0002	***
BCL2	*BCL2*	0.487	< 0.0001	****	-0.1836	0.0396	*
RANTES	*CCL5*	0.07816	0.3843	ns	-0.2627	0.003	**
BMP4	*BMP4*	-0.3192	0.0003	***	0.162	0.07	ns
Extracellular matrix/degradation							
MMP9	*MMP9*	-0.004226	0.9625	ns	-0.1193	0.1834	ns
TIMP1	*TIMP1*	-0.009881	0.9126	ns	-0.2127	0.0168	*
Sulfatase 1	*SULF1*	-0.2694	0.0023	**	-0.08024	0.3718	ns
Sulfatase 2	*SULF2*	-0.5118	< 0.0001	****	0.1538	0.0856	ns
Growth factors							
TGFB1	*TGFB1*	0.4135	< 0.0001	****	-0.3427	< 0.0001	****
TGFA	*TGFA*	0.4058	< 0.0001	****	0.123	0.17	ns
IGF1	*IGF1*	-0.2969	0.0007	***	0.1296	0.148	ns
PDGFA	*PDGFA*	-0.5459	< 0.0001	****	0.3771	< 0.0001	****
PDGFB	*PDGFB*	0.2634	0.0029	**	-0.1694	0.058	ns
PDGFC	*PDGFC*	-0.4801	< 0.0001	****	0.269	0.0023	**
PDGFD	*PDGFD*	-0.2303	0.0095	**	0.2796	0.0015	**
Chemokines and receptors							
CCR2	*CCR2*	-0.3749	< 0.0001	****	0.05098	0.5692	ns
CCR5	*CCR5*	-0.06846	0.4444	ns	-0.2076	0.0192	*
Interleukin 10	*IL10*	-0.1088	0.2254	ns	-0.1253	0.1622	ns
Interferon gamma	*INFG*	0.1622	0.0696	ns	-0.1909	0.0323	*
IL2	*IL2*	0.3133	0.0004	***	-0.2224	0.0123	*
IL6	*IL6*	-0.2154	0.015	*	-0.05445	0.5432	ns
IL4	*IL4*	0.4896	< 0.0001	****	-0.3051	0.0005	***
IL5	*IL5*	0.404	< 0.0001	****	0.02027	0.8218	ns
IL9	*IL9*	0.4855	< 0.0001	****	-0.2934	0.0009	***

		LPAR5			**LPAR6**		
	Gene Symbol	**Pearson r**	**p-value**	**Signi-ficance level**	**Pearson r**	**p-value**	**Signi-ficance level**
Cell type markers							
Smooth muscle cells							
Myosin heavy chain 11	*MYH11*	0.3653	< 0.0001	****	-0.2408	0.0066	**
Smoothelin	*SMTN*	-0.3695	< 0.0001	****	-0.5444	< 0.0001	****
Alpha smooth muscle actin	*ACTA2*	-0.3167	0.0003	***	-0.2255	0.0111	*

S2 Table continued

		LPAR5			LPAR6		
Myocardin	*MYOCD*	-0.407	< 0.0001	****	-0.2115	0.0174	*
Transgelin	*TAGLN*	-0.422	< 0.0001	****	-0.2844	0.0013	**
Endothelial cells							
von Willebrand factor	*VWF*	0.472	< 0.0001	****	0.6393	< 0.0001	****
PECAM-1 (CD31)	*PECAM1*	0.1995	0.0251	*	0.163	0.0683	ns
Dendritic cells							
ITGAX (CD11c)	*ITGAX*	0.4244	< 0.0001	****	-0.3362	0.0001	***
LY75 (CD205)	*LY75*	0.4917	< 0.0001	****	0.4139	< 0.0001	****
CD80	*CD80*	0.5655	< 0.0001	****	0.4392	< 0.0001	****
T Lymphocytes							
CD11b	*ITGAM*	0.3943	< 0.0001	****	0.3279	0.0002	***
ITGAL	*ITGAL*	0.5303	< 0.0001	****	0.511	< 0.0001	****
CD27	*CD27*	0.2685	0.0024	**	0.3421	< 0.0001	****
CD28	*CD28*	0.6452	< 0.0001	****	0.5678	< 0.0001	****
CD3 delta	*CD3D*	0.2161	0.0151	*	0.2485	0.005	**
CD4	*CD4*	0.1155	0.1977	ns	0.05921	0.5102	ns
CD8A	*CD8A*	0.2882	0.0011	**	0.3144	0.0003	***
PTPRC (CD45RA)	*PTPRC*	0.4544	< 0.0001	****	0.6948	< 0.0001	****
CD69	*CD69*	0.5144	< 0.0001	****	0.5524	< 0.0001	****
ITGAE	*ITGAE*	0.2536	0.004	**	0.2311	0.0089	**
FABP4	*FABP4*	0.1492	0.0942	ns	0.1873	0.035	*
Macrophages							
CD83	*CD83*	0.4243	< 0.0001	****	0.2171	0.0146	*
CD86	*CD86*	0.4769	< 0.0001	****	0.5662	< 0.0001	****
RANK	*TNFRSF11A*	0.5756	< 0.0001	****	0.4303	< 0.0001	****
CD163	*CD163*	0.4787	< 0.0001	****	0.5628	< 0.0001	****
TNFRSF9	*TNFRSF9*	0.2011	0.0239	*	-0.01977	0.8261	ns
CD40	*CD40*	0.4126	< 0.0001	****	0.2321	0.0089	**
CD36	*CD36*	0.3239	0.0002	***	0.3013	0.0006	***
Inflammation/ Apoptosis/ Calcification markers							
IL-1beta	*IL1B*	0.4214	< 0.0001	****	0.3324	0.0001	***
NFkB	*NFKB1*	0.2796	0.0015	**	0.3435	< 0.0001	****
TNF-alpha	*TNFA*	0.3693	< 0.0001	****	0.3589	< 0.0001	****
MCP-1	*CCL2*	0.1886	0.0344	*	0.334	0.0001	***
Caspase-3	*CASP3*	0.4408	< 0.0001	****	0.5471	< 0.0001	****
Caspase-7	*CASP7*	0.4525	< 0.0001	****	0.5657	< 0.0001	****
Caspase-9	*CASP9*	0.365	< 0.0001	****	0.3399	< 0.0001	****
BCL2	*BCL2*	-0.3679	< 0.0001	****	-0.2746	0.0019	**
RANTES	*CCL5*	0.4579	< 0.0001	****	0.3982	< 0.0001	****
BMP4	*BMP4*	0.2555	0.0039	**	-0.0006809	0.994	ns
Extracellular matrix/ degradation							
MMP9	*MMP9*	0.218	0.0142	*	0.1144	0.2023	ns
TIMP1	*TIMP1*	0.3771	< 0.0001	****	0.2514	0.0045	**
Sulfatase 1	*SULF1*	0.4413	< 0.0001	****	0.4089	< 0.0001	****
Sulfatase 2	*SULF2*	0.3157	0.0003	***	0.3571	< 0.0001	****

S2 Table continued

Growth factors							
TGFB1	*TGFB1*	-0.4978	< 0.0001	****	-0.2573	0.0036	**
TGFA	*TGFA*	0.5957	< 0.0001	****	0.57	< 0.0001	****
IGF1	*IGF1*	0.4343	< 0.0001	****	0.6966	< 0.0001	****
PDGFA	*PDGFA*	0.08341	0.3531	ns	0.2096	0.0185	*
PDGFB	*PDGFB*	0.1527	0.0878	ns	0.04191	0.6412	ns
PDGFC	*PDGFC*	0.06585	0.4638	ns	0.2563	0.0038	**
PDGFD	*PDGFD*	-0.2219	0.0125	*	0.1722	0.0539	ns
Chemokines and receptors							
CCR2	*CCR2*	0.5877	< 0.0001	****	0.7626	< 0.0001	****
CCR5	*CCR5*	0.4241	< 0.0001	****	0.4083	< 0.0001	****
Interleukin 10	*IL10*	0.4787	< 0.0001	****	0.3627	< 0.0001	****
Interferon gamma	*INFG*	-0.01569	0.8616	ns	0.01233	0.891	ns
IL2	*IL2*	-0.175	0.0501	ns	-0.1519	0.0895	ns
IL6	*IL6*	0.3142	0.0003	***	0.2623	0.0029	**
IL4	*IL4*	-0.1973	0.0268	*	-0.4218	< 0.0001	****
IL5	*IL5*	-0.4005	< 0.0001	****	-0.2844	0.0012	**
IL9	*IL9*	-0.2452	0.0056	**	-0.3565	< 0.0001	****

Table S3. Expression correlation analyses between PPAP2B and various LPARs in plaques.
Pearson correlation analyses were calculated from n=127 human plaque microarrays, p-values are corrected for multiple comparisons according to the Bonferroni method. Correlation considered weak if r < 0.3, moderate if 0.3 < r < 0.5 and strong if r > 0.5. **p<0.01 and ****p<0.0001.

Correlation	Pearson r	95% confidence interval	P (two-tailed)	Significance level
PPAP2B vs. LPAR1	0.1586	-0.01608 to 0.3239	0.0749	ns
PPAP2B vs. LPAR2	-0.4009	-0.5376 to -0.2437	< 0.0001	****
PPAP2B vs. LPAR3	-0.2321	-0.3906 to -0.06033	0.0086	**
PPAP2B vs. LPAR4	-0.1145	-0.2831 to 0.06098	0.1999	ns
PPAP2B vs. LPAR5	-0.09513	-0.2650 to 0.08046	0.2874	ns
PPAP2B vs. LPAR6	0.366	0.2048 to 0.5079	< 0.0001	****

Table S4. Analysis of lipid species in human carotid plaques.
Molecular species (a) observed in human plaque by positive and negative ionization detection mode based on m/z value. (b) m/z calculated from molecular formula and (c) m/z measured using FTICR-MS (d) mass accuracy error unit in part per million (ppm).

[a]Compound Name		Ion Cluster	[b]*m/z calcd*	[c]*m/z measd*	[d]Error (ppm)
CE (18:2)	Cholesteryl linoleate	$[M+K]^+$	687.5477	687.5486	1.3
CE (18:1)	Cholesteryl oleate	$[M+K]^+$	689.5633	689.5639	0.9
CE (18:0)	Cholesteryl stearate	$[M+K]^+$	691.5790	691.5798	1.2
PC (34:1)	Phophatydilcholine	$[M+Na]^+$	782.5670	782.5673	0.3
PC (34:2)	Phophatydilcholine	$[M+H]^+$	758.5694	758.5696	0.2
LPC (18:2)	LysoPC	$[M+H]^+$	520.3409	520.3412	0.6
LPC (18:1)	LysoPC	$[M+H]^+$	522.3554	522.3559	1.0
LPC (18:0)	LysoPC	$[M+H]^+$	524.3710	524.3714	0.8
PA (36:2)	Phosphatidic acid	$[M-H]^-$	699.4970	699.4976	0.9
LPA (18:0)	LysoPA	$[M-H2O-H]^-$	419.2568	419.2572	1.0

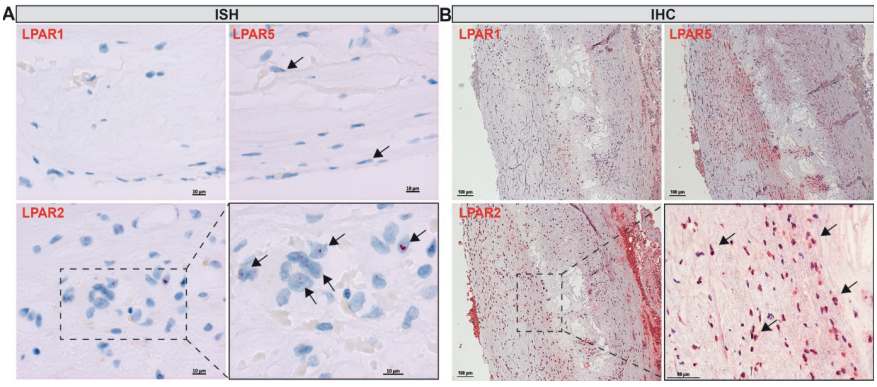

Figure S1. Localisation of LPARs in plaques.
In situ hybridization detection of LPARs 1, 5 and 2 mRNA transcripts in human lesions. Arrows indicate the RNA probe signals (red) (A). Immunohistochemistry staining of LPARs 1, 5 and 2 in plaques (red signal). Arrows in the enlarged LPAR2 image indicate positive signal in the cells within the necrotic core. Nuclei (purple) are stained with hematoxylin (B). Images taken with 20x, 40x and 63x objectives.

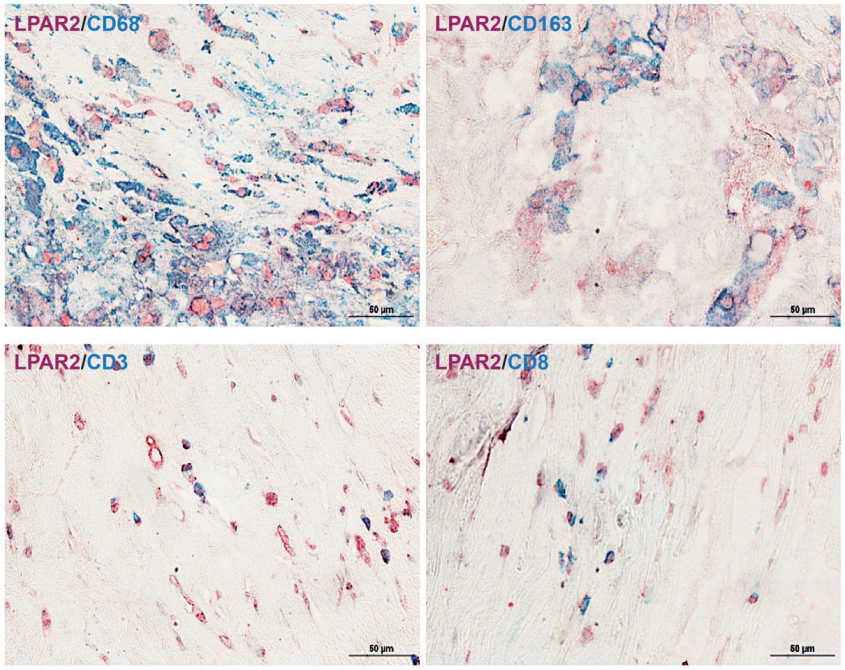

Figure S2. Immunolocalisation of LPAR2 in plaque inflammatory cells.
Double immunohistochemistry stainings shwing the colocalisation of LPAR2 (red signal) with macrophage cell markers (CD68 and CD163, green signal) and lymphocyte markers (CD3 and CD8, green signal). Images taken with the 40x objective.

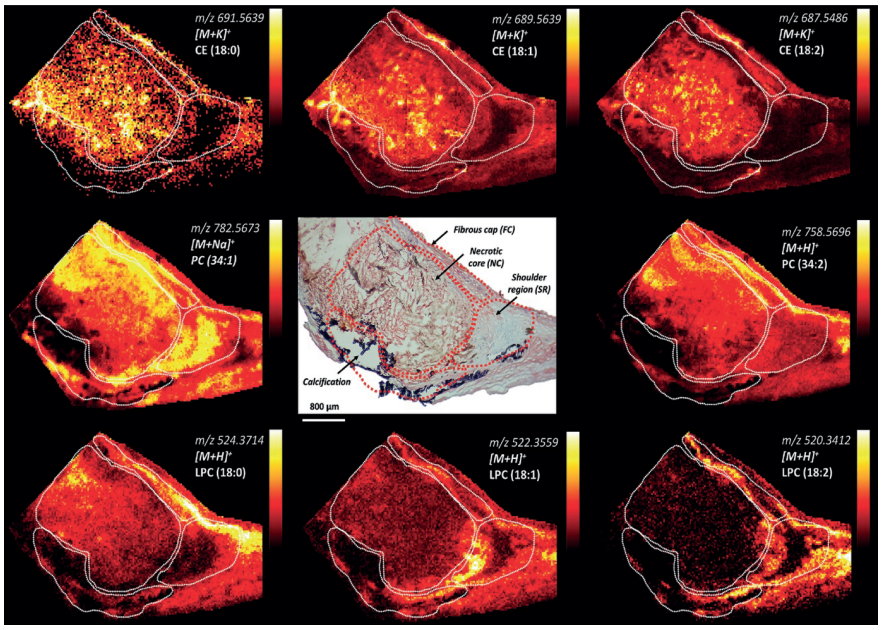

Figure S3. Molecular histology of the human atherosclerotic plaque based on its lipid fingerprint. Molecular distribution of the main lipid species from different classes (CE: Cholesteryl ester, PC: Phophatydilcholine and LPC: lysophosphatidylcholine) detected in human plaque tissue by mass spectrometry (MS) imaging using MALDI-FTICR in positive detection mode at 30 µm of spatial resolution. Identification of molecular species was performed by accurate MS match with database (<1 ppm) and MS/MS measurement. The different lipid classes are reported on the figure. Relative intensity scale (volcano intensity scale, 0-100%) is indicated on the side of each image. Histological regions of interest were identified by Oil-red-O staining (central picture, arrows).

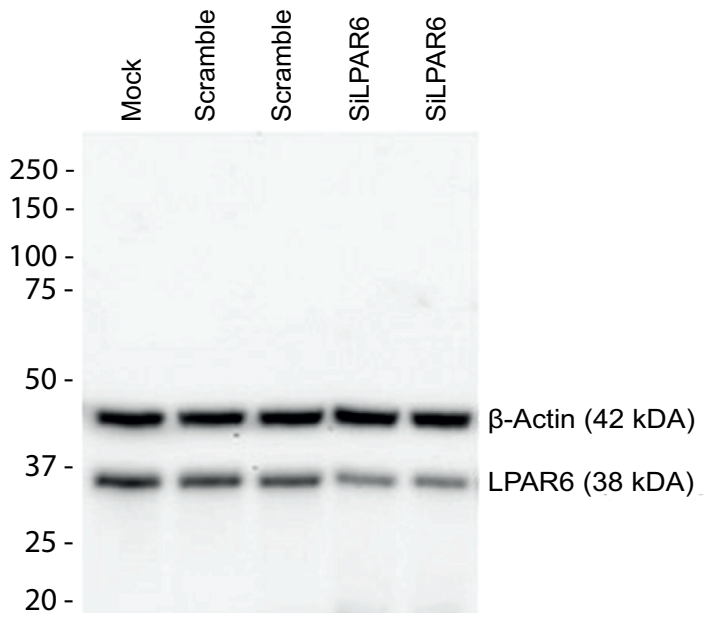

Figure S4. Western blot analysis of LPAR6 protein levels in HUVEC lysates.
LPAR6 mRNA silencing resulted in repression of the protein levels compared to non-targeting scramble oligos and mock control. Protein levels of beta-actin were used as loading control. Ladder marks indicated on the left.

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PART THREE

CAT EYE SYNDROME CRITICAL REGION PROTEIN 1

CHAPTER 6

CECR1 diminishes adenosine induced neutrophil activity and is associated with decreased survival probability in humans

In preparation

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ABSTRACT

Introduction

In recent years, many studies have implicated activation of neutrophils in the process of atherosclerosis. Neutrophils can be activated by adenosine which is commonly found in atherosclerotic lesions. Patients afflicted by loss-of-function mutations in Adenosine Deaminase 2, which is encoded by the gene Cat eye syndrome critical region protein 1 (CECR1) and converts adenosine into inosine, suffer from various vascular and inflammatory phenotypes related to disturbed neutrophil activity. We hypothesized that CECR1 plays a role in atherosclerosis development by regulating neutrophil activation.

Methods and Results

Immunohistochemical (IHC) staining of human carotid arteries showed that CECR1 protein is present in human atherosclerotic lesions. Subsequent whole mount in situ hybridization (ISH) indicated that *CECR1* mRNA is produced in atherosclerotic lesions. *In situ* mass spectrometry imaging was combined with IHC and ISH of CECR1 and showed that regions with high CECR1 expression were devoid of adenosine but were highly enriched in inosine, indicating that CECR1 converts adenosine into inosine in human atherosclerotic lesions. Slow Off-Rate Modified Aptamer Arrays on human serum showed that CECR1 levels correlate with lower levels of circulating neutrophils, are increased in patients suffering from coronary heart disease and myocardial infarction and that high levels of CECR1 correlate with a lower post incident coronary heart disease survival probability in humans. Furthermore, it was shown that adenosine strongly induced *in vitro* neutrophil activation as seen by enhanced NETosis and subsequent *in vitro* monocyte activation as seen by enhanced MCP-1 and IL-1β gene expression. NETosis and monocyte activation were inhibited upon costimulation with CECR1.

Conclusion

These results indicate, for the first time, that CECR1 is present and active in atherosclerotic patients and suggest that modulation of adenosine-mediated neutrophil activation and inflammation may contribute to a novel and directed therapeutic approach in the treatment of cardiovascular disease.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease[1,2] characterized by the progressive neointimal lesion formation and lumen narrowing of affected arteries[3]. This multi-factorial inflammatory process[4] results in diverse cerebrovascular and cardiovascular complications associated with high mortality and morbidity where acute myocardial infarction, stroke and peripheral artery disease are the main clinical manifestations[5–8].

Much effort has been committed to elucidating the contributory role of various immune cells including monocytes, macrophages, lymphocyte subsets, dendritic cells and platelets. Because of the scarce detection of neutrophils in atherosclerotic plaques compared to other immune cells, their contribution was largely neglected. However, in the last years studies pointing towards the contribution of neutrophils to atherogenesis have accumulated[9–12]. Neutrophils have been implicated in the activation and recruitment of monocytes/macrophages[13–15], which are known to be involved in the process of atherogenesis[16]. In addition, studies that imply a role for neutrophils in advanced atherosclerosis by promoting plaque destabilization and necrotic core formation are emerging[17,18]. Neutrophils influence these various processes by the release of cytokines and proteases and a process called NETosis (i.e. the formation of neutrophil extracellular traps) upon activation[19–22]. The release and generation of adenosine is greatly enhanced in the setting of inflammation[23] and adenosine has been implicated to play a crucial role in the modulation of neutrophil function in various diseases, including ischemia reperfusion injury, sepsis, and non-infectious acute lung injury[23–25].

Recently, adenosine deaminase 2 (ADA2), encoded by the gene Cat eye syndrome critical region protein 1 (CECR1)[26], has been implicated in the regulation of extracellular adenosine levels[27,28] and to play a role in regulating neutrophil activity[29]. A common feature for patients afflicted by loss-of-function mutations in CECR1 is a disturbed neutrophil biology, as seen a significant increase in neutrophil activity[28,29]. These patients suffer from a spectrum of vascular and inflammatory phenotypes, ranging from early-onset recurrent stroke to systemic vasculopathy or vasculitis[30]. Disturbed levels of CECR1 have also been associated with autoimmune diseases[31,32], which are known to be associated with a higher risk for the development of atherosclerosis[33]. Together, this suggests that CECR1 may play role in atherosclerosis development by regulating neutrophil activation.

The aim of this study is to investigate whether CECR1 may be involved in atherosclerosis development through the modulation of neutrophil activity. Therefore, we first investigated if CECR1, adenosine and inosine are present in human atherosclerotic plaques. Furthermore, we assessed if circulating CECR1 levels correlate with circulating neutrophil numbers and if circulating CECR1 levels associate with coronary heart disease (CHD), myocardial infarction (MI) and survival probability post coronary heart disease in humans. Finally, we explored the effect of adenosine and CECR1 treatment on human neutrophil and monocyte activation.

MATERIALS AND METHODS

Microarray on BiKE study material

Late stage/ advanced atherosclerotic plaques were obtained from patients undergoing surgery for high grade (>50%) carotid stenosis and retained within the human **Bi**obank of **K**arolinska **E**ndarterectomies (**BiKE**, Karolinska Institutet, Stockholm, Sweden). Normal artery controls were obtained from nine macroscopically disease-free iliac arteries and one aorta from organ donors without history of cardiovascular disease. All samples were collected with informed consent from patients or organ donor guardians. 127 plaques from BiKE patients and 10 normal arteries were analyzed by Affymetrix HGU133 plus 2.0 GeneChip microarrays. Robust multiarray average normalization was performed and processed gene expression data was transformed in log2-scale. The microarray dataset is available from Gene Expression Omnibus (GSE21545). The BiKE study cohort demographics, details of sample collection, processing, and analyses were previously described[34].

Multimodal analysis of human carotid plaques

Human carotid plaques obtained from BiKE were used as analytical material. Snap frozen plaque tissue was sectioned and thaw mounted on ITO/Superfrost glass slides to enable multimodal analysis.

Immunohistochemistry (IHC)

All IHC reagents were from Biocare Medical (Concord, CA). Isotype rabbit and mouse IgG were used as negative controls. In brief, 5 µm plaque sections were deparaffinized in Tissue Clear and rehydrated in graded ethanol. For antigen retrieval, slides were subjected to high-pressure boiling in DIVA buffer (pH 6.0). After blocking with Background Sniper (Biocare Medical, BS966), primary antibodies against CECR1 (ab67380, Abcam, Cambridge, UK) were diluted in Da Vinci Green solution (Biocare Medical, PD900), applied on slides and incubated at RT for 1h. For colocalisations, antibodies for cell-specific markers were used: CD163 (ab74604, Abcam), CD68 (NCL-1-CD68, Novocastra), CD163 (MAB1652, Abnova) and smooth muscle α-actin (SMA, M0851, DAKO). A double-stain probe-polymer system containing alkaline phosphatase and horseradish peroxidase was applied, with subsequent detection using Warp Red (Biocare Medical, WR806) and Vina green (Biocare Medical, BRR807A). Slides were counterstained dehydrated and mounted in Pertex (Histolab, Gothenburg, Sweden). Images were taken with a Nikon OPTIPHOT-2 microscope equipped with a digital camera and processed with NIS-Elements software[35].

In situ hybridization (ISH)

Chromogenic mRNA-ISH was essentially performed as previously described[36,37]. For detection of CECR1 mRNAs, ISH was performed in a Ventana Discovery ULTRA instrument (Ventana Medical Systems Inc., AZ, USA) using the ACD RNAscope® 2.5 Red Kit (Advanced Cell Diagnostics, Newark, CA, USA) and the mRNA Discovery ULTRA RED 4.0 procedure. RNAscope® 2.5 VS. Probes for CECR1 were designed by the probe manufacturer (Advanced Cell Diagnostics). FFPE sections (5 µm) were applied to Superfrost Plus (Thermo Fisher

Scientific) slides, and all operations including deparaffinization, pretreatment, ISH and counterstaining using hematoxylin were performed in a Ventana Discovery ULTRA instrument. Following the ISH-procedure in the Ventana instrument, slides were washed in lukewarm tap water with detergent until oil from the slides was fully removed. Subsequently, slides were washed in demineralized water, air dried and mounted in EcoMount mounting medium (Advanced Cell Diagnostics) prior to scanning in a bright-field whole-slide scanner (Axio Scan.Z1, Zeiss, Oberkochen Germany) using a 20x objective. The resulting digital images were inspected and regions of interest were selected.

In situ mass spectrometry imaging (MSI)

For the MALDI imaging experiment, two MALDI matrices were used to enable the detection of lipid species, the 2,5-Dihydroxybenzoic acid (2,5-DHB) and the 9-aminoacridine (9AA, Sigma-Aldrich, saint-quentin Fallavier, France) for positive and negative detection mode, respectively. 2,5-DHB powder (150 mg) was used and vaporized on tissue sample using a home-built sublimation apparatus (150°C, 8 min, 2.10–3 mbar). 9AA solution was prepared at 10 mg/mL in methanol (MeOH)/water (7:3). The 9AA matrix solution was sprayed onto tissue sections using the SunCollect automatic sprayer (SunChrom, Friedrichsdorf, Germany). MS images were obtained using a SolariX MALDI-FTICR 7.0T (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser used at a repetition rate of 1 kHz. Mass spectra were acquired in the 100–1,200 *m/z* range in positive and negative detection mode depending on MALDI matrix. The mass spectrum was obtained by mass spectra average of 300 consecutive laser shots on the same location, with a time domain of 1MWord, subsequent single zero filling, and sine wave apodization. An image curve reduction (ICR) noise threshold was fixed at 0.97 for imaging acquisition. An image raster size of 30 µm was selected for human plaque tissues analysis. FTMSControl 3.0 and FlexImaging 4.2 software packages (Bruker Daltonics, Bremen, Germany) were used to control the mass and set imaging parameters. The visualization of imaging data was performed using *MultiImaging* 1.1 software (ImaBiotech, Lille, France). To combine and compare IHC and ISH tissue staining with the adenosine/inosine distribution, MALDI imaging acquisition was performed on adjacent carotid tissue sections and scanned using a 40x magnification objective on an Olympus IX18 microscope (Olympus, Germany). Scans were then integrated in the *MultiImaging* software for a co-registration with imaging data to accurately correlate the distribution of molecular species with human plaque histological regions.

Proteomics on AGES-Reykjavik study material

Associations between circulating CECR1 levels and neutrophils, the presence of MI and CHD, and survival were explored in the AGES-Reykjavik cohort (n=5457)[38], a single-center prospective population-based study of deeply phenotyped elderly European Caucasians (aged 66 through 96, mean age 75±6 years) who survived the 50-year-long prospective Reykjavik study. Phenotype description, patient numbers and other details related to the present study have been described previously[39]. The AGES-Reykjavik study was approved by the NBC in Iceland (approval number VSN-00-063), the National Institute on Aging Intramural Institutional Review Board (USA), and the Data Protection Authority in Iceland.

We applied a custom version of the Slow Off-rate Modified Aptamer (SOMAmer) platform targeting proteins known or predicted to be found in the extracellular milieu, including the predicted extracellular domains of single- and certain multi-pass transmembrane proteins, as previously described[39].
For survival analysis post CHD, we used 1345 incident CHD cases during a survival follow-up period of 12 years. Follow-up time for survival post incident CHD was defined as the time from 28 days after an incident CHD event until death from any cause or end of follow-up time.

Neutrophil isolation and *in vitro* NETosis

Whole blood was obtained from 6 healthy donors with informed consent. EDTA-anticoagulated blood was used to isolate neutrophils using EasySep Direct Human Neutrophil Isolation Kit (StemCell Technologies) following the manufacturer's instructions. Isolated neutrophils were seeded in 5 x 10^4/ml in a black 96-wells plate in 100 µl PBS and stimulated in duplicate with 10µM Adenosine (ABCAM), 100 nM CECR1 or a combination of the two for 4 hours at 37°C. Non-stimulated neutrophils were treated as controls. NETosis was assessed by measuring cell free DNA in the remaining wells using a fluorometric assay for double-stranded DNA Quant-iT PicoGreen dsDNA Assay kit (Invitrogen) following the manufacturer's instructions. Fluorescence was measured using microplate reader with excitation at 480 nm and emission at 520 nm. 50µl of the culture supernatant per condition was collected in low binding tubes and stored at -80°C for stimulation of THP1 monocytes.

THP1 *in vitro* culture and mRNA expression analysis

THP1 monocytes were cultured in RPMI containing 1% glutamine and 10% FBS. To assess the effect of NETosis on THP1 monocytes, 0.5 x 10^6 cells/well were seeded in a 12-well plate and incubated for 24 hours at 37°C. After incubation, 50 µl supernatant of stimulated neutrophils was added to the cells in duplicate per condition and incubated for 4 hours at 37°C. After each experiment, cells and were collected for subsequent RNA isolation.
RNA was isolated with the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Isolated RNA (500 ng) was reverse transcribed into cDNA with the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA) and analyzed by real-time fluorescence assessment of SYBR Green signal (iQ™ SYBR® Green Supermix, Bio-Rad, Hercules, CA) in the CFX96™ Real-Time Detection System (Bio-Rad, Hercules, CA). Each sample was measured in duplicate. Primers were designed for the human genes of interest, sequences are listed in table 1. mRNA levels were analyzed and corrected for the housekeeping gene *ACTB,* which was arbitrarily set on 1.

Statistics

BiKE transcriptomic dataset analyses were performed with GraphPad Prism 6 and Bioconductor software using a linear regression model adjusted for age and gender and a two-sided Student's t-test assuming non-equal deviation, with correction for multiple comparisons according to Bonferroni, as previously described[34]. Data is presented as mean ± SD and adjusted $p<0.05$ was considered to indicate statistical significance.

Table 1. Primer sets for qPCR analysis.

Gene	Species	Direction	Primer sequence (5'-3')
ACTB	Human	Forward	GATCGGCGGCTCCATCCTG
		Reverse	GACTCGTCATACTCCTGCTTGC
IL-6	Human	Forward	AGTGAGGAACAAGCCAGAGC
		Reverse	GTCAGGGGTGGTTATTGCAT
IL-1β	Human	Forward	GAGCTCGCCAGTGAAATGAT
		Reverse	GGAGATTCGTAGCTGGATGC

Prior to circulating CECR1 protein analyses, we applied a Box-Cox transformation on the proteins to improve normality, symmetry and to maintain all protein variables on a similar scale[39]. Given consistency in terms of sample handling including time from blood draw to processing, same personnel handling all specimens and the ethnic homogeneity of the Icelandic population we adjusted only for age and sex in all our regression analyses. For protein to neutrophil correlation we used linear regression analysis and a one-way ANOVA was used to test for significance of circulating CECR1 levels in healthy individuals vs MI and CHD patients. Cox proportional hazards regression was used to test for post incident CHD survival and Kaplan-Meier plots were applied to display survival data.
qPCR data was analyzed according to the ΔΔCt method, statistical tests were performed on ΔCt values. Two-way ANOVA was used to analyze *in vitro* data to take into account day-to-day variation of the experiments. All statistical analyses were performed in SPSS statistics version 21.0. A two-tailed p-value of 0.05 was regarded statistically significant in all analyses.

RESULTS

CECR1 mRNA and protein are present in human atherosclerotic plaques

To explore if CECR1 signaling can be involved in human plaque development, we first investigated if CECR1 could be detected in late-stage human carotid plaques from the BiKE study. Immunohistochemistry revealed that CECR1 was indeed expressed in human atherosclerotic lesions (Figure 1A-C). Double staining with either αSMA, CD68 or CD163 showed that CECR1 is predominantly expressed in plaques near CD68+ positive cells, which are of the monocyte/macrophage lineage.
Gene expression analysis in BiKE revealed that *CECR1* mRNA expression was significantly upregulated in plaques ($p<0.0001$) compared to normal arteries (Figure 1D). Interestingly, *CECR1* mRNA levels were also increased in plaques harvested from symptomatic patients ($p=0.01$) compared to plaques from asymptomatic patients.

CECR1 mRNA and protein expression correlate with low adenosine and high inosine levels in human atherosclerotic lesions

Since it is established that CECR1 can convert adenosine into inosine[28], multimodal imaging was used to visualize the location of CECR1 mRNA or protein expression in relation to the

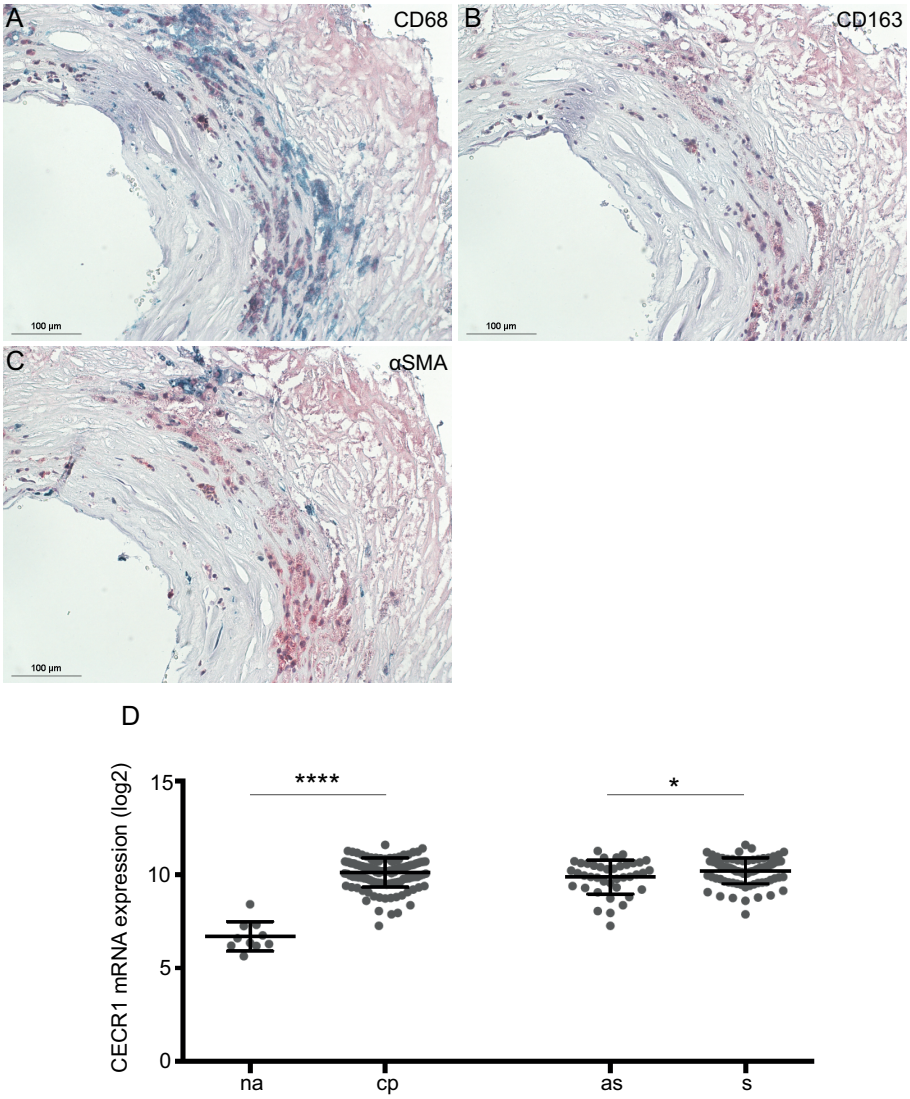

Figure 1. *CECR1* protein and mRNA expression is present in human atherosclerotic plaques.
IHC was used to visualize *CECR1* protein expression in human atherosclerotic plaques (red spots) and combined with IHC staining (blue) for αSMA (A), CD68 (B) and CD163 (C). *CECR1* mRNA expression was measured in normal arteries (na) and in carotid plaques (cp) of asymptomatic (as) and symptomatic patients (s) by microarray analysis (D). A two-sided Student's t-test assuming non-equal deviation was used to test for significance between normal and atherosclerotic arteries and between asymptomatic and symptomatic patients. *p<0.05, ****p<0.0001

presence of adenosine and inosine in human atherosclerotic lesions. ISH was used to visualize *CECR1* mRNA expression in atherosclerotic lesions and adjacent sections were processed for MSI, to measure adenosine and inosine (Figure 2A-E). When both ISH and MSI images were combined it revealed that inosine (Figure 2B and D) was highly present in regions that showed high *CECR1* mRNA expression (Figure 2A), whereas adenosine (Figure 2C and E) was clearly absent or lowered in these regions. Similar findings were obtained when MSI images were combined with images obtained after IHC targeting CECR1 protein (Figure 2F). Inosine (Figure 2G) presence was high in regions that showed the highest CECR1 protein expression, and adenosine (Figure 2H) was again found to be lower or absent in these same regions.

Serum CECR1 levels are associated with decreased numbers of circulating neutrophils, increased presence of MI and CHD and decreased post incident CHD survival probability in humans

Since rare loss-of-function mutations in the CECR1 gene are associated with increased neutrophil numbers and activity in patients, we investigated the relation between CECR1 levels and neutrophil numbers in patients with normal functional CECR1. Data from the AGES-Reykjavik study revealed that circulating CECR1 levels are negatively correlated with the number of circulating neutrophils in human subjects (Figure 3A). Serum measurements in the AGES-Reykjavik study also showed increased levels of circulating CECR1 in MI ($p=1e-08$) and CHD ($p<0.001$) compared to healthy controls (Figure 3B). We next explored if variable levels of CECR1 in the human circulation were associated with survival probability in the AGES-Reykjavik study. We found that higher serum CECR1 levels were associated with decreased survival probability post incident CHD ($p=0.0001$) (Figure 3C).

CECR1 inhibits adenosine induced NETosis in human neutrophils *in vitro* and diminishes the inflammatory response of monocytes indirectly through inhibition of neutrophil activation

Isolated human neutrophils showed an increase of NETosis upon adenosine stimulation ($p<0.05$) compared to unstimulated neutrophils (Figure 4A). CECR1 was able to normalize the adenosine induced NETosis ($p<0.05$), whereas CECR1 alone did not affect NETosis in control neutrophils. To investigate whether adenosine induced neutrophil activation can trigger the inflammatory response of monocytes, we examined mRNA expression in THP1 cells stimulated with the supernatant of neutrophils treated with 10μM Adenosine, 100 nM CECR1 or a combination of the two. The supernatant of adenosine treated neutrophils was able to increase both IL-6 and IL-1β mRNA expression (both $p<0.05$) in THP1 cells compared to the supernatant of untreated neutrophils (Figure 4B). Both the supernatant of only CECR1 treated and the CECR1/adenosine treated neutrophils did not result in a change of expression of these inflammatory cytokines in THP1 (Figure 4B).

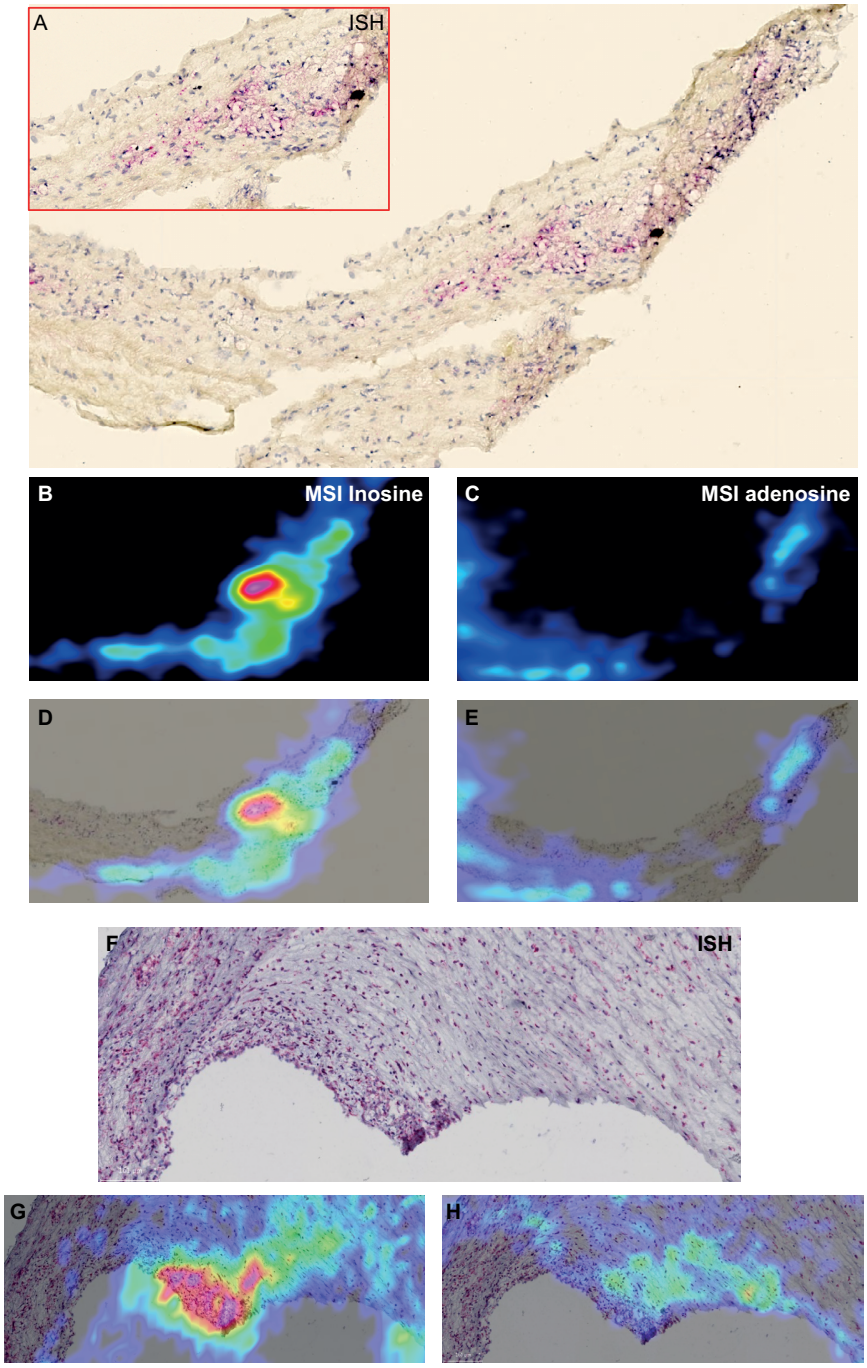

Figure 2. *CECR1* mRNA expression colocalizes with increased inosine levels and decreased adenosine levels. ISH and IHC were used to visualize *CECR1* mRNA and protein expression respectively (A and F, red spots) ►►

◀◀ and *in situ* MSI was used to visualize inosine (B) and adenosine (C) in human atherosclerotic plaques. An overlay of *in situ* MSI visualized inosine and adenosine with ISH visualized *CECR1* mRNA expression (D and E) or IHC visualized CECR1 protein expression (G and H) was made to assess colocalization.

DISCUSSION

In the present study, we showed that *CECR1* mRNA is expressed in human atherosclerotic lesions and that regions expressing *CECR1* mRNA colocalize with high inosine and low adenosine levels. Since adenosine is known to induce NETosis[40] and we observed that circulating CECR1 protein levels are negatively correlated with circulating neutrophil numbers[29], we hypothesized that CECR1 affects atherosclerosis development by regulating neutrophil activation. Indeed, our *in vitro* studies showed that adenosine induces NETosis in neutrophils isolated from healthy donors and that CECR1 represses adenosine induced NETosis. Moreover, the conditioned medium of adenosine stimulated neutrophils induced THP1 monocyte activation by upregulating *MCP-1* and *IL-1β* mRNA expression. While *MCP-1* and *IL-1β* mRNA expression in THP1 monocytes stimulated with conditioned medium from CECR1 or simultaneous adenosine and CECR1 stimulated neutrophils was comparable to non-stimulated control THP1 monocytes. Finally, we showed that circulating CECR1 levels are increased in patients with a myocardial infarction and coronary heart disease and that high circulating CECR1 levels are associated with decreased post CHD survival probability in humans.

Extending the previous finding by Zavialov *et al,* who showed that CECR1 is secreted by monocytes undergoing differentiation into macrophages[41], we here demonstrate that CECR1 is expressed in human atherosclerotic plaques in close proximity of CD68+ monocytic cells, suggesting that CECR1 is expressed in close proximity of inflammatory regions. The relatively higher *CECR1* mRNA expression in atherosclerotic arteries compared to normal arteries may be explained by this specific inflammatory expression. Cells from the monocytic lineage make up a relatively large proportion of the atherosclerotic plaque through the influx and proliferation in response to inflammatory cytokines produced by these plaques[42,43], which will increase CECR1 expression. The higher expression observed in asymptomatic patients compared to symptomatic patients is also in line with these findings since these lesions are more instable partially due to a higher presence of macrophages and macrophage activity[44,45].

Adenosine, a purine nucleoside normally found at low concentrations in human tissues, is released into the extracellular space in response to metabolic stress such as that encountered during inflammatory events or during tissue hypoxia or ischemia[46,47]. Adenosine acts as an immunomodulator on monocytes and neutrophils[47], regulates the expression of proteins involved in cholesterol flux[48], plays a role in vasculogenesis/ angiogenesis and vascular remodeling[49,50], and has platelet inhibitory effects[51], all of which may affect atherosclerosis development.

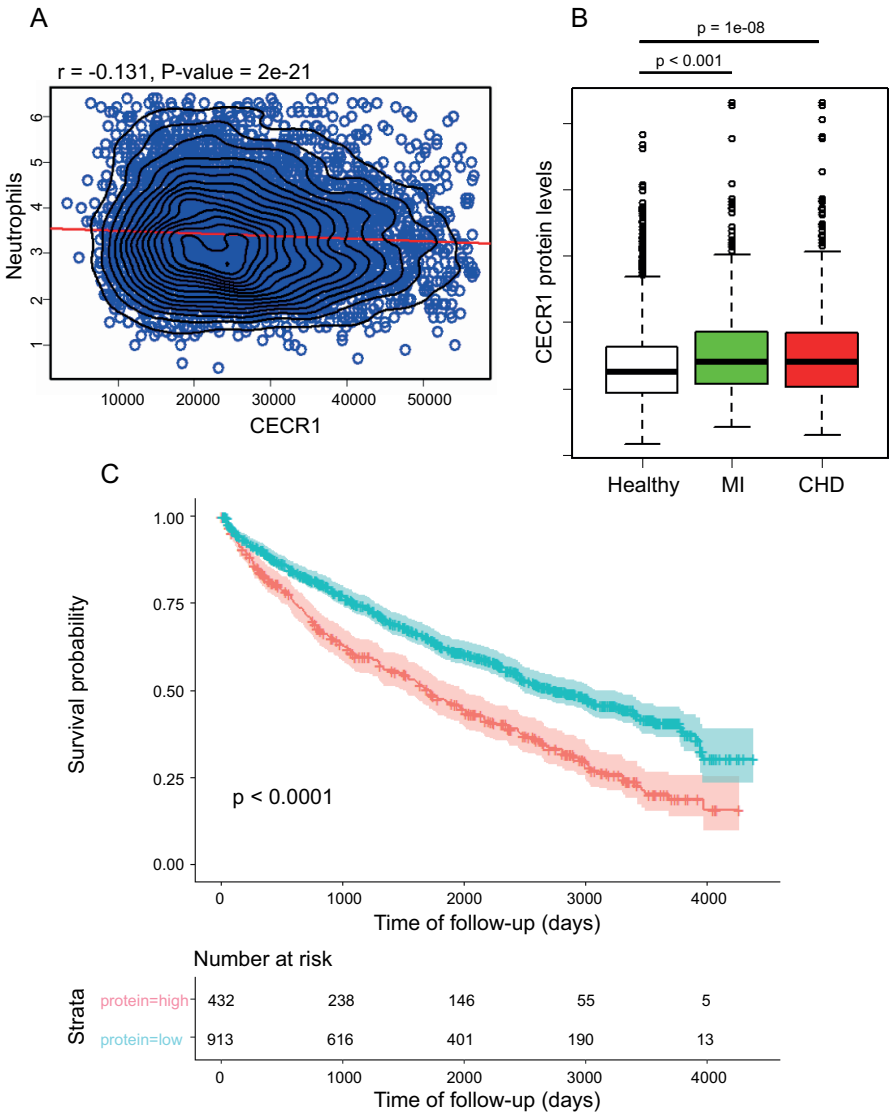

Figure 3. Serum CECR1 levels are associated with decreased circulating neutrophils numbers, presence of MI and CHD and decreased post incident CHD survival probability in humans.
Circulating CECR1 protein levels were correlated with circulating neutrophils numbers in humans (A). Next, circulating CECR1 protein levels were compared between healthy controls and patients with MI and CHD (B). Furthermore, circulating CECR1 protein levels were correlated with survival probability (C). Linear regression analysis was used to test for CECR1 protein to neutrophil correlation and a one-way ANOVA to test for significance of circulating CECR1 levels in healthy individuals vs MI and CHD patients. Cox proportional hazards regression was used to test for post incident CHD survival and Kaplan-Meier plots were applied to display survival data.

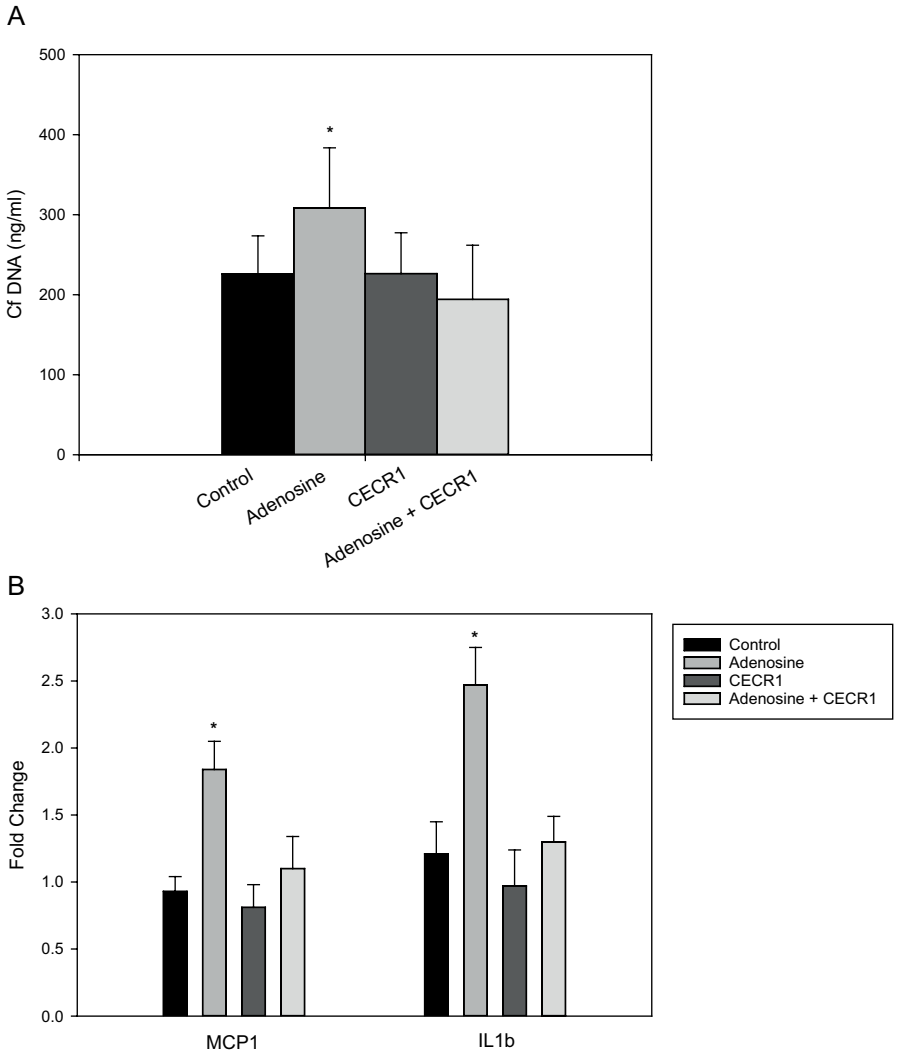

Figure 4. CECR1 inhibits adenosine induced NETosis in human neutrophils *in vitro* and diminishes the inflammatory response of monocytes indirectly through inhibition of neutrophil activation.
Isolated neutrophils from healthy donors were treated with adenosine, CECR1 or a combination of the two to assess neutrophil activation by measuring NETosis (A). The conditioned medium of the neutrophils was subsequently added to THP1 monocytes to asses monocyte activation by examining MCP-1 and IL-1β mRNA expression (B). A two-way ANOVA was used to test for significance of *in vitro* experiments. qPCR data was analyzed according to the ΔΔCt method, statistical tests were performed on ΔCt values. *p<0.05

The level of adenosine is regulated by proteins called adenosine deaminases (ADAs), of which ADA1 and CECR1 are the most described members. ADAs decrease the level of adenosine by converting it into inosine[28]. CECR1 was first described as the residual source of adenosine deaminase activity in the spleen of a patient with severe combined immunodeficiency (SCID) due to ADA1 deficiency[52].

Although CECR1 has long been regarded as an isozyme of ADA1, they differ in structure, cellular localization, and expression. ADA1 is a 41-kDa monomer protein that is present in all human tissues and has a critical function in adaptive immune system development, while CECR1 is a 59-kDa protein that forms homodimers and is secreted into the extracellular space[53,54]. CECR1 has a 100-fold higher Michaelis Konstant for adenosine than ADA1 and which is several orders higher than the concentration of adenosine in plasma[55]. This implies that the rate of adenosine conversion by CECR1 is close to zero at the physiological adenosine concentrations. Moreover, CECR1 activity appears to increase with disease activity and relapse[31,32,56–59]. This correlation also seems to be true in atherosclerotic patients with a more mild inflammatory background since we showed that circulating CECR1 levels were enhanced in patients suffering from MI and CHD.

As adenosine signaling must be tightly regulated to control the immune response, CECR1 may have a role in the degradation of extracellular adenosine at the site of inflammation. Our findings using multimodal imaging in atherosclerotic lesions are in line with this hypothesis as we observed that CECR1 protein and RNA expression colocalize with higher levels of inosine and lower levels of adenosine.

Patients with loss-of-function mutations in CECR1 often suffer from chronic or recurrent systemic inflammation and are at risk of developing ischemic or haemorrhagic cerebral stroke and other vasculopathy-related manifestations including hypertension[60]. Moreover, CECR1 deficiency is associated with a reduction in anti-inflammatory M2 macrophages and an increase in pro-inflammatory cytokines[60]. Since it has been suggested that the observed vasculitis in CECR1 deficient patients is caused by chronic neutrophil activity[29], we investigated if circulating CECR1 levels are associated with neutrophil levels. In line with literature, we indeed found a negative correlation between circulating CECR1 protein levels and circulating neutrophil numbers, indicating that CECR1 indeed may affect neutrophil activity.

Interestingly, we observed that high circulating CECR1 levels are associated with decreased post CHD survival probability in humans, which would suggest that CECR1 may have negative impact on atherosclerosis. However, the presence of a protein within an atherosclerotic lesion or the circulation does not automatically imply a pro-atherogenic role to that specific protein. CECR1 might be a compensatory mechanism that tries to dampen the immune response and only becomes upregulated upon extreme inflammation and increasing levels of adenosine.

Since CECR1 deficiency results in neutrophilia[29] and increased activity and neutrophils express all four adenosine receptors[61], we hypothesized that CECR1 might reduce neutrophil activation by decreasing adenosine levels. Our *in vitro* studies showed that adenosine indeed induces neutrophil activation by NETosis in neutrophils isolated from healthy donors and that CECR1 represses adenosine induced NETosis. Moreover, the conditioned medium of

adenosine stimulated neutrophils induced THP1 monocyte activation by upregulating *MCP-1* and *IL-1β* mRNA expression. While *MCP-1* and *IL-1β* mRNA expression in THP1 monocytes stimulated with conditioned medium from CECR1 or simultaneous adenosine and CECR1 stimulated neutrophils was comparable to the non-stimulated control. Our observations that adenosine induces NETosis and subsequent monocyte activation in combination with the inhibitory effect of CECR1 on adenosine induced NETosis is in line with a recent study performed by Carmona-Rivera *et al.,* who showed that adenosine triggers NETosis in neutrophils isolated from CECR1 deficient patients and that this can be inhibited by CECR1[62]. However, this seemingly atheroprotective effect of CECR1 opposes the low survival probability in individuals with high CECR1 levels. There are several possible explanations for these opposing observations. Firstly, these opposing effects could be caused by different concentrations of adenosine since low adenosine levels mainly engage the A_1 and A_3 adenosine receptors, thereby promoting chemotaxis and phagocytosis, while high adenosine levels mainly signal through A_{2A} and A_{2B} adenosine receptors, thereby inhibiting neutrophil trafficking and granule release[61]. Secondly, neutrophils are not the only cells in the human body that react to either adenosine or CECR1. Although adenosine seems to have a disease deteriorating effect on neutrophils, it was previously described to have protective effects on monocytes and endothelial cells by downregulating thrombin induced VCAM-1, ICAM-1 and E-selectin cell surface expression and inhibiting thrombin induced expression of pro-inflammatory cytokines[63]. If the protective effects of adenosine on endothelial cell activation take precedence over the disease deteriorating effects of adenosine on neutrophil activation, the net effect of reducing adenosine levels might be disease deteriorating and proatherogenic.

Taken together, our study provides more insight into the role of CECR1 in adenosine-mediated neutrophil activation and atherosclerosis development. Although not conclusively, these findings suggest that modulation of adenosine levels may contribute to a novel and directed therapeutic approach in the treatment of cardiovascular disease by regulating the inflammatory drive behind the pathology.

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SUMMARY AND DISCUSSION

CHAPTER 7

General summary and discussion

INTRODUCTION

The enormous rise in cardiovascular disease (CVD) incidence and death rate in the first half of the 20th century in industrialized countries[1,2] augmented CVD related research[3]. The increased understanding of the disease pathology and risk factors led to a reduced CVD death rate in the second half of the 20th century in developed countries[1], but the simultaneous rise in middle- and low-income countries[4] has made it the leading cause of death worldwide[5]. A healthy lifestyle, which includes a healthy diet, smoking cessation, physical activity and weight loss massively decrease the risk of CVD[6–8]. Unfortunately, a number of intervention studies have shown that it is difficult to adhere to a healthy lifestyle[9]. A Finnish counselling intervention study showed that, although participants were very motivated to adapt a healthier lifestyle, adherence to these changes (which was 52% at baseline) had dropped to 8% and 4% after 6 and 12 months after counselling intervention respectively[10]. Several studies that focussed on weight loss showed that, despite being initially successful, many participants regain half of their lost weight within a year and return to baseline weight within 3 to 5 years[9]. Currently, CVD accounts for approximately one-third of all deaths[5] and is a huge economic burden as only in the US, 396 billion dollars are spend on direct medical costs of CVD annually[11]. Coronary heart disease and stroke[12–14] together are responsible for more than half of all CVD related deaths[11]. Therefore, it is of great importance to develop novel therapies against atherosclerosis, which is the underlying disease pathology of these disorders[12–14].

However, the development of a new therapeutic drug is a long and costly process[15]. The current development costs of a single drug are estimated around 2.6 billion dollars[15] and these costs are expected to rise in the near future[15,16]. One of the reasons of the high costs in drug development is its high attrition rate[15]. Of all 24 potential drugs that result from pre-clinical research, only 8 enrol into clinical trials and only 1 drug will eventually make it to the market[17]. To improve drug development efficacy and productivity, AstraZenca recently reviewed its own drug development projects to gain insight into the main reasons for failure[18]. They found that, especially in the preclinical and phase I clinical trials, most projects were closed due to safety issues[18]. These safety issues were mostly caused by the off-target effects of the tested compounds and mainly affected the cardiovascular system and the liver[18]. The percentage of projects closed due to safety issues declined in each of the succeeding clinical phases and was substituted by projects closed due to a lack of efficacy, making efficacy problems the second most important reason for drug development failure[18]. Other important findings in this study are that projects were more likely to be successful if they obtained a good target validation, a genetic link exists between the target and the disease, and a good understanding of the role of the target in the disease was present[18]. Based on these findings, AstraZeneca came up with five key factors in a 5R strategy that are important determinants to a successful project and implemented these factors to improve their drug development productivity[18].

Inspired by AstraZeneca's strategy, we developed a five-factor approach within the CarTarDis consortium to identify druggable targets in CVD. Since the five factors defined by AstraZeneca apply to the entire process of drug discovery, from the preclinical phase to phase II clinical

trials (phase III clinical trials were excluded as the attrition rate is much higher in phase II and as there were too little phase III trials to draw valid conclusions), and our focus concentrates on the preclinical phase, we slightly adjusted their five key factors to five factors that are more applicable to our project, namely: druggability, novelty, genetics, feasibility and biological pathways.
In order to identify potentially novel druggable targets in CVD, we first created a shortlist of potential targets based on already existing data. This could either be data that was available through publications or data that was obtained from the population-based human cohorts (KORA, AGES and REFINE) or case-control studies (CardioGenX, BiKE and Socrates) that we had access to within the CarTarDis consortium. Next, we scored the potential targets on the shortlist on druggability, novelty, genetics, feasibility and pathways. This helped us to identify the top candidate targets and simultaneously revealed the existing knowledge gaps of each potential target. Since a good understanding of the role of the target in the disease improves its chances on being successful in drug development, we subsequently validated the involvement of these targets in CVD in multiple model systems. With this approach, we not only aimed to identify promising targets in CVD, but also tried to augment these targets with sufficient information to be interesting targets for drug development companies. The validation studies on three of our shortlisted targets were described in this thesis.

PART ONE - ONCOSTATIN M

Oncostatin M (OSM) was added to our shortlist of potential targets based on the existing literature and our own preliminary *in vitro* experiments. We hypothesized that OSM induces endothelial cell activation and thereby accelerates atherosclerosis development. In **chapter 2**, we showed that OSM indeed induces endothelial cell activation in cultured human endothelial cells. This finding was backed up by our *in vivo* data as we also observed signs of endothelial cell activation in mice that were treated with OSM for 3 weeks. We further strengthened this hypothesis by showing that OSM as well as its receptors are present in human atherosclerotic plaques in **chapter 3**, suggesting that OSM might indeed play a role in atherosclerosis development in humans. However, our long-term *in vivo* study, in which we treated ApoE3*Leiden.CETP mice for 16 weeks with OSM, showed surprising results as atherosclerotic lesions were smaller in OSM-treated mice. The observed anti-atherogenic effects of OSM in the ApoE3*Leiden.CETP model are in line with the improved survival of coronary heart disease patients with higher serum OSM levels. Correspondingly, **chapter 4** showed that a common genetic variant in OSMR (one of the receptors for OSM), that is associated with reduced *OSMR* expression in arterial tissue, correlates with a more vulnerable plaque phenotype in human carotid plaques from the Athero-Express Biobank Study.
Although our observation that OSM induces endothelial cell activation is in line with our hypothesis, the observed anti-atherogenic effects of OSM oppose the second part of our hypothesis in which we hypothesized that OSM-induced endothelial cell activation enhances atherosclerosis development. As we did not find a solid explanation for these opposing

results, we can only speculate about the mechanisms that led to these observations. Looking back at our *in vitro* endothelial cell activation experiments, we only observed an increase in intercellular adhesion molecule 1 (ICAM-1) membrane expression and not of vascular cell adhesion molecule 1 (VCAM-1), P-selectin or E-selectin, despite the increase in mRNA expression that was observed for all of these adhesion molecules. Instead, we observed an increase in soluble E-selectin and VCAM-1, indicating that there is less expression on the membrane. But, as also circulating levels of adhesion molecules are associated with atherosclerosis development in patients[19–22], we did not further investigate this. However, specific adhesion molecules bind specific integrins and the composition of expressed adhesion molecules on the endothelial cell surface can therefore influence binding of specific leukocyte subsets[23]. Specifically, in the case of OSM-induced endothelial cell activation, one would expect a preference for binding non-classical monocytes above classical monocytes as ICAM-1 mainly interacts with integrins that are present on non-classical monocytes[24–26], while VCAM-1 mainly interacts with integrins expressed on classical monocytes[24,27]. This theory is in line with our observations in **chapter 3**, where we demonstrated that mice treated with OSM have a lower percentage of circulating classical monocytes. The non-classical monocytes distinguish themselves from the classical monocytes by patrolling the luminal site of the vasculature where they remove debris and apoptotic cells[26], by their involvement in wound healing[26] and their preference to differentiate into M2 macrophages, which have been reported to protect the vasculature and to inversely correlate with atherosclerotic disease progression in humans[27–29]. The classical monocytes on the other hand, are rapidly recruited to sites of inflammation where they are likely to extravagate and differentiate into M1 macrophages[26], a well-known pro-inflammatory subset of macrophages that is linked to atherosclerotic plaque progression[27,30]. So, the adhesion molecule expression profile on the activated endothelial cells suggests that mainly non-classical monocytes will bind to the endothelial cells that were activated by OSM. This might explain why there is more monocyte adhesion in mice treated with OSM, but smaller atherosclerotic plaques. It should be noted that this hypothesis is solely based on reasoning and further research is necessary to test this hypothesis.

Another explanation of the anti-atherogenic effect of OSM might be that OSM is not as pro-inflammatory as generally assumed. Although we, and others, have observed an OSM-induced increase in the release of inflammatory cytokines interleukin(IL)-6 and monocyte chemoattractant protein-1 (MCP-1), there are also reports that show the anti-inflammatory properties of OSM as it suppresses tumor necrosis factor(TNF)α[31] and IL-1β[32] production. Like IL-6 and MCP-1[33,34], these cytokines are also considered as drivers of atherosclerosis[35–37]. Since we selectively measured IL-6 and MCP-1 levels and did not analyze a more general immune profile, we can only speculate that TNFα and IL-1β might have been decreased in our study as well, but we cannot draw hard conclusions regarding the role of OSM in the general immune response from our studies.

Furthermore, the suggested atherogenic role of OSM in literature is mostly based on its presence in atherosclerotic lesions[38] and on either its elevated levels in inflammatory diseases like rheumatoid arthritis[39,40] and periodontal disease patients[41] or its stimulating role on processes like angiogenesis[42], smooth muscle cell proliferation[38] and tissue factor

expression[43], which are all risk factors for atherosclerosis[38,41–44]. The presence of OSM in atherosclerotic plaques described in this thesis and by others makes it logical to ascribe an atherogenic role to OSM. However, we should be careful drawing conclusions from the presence of certain proteins within a disease as epidemiological studies are often biased by reverse causality[45]. Recent examples of reverse causality in CVD are drugs increasing HDL levels, hormone therapy in post-menopausal women and vitamin E supplementation. Based on epidemiologic studies, it was hypothesized that these treatments would reduce CVD, but randomized controlled trials could not confirm these hypotheses[46,47]. The observed protective effects of hormones and vitamin E were probably related to socioeconomic and lifestyle factors that confounded their associations with CVD[46]. To our knowledge, only one intervention study regarding the role of OSM signaling in atherosclerosis has been published. Although, this study showed that OSMR deficient ApoE$^{-/-}$ mice have smaller and less severe lesions, this is no hard evidence for a role of OSM signaling in atherosclerosis as IL-31 also signals through OSMR[48,49].

Finally, OSM is a complex protein as it exerts dual effects on different cell types and even dual effects in one and the same cell type. Depending on the cell type, OSM has both antiproliferative and proliferative effects[38,42,50,51], which makes it difficult to predict the effects of OSM in an *in vivo* setting, which does not consist of one single cell type, but of many different cell types. Also, OSM can have different time-dependent effects[52]. In human proximal tubular cells for example, OSM first promotes and subsequently inhibits connective tissue growth factor mRNA expression. Apart from the likely disadvantageous effect of OSM on endothelial cell activation, OSM generally seems to have a beneficial effect in mouse models of acute diseases, including acute liver failure[53], cardiac ischemia/reperfusion injury[54] and myocardial infarction[55,56] while it has disease deteriorating effects OSM in chronic diseases, including chronic liver injury[57] and chronic heart failure[58]. The effect of OSM on endothelial activation and the different faces of OSM in acute and chronic disease states are similar to the different faces of its family member IL-6, which is also described to induce endothelial cell activation[59,60] and be protective in the acute phase and disease causing in the chronic phase[61].

Despite our efforts, a lot of questions on the role of OSM in atherosclerosis are still remaining. Therefore, additional research needs to be performed before OSM can be considered as a target in CVD. Firstly, as the anti-atherogenic effects we observed in our studies oppose the pro-atherogenic effects observed by Zhang *et al.,*[48] further studies are needed to verify how OSM affects atherosclerosis development. Secondly, the mechanism behind the OSM-induced effects on atherosclerosis needs to be elucidated in more detail. Thirdly, it should be investigated if modification of OSM signaling by targeting either OSM or any of its downstream targets is safe and if this can efficiently reduce cardiovascular events. Although further research on the role of OSM in atherosclerosis is essential, the data provided in this thesis does provide a warning to be cautious with OSM inhibiting antibodies as inhibition of OSM signaling might increase the risk on CVD. Especially since higher serum OSM levels in humans are associated with improved survival in coronary heart disease patients.

PART TWO - PHOSPHOLIPID PHOSPHATASE 3

Phospholipid Phosphatase 3 (PLPP3) was added to the shortlist of candidate targets mainly due to its strong genetic evidence to CVD. In **chapter 5,** we show that higher *PLPP3* mRNA expression levels are associated with improved major adverse cardiovascular, cerebrovascular, and vascular event-free survival. So, hypothetically, PLPP3 expression should be increased to prevent CVD. Since PLPP3 is a membrane protein, increasing PLPP3 expression or activity in patients is not something that can easily be achieved. Therefore, we looked into downstream targets of PLPP3 that are better druggable to explore if these are promising targets. Lysophosphatidic acid (LPA) is one of the bioactive lipids that is inactivated by PLPP3[62], thereby preventing LPA from binding to one of the six specific LPA receptors (LPAR1-6)[62,63]. Silencing of *PLPP3* in endothelial cells induced endothelial cell activation in a similar manner as LPA does, suggesting that reduced *PLPP3* expression in endothelial cells induces endothelial cell activation via elevated LPA levels. Of all LPA receptors, LPAR6 showed the highest expression in human endothelial cells. We therefore investigated whether targeting LPAR6 attenuates LPA induced endothelial cell activation. Markers of endothelial cell activation were indeed reduced in LPA-treated endothelial cells with reduced *LPAR6* expression, but did not reach baseline levels, indicating redundancy, i.e. that other LPARs might be involved in LPA-induced endothelial cell activation as well. Furthermore, silencing of *LPAR6* did not attenuate the state of endothelial cell activation that was induced by silencing PLPP3, indicating that downstream targets of PLPP3, other than LPA, might have an important role in endothelial cell activation. One of the factors that is also inactivated by PLPP3 and could in parallel to LPA induce endothelial activation, is sphingosine 1-phosphate (S1P)[64]. This is a bioactive lipid that, like LPA, is dephosphorylated by PLPP3 and is involved in inflammation by recruiting lymphocytes from lymphoid organs into the blood and subsequently to sites of inflammation[65–68]. Moreover, S1P enhances endothelial E-selectin and VCAM-1 membrane expression in a similar fashion as TNFα[69], indicating that S1P, like LPA, is able to induce endothelial activation. Furthermore, S1P enhances tissue factor expression in endothelial cells, suggesting that S1P has pro-thrombotic properties[66]. However, others report that S1P enhances the endothelial barrier function[70], inhibits monocyte adhesion to the endothelium[71] and administration of a S1P analogue in a mouse model for atherosclerosis reduces atherosclerosis development[72]. So, although our studies shed some light on the protective effect of PLPP3 on endothelial cell activation, there are a lot of questions remaining on the mechanisms behind these observed results. Therefore, additional research is required before PLPP3 can be considered as a genuine target in CVD.

PART THREE - CAT EYE SYNDROME CRITICAL REGION PROTEIN 1

Cat eye syndrome critical region protein 1 (CECR1), which reduces adenosine levels by converting it into inosine[73], was added to the shortlist of candidate targets mainly due to its high score on novelty and pathways. In **chapter 6**, we show that CECR1 mRNA and protein is expressed in human atherosclerotic lesions and that CECR1 is active in atherosclerotic lesions since regions expressing *CECR1* mRNA colocalize with high inosine and low adenosine levels. These observations suggest that that CECR1 might have a role in atherosclerosis development. Patients with loss-of-function mutations in CECR1 often suffer from chronic or recurrent systemic inflammation and are at risk of developing ischemic or haemorrhagic cerebral stroke and other vasculopathy-related manifestations including hypertension and vasculitis[74,75]. Moreover, CECR1 deficiency is associated with a reduction in anti-inflammatory M2 macrophages and an increase in pro-inflammatory cytokines[74]. As it has been suggested that the observed vasculitis in CECR1 deficient patients is caused by chronic neutrophil activity[75], we first investigated if circulating CECR1 levels are associated with neutrophil levels. In line with literature, we indeed found a negative correlation between circulating CECR1 protein levels and circulating neutrophil numbers, indicating that CECR1 indeed affects neutrophil numbers. Since systemic inflammation is a driver of atherosclerosis[76], CECR1 converts adenosine into inosine[73] and neutrophils express all four adenosine receptors[77], we hypothesized that CECR1 reduces atherosclerosis development by reducing adenosine levels and thereby neutrophil activation. Our *in vitro* studies showed that adenosine indeed induces neutrophil activation by NETosis in neutrophils isolated from healthy donors and that CECR1 represses adenosine induced NETosis. This is in line with a recent study performed by Carmona-Rivera *et al.,* who showed that adenosine triggers NETosis in neutrophils isolated from CECR1 deficient patients and that this can be inhibited by CECR1[78]. Moreover, we showed that the conditioned medium of adenosine stimulated neutrophils induces THP1 monocyte activation by upregulating *MCP-1* and *IL-1β* mRNA expression. While *MCP-1* and *IL-1β* mRNA expression in THP1 monocytes stimulated with conditioned medium from CECR1 or simultaneous adenosine and CECR1 stimulated neutrophils was comparable to the non-stimulated control. Finally, we show that circulating CECR1 levels are increased in myocardial infarction and coronary heart disease patients and that high circulating CECR1 levels are associated with decreased post CHD survival probability in humans. The latter observation suggests that CECR1 has proatherogenic, which opposes the seemingly atheroprotective effects that CECR1 might exert by inhibiting adenosine induced neutrophil activation. There are several possible explanations for these opposing observations. Firstly, these opposing effects could be caused by different concentrations of adenosine since low adenosine levels mainly engage the A_1 and A_3 adenosine receptors, that for example, promotes chemotaxis and phagocytosis, while high adenosine levels mainly signal through A_{2A} and A_{2B} adenosine receptors, that for example, inhibit neutrophil trafficking and granule release[77]. Secondly, neutrophils are not the only cells in the human body that react to either adenosine or CECR1. Although adenosine seems to have a disease deteriorating effect on neutrophils, it was previously described to have

protective effects on endothelial cells by downregulating thrombin induced VCAM-1, ICAM-1 and E-selectin cell surface expression and inhibiting thrombin induced expression of pro-inflammatory cytokines[79]. If the protective effects of adenosine on endothelial cell activation take precedence over the disease deteriorating effects of adenosine on neutrophil activation, the net effect of reducing adenosine levels might be disease deteriorating and proatherogenic. Finally, as already touched upon earlier, the presence of a protein within an atherosclerotic lesion does not assign a pro-atherogenic role to that specific protein. Like IL-10, which is expressed in a substantial number of advanced plaques and has anti-inflammatory effects and is therefore suggested to be a modulator of the local immune response[80], CECR1 might be a compensatory mechanism that tries to dampen the immune response and only becomes upregulated upon extreme inflammation. To conclude, despite the inhibiting effects of CECR1 on adenosine induced neutrophil activation, which suggests a protective effect, high CECR1 serum levels are associated with reduced survival in CHD patients. Additional research is needed to shed light onto these seemingly opposing effects and is necessary before CECR1 can be considered as a therapeutic target in CVD.

REFLECTION OF OUR APPROACH

One of the strengths of our five-factor approach is the scoring on genetic evidence that we applied since the presence of a genetic link between the target and the disease was shown to greatly enhance the successfulness of the target[18,45,81]. One of the success stories of applying genetics in drug development is the discovery of the gain of function mutation in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene. This mutation was shown to cause hypercholesterolemia, while loss of function mutations in this gene decreases low density lipoprotein (LDL) cholesterol levels and coronary heart disease incidence[82]. These observations led to the development of a novel mouse model for atherosclerosis, which develops atherosclerosis by adenoviral mediated overexpression of *PCSK9* in combination with a high fat diet[83]. Mouse studies further emphasized the pronounced effects of *PCSK9* gene expression alterations as a single injection of an adeno associated virus encoding *PCSK9* in a wildtype mouse is sufficient to induce atherosclerosis[83], while most genes that are suspected to have a role in atherosclerosis development have a less pronounced effect and need to be investigated in double knockout mice, which lack both the gene of interest and a known atheroprotective gene, like Apolipoprotein E (ApoE) or the LDL receptor[83]. Since the generation of a double knock out mouse is often difficult and time consuming, the creation of an atherosclerotic mouse model by the single injection of a PCSK9 adeno associated virus is already a success of the discovery of the PCSK9 mutation[83]. Further success was obtained with the development of PCSK9 inhibitors. Alirocumab and evolocumab, monoclonal antibodies against human PCSK9, not only lowered plasma cholesterol levels in ApoE3*Leiden.CETP mice, but also dramatically decreased atherosclerotic lesion size and severity when administered as monotherapy and on top of statins[84,85]. A more recent study in ApoE3*Leiden.CETP mice showed that these atheroprotective effects can also be obtained by treatment with a vaccine against PCSK9,

which could potentially replace the expensive PCSK9 antibody therapy, which needs to be applied much more frequently[86]. The impact of the discovery of this specific *PCSK9* mutation is further emphasized by its rapid introduction onto the market as less than 12 years passed between the initial discovery in 2003 and FDA approval of PCSK9 inhibitors in 2015[87]. Even though the effects of PCSK9 antibodies on CVD incidence in humans still needed to be investigated in long-term clinical trials at the time of FDA approval, both evolocumab and alirocumab were already approved as it had already been shown that they both decrease LDL cholesterol levels in humans[88,89]. More recently, the effects of evolocumab and alirocumab on cardiovascular outcomes were published. Evolocumab significantly reduced the risk of the primary end point (composite of cardiovascular death, myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization) from 11.3% to 9.8% and the key secondary end point (composite of cardiovascular death, myocardial infarction, or stroke) from 7.4% to 5.9%[90] and alirocumab reduced occurrence of primary end point (composite of death from coronary heart disease, nonfatal myocardial infarction, fatal or nonfatal ischemic stroke, or unstable angina requiring hospitalization) from 11.1% to 9.5% and all-cause mortality from 4.1% to 3.5%[91].

Although the scoring on feasibility, which was based on our in-house knowledge, biobanks, techniques and *in vitro* and *in vivo* models, helped us getting a quick start with all selected targets, it might be a weakness of our approach as this scoring could exclude promising targets in drug discovery due to our lack of knowledge or techniques in specific areas. For example, our strong background with endothelial cell culture might have slightly biased our target selection to favour endothelial cell affecting targets above other cell types that are involved in atherosclerosis development, like smooth muscle cells[38] and macrophages[92].

Even though we had access to a broad source of data and *in vitro* and *in vivo* techniques, it is important to realize that each of these techniques have their own strengths and limitations. Human biobanks, for example, are a great source for correlative studies between the target of interest and the disease in humans as the human disease state is often very complex and difficult to mimic in cell, tissue or animal models[93]. However, these biobanks cannot be used for novel drug screening and the identification of a lead compound, for these purposes, the application of cell culture is much more appropriate[94]. An intermediate model, which includes some of the disease complexity observed in humans and the possibility to investigate the safety and efficacy of interventions, are animal models[95]. In atherosclerosis, mouse models are most extensively used to investigate disease pathology and possible intervention therapies[96]. But, although mouse models are a powerful tool in drug development research, it should be noted that mice are different from men and there are also differences in the pathology of atherosclerosis. Firstly, in the majority of humans with atherosclerosis, the disease is caused by a mix of multiple factors (including genetic susceptibility, elevated lipoprotein levels, diabetes, hypertension and smoking), while mouse models are much more monogenic as the multifactorial complexity that is present in humans is difficult to mimic in a mouse model[97]. Secondly, the time span in which atherosclerotic lesions develop greatly differs between humans and mouse models. In humans, the formation of atherosclerotic lesions takes decades, while atherosclerotic lesions in mouse models develop in a couple of months[97]. Thirdly, although plaques are located at sites that

are subjected to low or oscillatory shear stress both in mice and man, most atherosclerosis in mice is located in the aortic root, while this first segment of the artery is usually protected from atherosclerosis in humans[96,97]. Lastly, and possibly also most important, plaque rupture and subsequent thrombosis, which are important clinical features of atherosclerosis in humans, barely occur in mouse models[96,97]. Therefore, it is important to realize that, although mouse models are a very powerful tool to study atherosclerosis development, observations from mouse studies cannot directly be translated to the human situation.

FUTURE PERSPECTIVES

Anti-inflammatory therapies

All three potential targets that were described in this thesis have in common that they affect the inflammatory response. Despite the current lack of CVD drugs that primarily target the immune system, recent studies provide a promising future for this class of drugs in CVD. Multiple anti-inflammatory therapies that have been tested in atherosclerotic rabbit and mouse models were shown to have athero-protective effects[98]. Moreover, a number of these anti-inflammatory therapies, also seem to have a protective effect in humans as they are related to less myocardial infarctions, reduced cardiovascular risk and lower levels of inflammatory markers[98]. Two of these anti-inflammatory therapies, colchicine and canakinumab, have even proven to reduce the occurrence of cardiovascular events in clinical trials. Colchicine, which is prescribed to prevent gout and familial Mediterranean fever, was the first anti-inflammatory therapy targeting CVD that was tested clinically (in the small LoDoCo trial). It was administered to coronary disease patients on top of statins and other standard secondary prevention therapies and reduced cardiovascular events in patients with stable coronary disease with 72%[99]. Although 11% of the colchicine treated patients withdrew early from the study due to intestinal intolerance and another 5% due to a range of possible side effects, colchicine remains an interesting therapy for secondary prevention of CVD due to its high effectiveness and inexpensiveness[99]. However, the observed results still need to be confirmed in a clinical trial with a larger group of patients[99], which is currently ongoing. More recently, the results of the CANTOS trial were revealed. Every three months, patients with a history of myocardial infarction, subcutaneously received canakinumab, a monoclonal antibody targeting the inflammatory cytokine IL-1β[100]. Canakinumab therapy reduced the occurrence of recurrent cardiovascular events by approximately 20%, independent of lipid-lowering[100]. A major complication of canakinumab is the increased death rate due to infections or sepsis that was observed in this study[100], which made Novartis decide not to introduce canakinumab on the market as a cardiovascular drug[101]. Not all investigated anti-inflammatory therapies have atheroprotective effects. The CIRT trial, which investigated the effects of low dose methotrexate, a drug that is prescribed in inflammatory conditions, including rheumatoid arthritis, psoriatic arthritis, and juvenile idiopathic arthritis, for secondary prevention of CVD was stopped as methotrexate did not lower levels of inflammatory proteins and did neither affect the incidence of cardiovascular events[102]. Despite the absence of cardioprotective effects in the CIRT trial and the increased chance of infections that comes

along with anti-inflammatory therapies, the studies mentioned above show that targeting inflammation for future treatment of CVD patients is worth further investigation.

Drug development

The main reason for failure in the early stages of drug development is safety as most preclinical studies are closed due to cardiovascular toxicity, followed by hepato- and renal toxicity[18]. Interestingly, even after market approval, organ-specific toxicity remains an important problem as approximately 90% of the withdrawn drugs have these safety issues[103,104]. Of the nineteen drugs that were withdrawn from the European market from 2002 to 2011, most were withdrawn because of cardiovascular (9 out of 19) or hepatic (4 out of 19) disorders[105]. To limit the amount of time and money spend on developing drugs with adverse toxic side effects, any potential drug is first tested in *in vitro* studies. However, these *in vitro* tests are performed on a monolayer of cells cultured on plastics that lack the cell-cell interactions that are present *in vivo*[106]. Recent developments resulted in the fabrication of 3D tissues that mimic the *in vivo* situation much better[106]. Indeed, *in vitro* drug testing of 8 clinical drugs (of which 6 were reported to have potential cardiotoxic effects) in a 3D human cardiac tissue model showed results that were in line with clinical observations and predicted cardiotoxic effects much better than conventional testing[107]. In another study, 3D human hepatocyte spheroids were used to test the hepatotoxicity of 123 drugs, of which 70 were reported to have hepatotoxic effects[108]. The model correctly identified 69% of all reported hepatotoxic drugs, thereby performing better than any other *in vitro* model[108]. Development of more comprehensive models of other tissues, including kidney, lung, musculoskeletal, skin and adipose tissue, and the development of organs-on-chips are also rapidly progressing[109]. The incorporation of these models in drug development might contribute to improved efficacy and safety of drug development in the future.

Currently, small-molecule drugs and proteins are the two major structural classes of FDA-approved drugs, but these two drug classes have their limitations. It has been estimated that small molecule drugs are only able to bind 2-5% of the human proteins and the use of protein-based drugs is limited by the size and the stability of the proteins[110]. Within the CarTarDis consortium, several potential drug targets were excluded from the shortlist due to the low druggability score as they were not targetable by either small molecules or proteins. However, advances in RNA delivery, which enables protein downregulation, protein upregulation and even gene editing through the RNA-guided CRISPR-Cas system, has massively increased therapeutic opportunities[110]. A great example of RNA-based therapeutics in CVD is mipomersen[111], which was approved by the FDA in 2013 to treat familial hypercholesterolemia patients[112]. Mipomersen is an antisense oligonucleotide that targets apolipoprotein B mRNA and thereby inhibits synthesis of the protein, resulting in a lower number of atherogenic lipoproteins[111]. Examples of other RNA based therapeutics in CVD that are currently being investigated are inclisiran, which is a RNAi that inhibits PCSK9 protein synthesis and volanesorsen, which targets apolipoprotein C3 mRNA[111]. Both therapeutics have shown promising results in phase II clinical trials, which may enhance further investigation of other possible targets in CVD[111], that were not yet targetable before the RNA-based therapeutics era.

Application of personalized medicine in CVD

Personalized medicine has emerged across the pharmaceutical drug development field as an attractive opportunity to improve the safety and efficacy of both existing and novel drugs[113]. It was first introduced in the cancer field, where genetic testing was applied to identify and subsequently successfully treat HER2 overexpressing breast cancer patients with a specific HER2-targeting antibody therapy[114]. This was followed by several strong examples such as Imatinib in the cancer field, which specifically targets the fusion protein Bcr-Abl1 tyrosine kinase in chronic myelogenous leukemia patients that have a mutation leading to the creation of this fusion protein, which is much more active than the unaltered Abl protein and promotes dysregulated cell growth and cancer[114]. Also, in cystic fibrosis personalized medicine is successfully applied in patients carrying the delta F508 mutation in CFTR, who are treated with Ivacaftor, a drug that targets this specific mutation[114]. By identifying the most appropriate target population for a specific treatment and by omitting treatment in patients that are not likely to benefit from treatment, the overall efficacy of that treatment increases[113]. Furthermore, it reduces healthcare costs as patients that will not benefit will not be prescribed these personalized and target-specific drugs, which are usually quite expensive[115]. Moreover, personalized medicine approaches can also be used to identify patients with a high risk of major side effects, which can improve the safety of therapies[113]. In CVD, personalized medicine is not clinically applied yet, but there are several interesting future perspectives how this may be achieved. Firstly, if development of the previously mentioned vaccine against PCSK9 does not succeed, specific targeting of PCSK9 by antibody therapy, particularly in patients that have increased PCSK9 expression might be an attractive option[82,114]. Secondly, genotype-guided dosing of drugs to limit the side effects that are associated with a particular drug or mechanism of action is attractive. As an example, Warfarin is a therapeutic that prevents thromboembolic disease but also causes bleedings upon overdosing, which makes it important to determine the appropriate dose for an individual patient as fast as possible[116]. The EU-PACT warfarin trial showed that taking into account the patient's CYP2C9 and VKORC1 genotype (two genes that have a relatively big impact on the variability in the individual daily dose requirement) reduces excessive anticoagulation incidence, the adjustment time to reach a stable dose and the number of Warfarin dose adjustments compared to standard warfarin dosing[116]. Thirdly, the identification of responders and non-responders to a given therapy holds high potential[117]. In the CANTOS trial for example, researchers showed that the amount of high sensitivity C-reactive protein (hsCRP) reduction predicts the efficacy of IL-1β targeted treatment since patients with higher reduction in hsCRP levels have an increased reduction in cardiovascular events than the patients with less reduction in hsCRP levels. This observation suggests that the reduction in hsCRP levels could be used as an indicator whether to continue or not with this specific treatment[117]. Finally, personalized medicine approaches could be used in CVD to identify patients that have a high risk of developing side effects. This could, for example, be used to identify patients that have a genetic and immunologic susceptibility to develop myopathy due to statin treatment[118]. Especially since patients that develop myopathy are more likely to stop taking their medication[118], it would be beneficial to identify these patients before they develop myopathy and prescribe them another lipid-lowering drug.

Improvement of current therapies

Although development of new drugs and the identification of patients that benefit most from a certain therapy are significant contributors in our fight to diminish CVD, it is important to realize that development of novel therapies is not the only solution to reduce cardiovascular events. It is very valuable to sometimes take a step back and to look at the complete picture to be able to identify the current problems and to come up with other possible solutions that might improve current therapy as well.

One of the current problems that holds back effective CVD therapy is low drug adherence as overall adherence to primary and secondary prevention therapy for CVD was shown to be only 57% over a median treatment period of 24 months[119]. Considering that combination therapy (mostly treatment with aspirin and blood pressure and lipid-lowering drugs) reduces the risk of a cardiovascular event by approximately 80%, it was estimated that, based on these numbers, approximately 130,000 deaths could be avoided yearly in the USA only by adherence to therapy[119]. One of the proposed possible solutions to low drug adherence is the polypill, in which multiple drugs are combined in one single pill[120]. This would lower the medication burden and since patients are more likely to adhere to therapies with less frequent dosing[121], it was hypothesized that the polypill would improve drug adherence[120]. Indeed, several clinical trials showed that patients were more likely to adhere to the polypill (adherence ranged from 70 – 86%)[122–124] than to usual care (adherence ranged from 46 – 65%)[122–124] after 12 – 18 months of treatment[120,122–124], resulting in lower blood pressure and LDL cholesterol levels[120]. Another possible future approach that may further improve drug adherence is the incorporation of healthcare services provided via the internet, also called electronic health or eHealth. Especially the use of mobile health (mHealth) interventions is massively being investigated at the moment[125]. Initial studies that focused on adherence to pharmacological therapy in secondary CVD prevention showed promising results[125], but further studies are needed to provide more information on the efficacy and adherence to these novel approaches. Next to drug adherence, eHealth interventions can also be used to promote adherence to a healthier lifestyle. It has already been shown that eHealth interventions positively affect blood pressure control by reducing both systolic and diastolic blood pressure in patients with hypertension[126] and that web-based digital health intervention increases short-term weight loss in overweight and obese adults[127]. Despite the promising results of eHealth and mHealth studies, currently ongoing and future studies will further extend on these findings and will provide a more information on the efficacy and long-term adherence to these novel approaches.

Additional eHealth opportunities

Besides providing patients with information and motivating them to adhere to their medication and a healthy lifestyle, eHealth can also be used for data collection, giving researchers and physicians access to real-world data. Relatively simple devices, such as smart watches, are able to monitor and record parameters like physiological activity and sleep while slightly more advanced sensors are able to measure physiological parameters including heart rate, blood pressure and respiration rate[128,129]. This data can be used to amongst others, identify target populations, develop new treatments or predict efficacy of

a particular treatment in a specific treatment group[130], thereby contributing to personalized medicine. Lv *et al*., recently developed a personalized-care model that provides personalized feedback to hypertension patients based on patient generated health data, including home-monitored blood pressure, weight and lifestyle behavior and showed that this approach significantly reduced patients' blood pressure[131]. Furthermore, Muhlestein *et al.,* showed that smartphone ECG has an excellent correlation with the current golden standard 12-lead ECG[132], which further raises hope on successful implementation of eHealth for at least some physical parameters. The incorporation of eHealth to improve personalized medicine is also a promising approach in other medical fields including mental health, where continuous monitoring of sleep, mood and appetite can be used in clinical decision making[133]. Moreover, implementation of eHealth might improve healthcare in developing countries since access to specialized care is often limited in remote areas and eHealth might cost-effectively extend the reach of specialized care to remote areas in developing countries[133,134]. However, although implementation of eHealth looks promising, only time will tell whether eHealth will truly impact future healthcare.

CONCLUSION

The high death rate due to CVD demands the development of novel cardiovascular therapies. However, the high costs and the high number of failures in drug development, make careful considerations whether to invest in a potential drug or not mandatory. In this thesis, the five-factor approach that we used to identify potentially novel druggable targets in CVD is briefly described, as well as some of the validation studies that were performed to gain more insight into the role of three of the shortlisted potential targets in CVD. Next to extensively collecting information on a potential target before introducing it into the process of drug development, current improvements in 3D tissue culture might further improve the process of drug development since these tissues predict potential toxic effects much better than conventional tests. Furthermore, efficacy of current and future CVD therapies might be improved by the incorporation of personalized medicine, which has already been proven to be successful in cancer and cystic fibrosis patients, and novel eHealth opportunities. However, although there are plenty of options to implement personalized medicine and eHealth in CVD, only time will tell whether these novel approaches will truly impact future CVD healthcare.

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CHAPTER 8

Dutch summary and discussion

INTRODUCTIE

De toename van het aantal patiënten met hart- en vaatziekten en het aantal patiënten dat hieraan overleed in de eerste helft van de 20e eeuw in hoge-inkomenslanden[1,2] was een stimulans voor hart- en vaatziekte gerelateerd onderzoek[3]. Hierdoor nam de kennis over het ontstaan van hart- en vaatziekten en de risicofactoren die bij deze ziekten een rol spelen toe, wat resulteerde in een afname van het aantal overledenen als gevolg van hart- en vaatziekten in hoge-inkomenslanden[1]. Echter, de gelijktijdige toename van het aantal patiënten met hart- en vaatziekten in lage- en midden-inkomenslanden[4] zorgde ervoor dat hart- en vaatziekten de grootste doodsoorzaak wereldwijd is geworden[5]. Een gezonde levensstijl, bestaande uit factoren als een gezond dieet, stoppen met roken, voldoende lichamelijke inspanning en gewichtsverlies kunnen het risico op hart- en vaatziekten sterk verminderen[6–8]. Helaas hebben een aantal interventiestudies aangetoond dat het vaak lastig is om deze gezondere levensstijl vast te houden[9]. Een Finse interventiestudie heeft laten zien dat, ondanks de sterke motivatie van deelnemers om een gezondere levensstijl aan te nemen, trouw aan een gezondere levensstijl (welke 52% was bij aanvang van de studie) 6 en 12 maanden na interventie afgenomen was tot respectievelijk 8% en 4%[10]. Een aantal studies dat heeft gekeken naar gewichtsverlies liet zien dat, ondanks het initiële gewichtsverlies, veel deelnemers een jaar na het begin van de studie alweer aangekomen waren en na 3 tot 5 jaar zelfs weer op hun oude gewicht zaten[9]. Momenteel overlijdt ongeveer één op de drie mensen aan hart- en vaatziekten[5], maar daarnaast is ook de economische impact groot. Alleen al in de Verenigde Staten wordt jaarlijks 396 miljard dollar besteed aan directe kosten verbonden aan hart- en vaatzieken[11]. Hart- en herseninfarcten zijn samen verantwoordelijk voor meer dan de helft van alle aan hart- en vaatziekten gerelateerde overledenen[11]. Daarom is het van groot belang om nieuwe therapieën te ontwikkelen tegen atherosclerose, wat de onderliggende oorzaak is van hart- en herseninfarcten[12–14].

Atherosclerose

Atherosclerose, ook wel bekend als aderverkalking, is een complexe ziekte die wordt veroorzaakt door een samenspel van verschillende processen, namelijk: veranderingen in het lipiden en lipoproteïnen metabolisme, het ontstaan van ontstekingsreacties en endotheeldysfunctie[15]. Als de endotheellaag (een monolaag van cellen die de binnenkant van de bloedvatwand bekleedt en daardoor een barrière vormt tussen de bloedstroom en het omliggende weefsel[16]) dysfunctioneel of geactiveerd raakt[17,18], vermindert de barrièrefunctie van het endotheel[17–19] waardoor lage-dichtheids-lipoproteïnen (LDL), welke cholesterol transporteren naar cellen[20], gemakkelijker vanuit het bloedvat het omliggende weefsel binnen kunnen treden waar ze ophopen in de subendotheliale matrix[14,21] en er een vetlaag met cholesterol ontstaat in de bloedvatwand. In de bloedvatwand wordt het LDL geoxideerd en proteolytisch gemodificeerd waardoor het ontstekingsproces, dat reeds geïnitieerd is door de geactiveerde endotheelcellen[22], verder toeneemt[23]. Het geoxideerde LDL wordt opgenomen door macrofagen die al in het weefsel aanwezig zijn of door monocyten die door de geactiveerde endotheelcellen vanuit het bloed gerekruteerd worden

en bij het uittreden differentiëren in macrofagen[21]. De geactiveerde endotheelcellen rekruteren de monocyten door deze eerst aan te trekken middels uitscheiding van het cytokine MCP-1 en vervolgens de snelheid waarmee deze monocyten door het bloedvat stromen af te remmen met behulp van selectines die tot expressie komen op de endotheelcellen[24]. Wanneer deze snelheid voldoende is afgenomen kunnen de monocyten binden aan de zogeheten adhesiemoleculen die tot expressie komen op de endotheelcellen en welke zorgen voor een sterke binding tussen de twee celtypen waarna de monocyt door de endotheellaag heen kan migreren[24]. Na migratie door de endotheellaag differentieert de monocyt in een macrofaag[25,26], welke in staat is grote hoeveelheden lipiden op te ruimen[27]. In deze eerste stadia zijn hoge-dichtheids-lipoproteïnen (HDL) nog in staat het proces van atherosclerose ongedaan te maken door het in de vaatwand opgehoopte cholesterol uit de vaatwand weg te transporteren[20,21] en het ontstekingsproces te remmen[21,28]. Dit proces wordt echter onomkeerbaar naarmate de cholesterolophoping groter wordt[23,29,30] en de aanwezige macrofagen veranderen in schuimcellen[27,31] waardoor het ontstekingsproces verder toe neemt[27] en de vetophoping in een vroege atherosclerotische laesie verandert[23,29,30]. Het ontstekingsproces zorgt ervoor dat de cellen die zich in deze atherosclerotische laesie bevinden, waaronder gladde spiercellen, endotheelcellen en macrofagen, afsterven (in apoptose gaan)[32]. In de latere stadia van atherosclerose zijn de macrofagen niet meer in staat om deze dode cellen op te ruimen en vormen deze dode cellen de zogenoemde necrotische kern[33,34]. Het continue ontstekingsproces zorgt samen met het tekort aan zuurstof (de hypoxie) die aanwezig is in de necrotische kern voor nieuwvorming van bloedvaatjes (angiogenese) aan de buitenkant van het bloedvat (vasa vasorum)[35–37]. Deze nieuw gevormde bloedvaten hebben een slechte barrièrefunctie en in ieder geval een gedeelte van deze vaten eindigt in de atherosclerotische laesie waardoor erythrocyten, leukocyten, bloedplaatjes en lipiden vanuit het bloedplasma gemakkelijk de laesie binnen kunnen komen en zo bijdragen aan verdere groei en instabiliteit van de laesie[36–39]. Een instabiele laesie is te herkennen aan de grote hoeveelheid aanwezige vetten en macrofagen en de dunne fibreuze kap[40,41]. Deze laesie heeft een grotere kans om te scheuren dan een stabiele laesie[42], welke te herkennen is aan de kleine necrotische kern, lage hoeveelheid aanwezige macrofagen en dikke fibreuze kap[40,41]. Als een laesie scheurt dan komt de inhoud van de laesie in contact met het bloed waardoor er een stolsel (trombus) kan ontstaan[43]. Afhankelijk van de grootte van de trombus, kan deze het lumen van een bloedvat en daarmee de toevoer van bloed naar het achterliggende weefsel blokkeren[43]. Afhankelijk van de locatie van deze blokkade kan dit leiden tot klinische complicaties zoals een hart- of herseninfarct[44,45].

Nieuwe therapieën tegen hart- en vaatziekten

Het mag duidelijk zijn dat het belangrijk is om nieuwe medicijnen te ontwikkelen die atherosclerose en daarmee hart- en vaatziekten tegengaan. Echter, het ontwikkelen van nieuwe medicijnen is een lang en duur proces[46]. De huidige ontwikkelingskosten van één enkel medicijn zijn ongeveer 2,6 miljard dollar[46] en deze kosten zullen in de toekomst waarschijnlijk alleen maar toenemen[46,47]. Eén van de oorzaken van deze hoge kosten is het lage slagingspercentage[46], van alle 24 studies die opgestart worden zijn er slechts 8 kandidaat

medicijnen die getest worden in klinische studies en is er slechts één medicijn dat daadwerkelijk op de markt komt[48]. Om het proces van medicijnontwikkeling te verbeteren heeft AstraZeneca een studie gedaan waarbij al haar eigen medicijnontwikkelingsprojecten onder de loep zijn genomen om erachter te komen waarom een groot gedeelte van de projecten niet tot het gewenste resultaat leidt[49]. De uitkomst van deze studie was dat de meeste projecten, voornamelijk preklinische en klinische fase I studies, gestopt werden vanwege veiligheidsoverwegingen. Veel kandidaat geneesmiddelen bleken namelijk ongewenste effecten op het hart- en vaatstelsel en de lever te hebben[49]. Naarmate het proces van medicijnontwikkeling verder vorderde, namen de veiligheidsproblemen af, maar werden er meer studies gestopt vanwege het gebrek aan effectiviteit van de ontwikkelde medicijnen[49]. Een andere belangrijke conclusie die uit dit onderzoek is voortgekomen is dat het slagingspercentage van projecten hoger is naarmate het doelwit waar het medicijn op aangrijpt goed gevalideerd is, als er een genetische link is tussen het doelwit en de ziekte van interesse en als de rol van dit doelwit in het ontstaan van de ziekte bekend is[49]. Gebaseerd op de bovengenoemde bevindingen heeft AstraZeneca vervolgens een strategie bestaande uit vijf sleutelfactoren ontwikkeld om het slagingspercentage van medicijnontwikkeling te verhogen[49].

Geïnspireerd door de strategie van AstraZeneca, hebben wij binnen het CarTarDis consortium onze eigen vijf-factor-bevattende strategie ontwikkeld om doelwitten te identificeren waartegen medicijnen ontwikkeld zouden kunnen worden om hart- en vaatziekten te bestrijden. De vijf factoren die wij hiervoor van belang achtten zijn: **druggability** (is het mogelijk om een medicijn te ontwikkelen dat effect heeft op het doelwit van interesse), **novelty** (hoe nieuw is het, hoeveel publicaties en patenten bestaan er al omtrent dit doelwit), **genetics** (is er bewijs van een genetische link tussen het doelwit en de ziekte van interesse), **feasibility** (hebben wij de juiste kennis en technieken in huis om de benodigde studies uit te voeren) en biologische **pathways** (bij welke signaleringsroutes is het doelwit van interesse betrokken).

Allereerst hebben wij een lijst gemaakt van doelwitten waar mogelijk medicijnen tegen ontwikkeld kunnen worden om hart- en vaatziekten te bestrijden. Deze lijst van potentiële doelwitten is gemaakt op basis van reeds gepubliceerde literatuur en op data afkomstig uit cohortstudies (KORA, AGES en REFINE) en case-control studies (CardioGenX, BiKE en Socrates) waar we toegang toe hadden binnen het CarTarDis consortium. De doelwitten op deze lijst hebben we vervolgens een score gegeven voor elk van de boven genoemde factoren. Dit heeft ons geholpen om een lijst van de in onze ogen meest veelbelovende kandidaat doelwitten te maken en heeft tegelijkertijd de hiaten in onze kennis over deze doelwitten blootgelegd. Aangezien het slagingspercentage van medicijnontwikkeling hoger is bij doelwitten waarvan de rol binnen het ziekteproces goed begrepen is, hebben wij vervolgens de betrokkenheid van deze doelwitten in hart- en vaatziekten geprobeerd te valideren. In dit proefschrift zijn de validatiestudies van drie van deze kandidaat doelwitten beschreven.

DEEL ÉÉN - ONCOSTATIN M

Oncostatin M (OSM) is aan onze lijst van mogelijke kandidaten toegevoegd vanwege de bestaande literatuur en onze eigen preliminaire *in vitro* experimenten. Onze hypothese was dat OSM endotheelactivatie induceert en daardoor bijdraagt aan de ontwikkeling van atherosclerose. In **hoofdstuk 2** hebben we laten zien dat OSM inderdaad endotheelactivatie induceert in zowel gekweekte menselijke endotheelcellen als in endotheelcellen van muizen die gedurende 3 weken met OSM zijn behandeld. In **hoofdstuk 3** hebben we laten zien dat OSM evenals de receptoren voor OSM, OSMR en LIFR, tot expressie komen in humane atherosclerotische laesies, dit leek onze veronderstelling dat OSM bijdraagt aan het proces van atherosclerose te versterken. Echter, in de 16 weken durende atherosclerosestudie die we hebben uitgevoerd in muizen zagen we juist kleinere atherosclerotische laesies. Deze observatie was dan wel weer in overeenstemming met onze bevinding dat patiënten met hogere OSM concentraties in het bloed een hogere overlevingskans hebben dan patiënten met lage OSM concentraties in het bloed. In analogie hebben we in **hoofdstuk 4** laten zien dat een genetische variant in OSMR, die zorgt voor een lagere expressie van OSMR en daardoor eventueel minder OSM signalering, correleert met een instabieler laesie fenotype. Zoals eerder al uitgelegd, hebben instabiele laesies een grotere kans om open te scheuren en is de kans op complicaties zoals een hart- of herseninfarct hierdoor groter.

Onze bevindingen zijn dus gedeeltelijk in overeenstemming met de vooraf gestelde hypothese. OSM zorgt namelijk wel voor activatie van endotheelcellen, maar lijkt de ontwikkeling van atherosclerose juist tegen te gaan. Aangezien we geen goed onderbouwde verklaring hebben kunnen vinden voor deze onverwachte resultaten kunnen we slechts speculeren over de mechanismen achter deze onverwachte effecten. Eén van de mogelijke verklaringen voor de verminderde atherosclerose ontwikkeling kan afgeleid worden uit de door ons uitgevoerde *in vitro* experimenten. Toediening van OSM aan endotheelcellen zorgde voornamelijk voor een toename van het adhesiemolecuul ICAM-1 aan de buitenkant van de endotheelcellen en niet voor toename van andere adhesiemoleculen zoals VCAM-1, P-selectin en E-selectin, ondanks de toename in mRNA expressie van al deze adhesiemoleculen. Daarentegen zagen wij juist een toename van VCAM-1 en E-selectin in het kweekmedium, wat erop wijst dat de endotheelcellen deze adhesiemoleculen juist losgelaten hebben in plaats van het aan de buitenkant van de cel tot expressie te brengen. Aangezien ook van de cel los gelaten adhesiemoleculen geassocieerd zijn met atherosclerose[50–53], was dit nog steeds in lijn met onze hypothese dat OSM atherosclerose verergert en hebben wij hier niet lang bij stil gestaan. Waar we op dat moment niet aan gedacht hebben is dat het type cellen dat door middel van deze adhesiemoleculen aan de endotheelcel bindt, afhankelijk is van het specifieke type adhesiemoleculen dat op de endotheelcel tot expressie komt[54]. In dit specifieke geval van voornamelijk hoge ICAM-1 expressie, zou men verwachten dat er veel meer niet-klassieke dan klassieke monocyten aan de endotheelcel hechten aangezien ICAM-1 een voorkeur heeft voor de niet-klassieke monocyten[24,55,56] en VCAM-1 juist een voorkeur heeft voor de voor de klassieke monocyten[24,57]. In **hoofdstuk 3** laten we zien dat muizen die behandeld zijn met OSM een lager percentage klassieke monocyten in hun bloed hebben. De niet-klassieke monocyten onderscheiden

zich van de klassieke monocyten door over de endotheellaag te patrouilleren, waarbij zij afbraakmateriaal en dode cellen opruimen[56], hun betrokkenheid bij het wondgenezingsproces[56] en hun voorkeur om zich te ontwikkelen tot M2 macrofagen, cellen die erom bekend staan dat ze de bloedvaten beschermen en het proces van atherosclerose in mensen lijken te remmen[57–59]. De klassieke monocyten daarentegen, worden snel gerekruteerd naar de plek van de ontsteking waar ze vanuit de bloedsomloop het ontstoken weefsel binnentreden en zich ontwikkelen tot voornamelijk M1 macrofagen[56]. Deze macrofagen stimuleren juist het onstekingsproces en zijn geassocieerd met atherosclerose progressie[57,60]. Samenvattend, het profiel van adhesiemoleculen dat tot expressie komt op endotheelcellen die gestimuleerd zijn met OSM suggereert dat er voornamelijk niet-klassieke monocyten aan de door OSM geactiveerde endotheelcellen zullen binden. Dit zou kunnen verklaren waarom we in muizen die behandeld zijn met OSM meer monocytadhesie zien maar tegelijkertijd ook kleinere atherosclerotische laesies. Deze hypothese is echter gebaseerd op aannames en verder onderzoek is dan ook noodzakelijk om deze hypothese te testen.

Een andere mogelijke verklaring voor de verminderde atherosclerosevorming zou kunnen zijn dat OSM minder ontstekingsstimulerend werkt dan algemeen gedacht. Ondanks dat wij, en anderen, hebben laten zien dat OSM zorgt voor het vrijgeven van de inflammatoire cytokinen IL-6 en MCP-1, zijn er ook studies die laten zien dat OSM ontstekingsremmende eigenschappen heeft aangezien het de productie van TNFα[61] en IL-1β[62] onderdrukt. Net als IL-6 en MCP-1[63,64], worden TNFα en IL-1β gezien als één van de drijvende krachten achter de ontwikkeling van atherosclerose[65–67]. Aangezien wij in onze studies slechts naar de concentraties van een beperkt aantal cytokinen hebben gekeken, kunnen wij op basis van onze onderzoeksresultaten geen sluitende conclusie trekken over de inflammatoire respons van OSM. Een mogelijke derde verklaring voor de onverwachte resultaten zou kunnen zijn dat de stimulerende rol van OSM op het proces van atherosclerose in de literatuur voornamelijk gebaseerd is op de aanwezigheid van OSM in atherosclerotische laesies[68] en op ofwel de verhoogde OSM concentraties in inflammatoire ziekten zoals reumatoïde artritis[69,70] en parodontitis[71] of op de rol van OSM in processen die een rol spelen in atherosclerose ontwikkeling zoals angiogenese[72], gladde spiercel proliferatie[68] en expressie van tissue factor[73], welke allemaal risicofactoren zijn voor de ontwikkeling van atherosclerose[68,71–74]. Ondanks dat de aanwezigheid van OSM in atherosclerotische laesies, die beschreven wordt in dit proefschrift en door anderen, wijst op de betrokkenheid van OSM bij atherosclerose ontwikkeling moeten we voorzichtig zijn om hier conclusies uit te trekken. Meerdere studies hebben namelijk al laten zien dat zulke aannames regelmatig beïnvloed worden door een omgekeerde oorzaak en gevolg[75], oftewel gelijkenis tonen met de vraag 'wat was er eerder, de kip of het ei?'. Recente voorbeelden hiervan op het gebied van hart- en vaatziekten zijn medicijnen die zorgen voor een toename van HDL, de toegepaste hormoontherapie in postmenopauzale vrouwen en de voorgeschreven vitamine E supplementen. Naar aanleiding van epidemiologische studies veronderstelde men dat deze behandelingen het risico op hart- en vaatziekten zouden verminderen, echter gerandomiseerde klinische studies konden deze hypothesen niet bevestigen[76,77]. De veronderstelde beschermende effecten van zowel hormonen als vitamine E waren

waarschijnlijk gerelateerd aan sociaaleconomische en levensstijl factoren, welke de gevonden associatie met hart- en vaatziekten vertroebeld hebben[76]. Voor zo ver wij weten is er slechts één interventiestudie gepubliceerd die heeft gekeken naar de rol van OSM signalering in atherosclerose ontwikkeling. Ondanks dat deze studie liet zien dat OSMR deficiënte ApoE$^{-/-}$ muizen kleinere en minder ernstige laesies hebben, is dit nog geen bewijs dat OSM signalering daadwerkelijk een rol speelt bij de ontwikkeling van atherosclerose aangezien IL-31 ook via OSMR signaleert[78,79]. Ten slotte is OSM een complex eiwit waarvan eerder al aangetoond is dat het tegenovergestelde effecten kan hebben afhankelijk van het celtype waarop het effect uitoefent en het moment in tijd waarop OSM in aanraking komt met een bepaald celtype. Afhankelijk van het celtype kan OSM namelijk een stimulerend, maar ook een remmend effect hebben op proliferatie[68,72,80,81], wat het moeilijk maakt om de *in vivo* effecten van OSM te voorspellen, aangezien een organisme niet uit één, maar uit een heleboel verschillende celtypen bestaat. Ook kan OSM tegenovergestelde effecten hebben op één en hetzelfde celtype op verschillende momenten in tijd[82]. In humane proximale tubulus cellen bijvoorbeeld, zorgt OSM in eerste instantie voor een toename van CTGF en op een later moment juist tot een afname van CTGF. Met uitzondering van het waarschijnlijk negatieve effect dat uitgaat van door OSM geïnduceerde endotheelactivatie, lijkt het erop dat OSM in muismodellen van acute aandoeningen zoals acuut leverfalen[83], reperfusieschade na cardiale ischemie[84] en een hartinfarct[85,86] vaak een beschermend effect heeft terwijl het in chronische ziekten zoals chronische leverschade[87] en chronisch hartfalen[88] het ziektebeeld juist verslechtert. Het effect dat OSM heeft op endotheelactivatie en de verschillende gezichten die OSM kan hebben in acute en chronische situaties tonen overeenkomsten met zijn familielid IL-6, waarvan ook beschreven is dat het beschermende effecten heeft in de acute fase en verslechterende effecten in de chronische fase[89].

Ondanks onze inspanningen zijn er nog veel vragen rondom de rol van OSM in atherosclerose en daarom is het noodzakelijk om eerst nog extra onderzoek te doen voordat OSM overwogen kan worden als mogelijk doelwit in hart- en vaatziekten. Ten eerste is het belangrijk om duidelijkheid te krijgen over de rol van OSM in atherosclerose aangezien het beschermende effect van OSM op atherosclerose tegenovergesteld is aan het stimulerende effect van OSM op atherosclerose ontwikkeling dat gevonden is door Zhang et al[78]. Ten tweede is het belangrijk om het mechanisme achter het door OSM geïnduceerde effect op atherosclerose te achterhalen en ten derde moet onderzocht worden of het remmen of stimuleren van de OSM signaleringsroute cardiovasculaire complicaties kan verminderen en veilig is. Ondanks dat aanvullend onderzoek naar de rol van OSM in atherosclerose ontwikkeling noodzakelijk is, gaat er van de data in dit proefschrift, en dan met name de associatie tussen hogere serum OSM concentraties en de hogere overlevingskans in patiënten met coronaire hartziekte, wel een waarschuwing uit dat men voorzichtig moet zijn met het remmen van OSM signalering.

DEEL TWEE - PHOSPHOLIPID PHOSPHATASE 3

Phospholipid Phosphatase 3 (PLPP3) is aan onze lijst van mogelijke kandidaat doelwitten toegevoegd vanwege de sterke genetische associatie met hart- en vaatziekten. In **hoofdstuk 5** laten wij zien dat een hogere *PLPP3* mRNA expressie geassocieerd is met een hogere kans op overleving zonder cardiovasculaire complicaties. Hypothetisch zou het verhogen van PLPP3 dus een beschermend effect moeten hebben op hart- en vaatziekten. Aangezien PLPP3 een membraaneiwit is, is het moeilijk om de expressie of de activiteit van PLPP3 in patiënten te verhogen. Daarom hebben wij gekeken of er factoren zijn die door PLPP3 beïnvloedt worden die als mogelijk doelwit zouden kunnen dienen. Lysophosphatidic acid (LPA) is één van de bioactieve lipiden die geïnactiveerd wordt door PLPP3[90], hierdoor wordt voorkomen dat LPA aan één van de zes LPA receptoren (genaamd LPAR1-6) bindt[90,91]. Onderdrukking van *PLPP3* in endotheelcellen zorgt voor activatie van endotheelcellen op eenzelfde manier als LPA, wat suggereert dat verminderde *PLPP3* expressie in endotheelcellen zorgt voor endotheelactivatie via verhoogde LPA hoeveelheden. Aangezien LPAR6 van alle LPA receptoren het hoogst tot expressie komt op humane endotheelcellen hebben wij gekeken of het remmen van LPAR6 zorgt voor een vermindering van LPA geïnduceerde endotheelactivatie. Markers van endotheelactivatie kwamen inderdaad verminderd tot expressie op cellen die zowel gestimuleerd waren met LPA als waarbij LPAR6 expressie was onderdrukt, echter onderdrukking van LPAR6 kon de door LPA geïnduceerde endotheelactivatie niet geheel teniet doen. Dit wijst erop dat andere LPA receptoren op de endotheelcellen waarschijnlijk ook een rol spelen bij de LPA geïnduceerde endotheelactivatie. Daarnaast zorgde onderdrukking van *LPAR6* niet voor een vermindering van de endotheelactivatie die was geïnduceerd door verminderde expressie van PLPP3, wat erop wijst dat er naast LPA ook andere door PLPP3 geïnactiveerde substraten betrokken zijn bij endotheelactivatie. Eén van de factoren die parallel aan LPA endotheelactivatie zou kunnen induceren en de mogelijke oorzaak zou kunnen zijn van de waargenomen endotheelactivatie is sphingosine 1-phosphate (S1P)[92]. Dit is een bioactief lipide dat net als LPA gedefosforyleerd wordt door PLPP3 en betrokken is in het ontstekingsproces door immuuncellen vanuit de lymfoïede organen (zoals de milt en lymfeknopen) naar de bloedsomloop te rekruteren en vervolgens naar de plek van de ontsteking[93–96]. Daarnaast zorgt S1P voor een verhoogde E-selectine en VCAM-1 expressie op het membraan van endotheelcellen op eenzelfde wijze als TNFα[97], wat suggereert dat S1P, net als LPA, endotheelactivatie kan induceren. Ook zorgt S1P voor een verhoging van tissue factor in endotheelcellen, wat er mogelijk toe kan leiden dat de endotheelcellen trombotische eigenschappen krijgen[94]. Andere studies daarentegen laten zien dat S1P de endotheelbarrière juist versterkt[98], monocytadhesie tegengaat[99], en dat S1P analogen atherosclerose ontwikkeling in muizen tegengaan[100]. Concluderend, ondanks dat deze studie heeft bijgedragen aan onze kennis betreffende het beschermende effect van PLPP3 op endotheelactivatie, zijn er ook hier nog veel vragen die beantwoord moeten worden over het mechanisme achter PLPP3 voordat PLPP3 werkelijk overwogen kan worden als kandidaat doelwit in de bestrijding van hart- en vaatziekten.

DEEL DRIE - CAT EYE SYNDROME CRITICAL REGION PROTEIN 1

Cat eye syndrome critical region protein 1 (CECR1), wat adenosine concentraties verlaagt door deze om te zetten in inosine[101], is aan onze lijst van mogelijke kandidaat doelwitten toegevoegd vanwege de hoge noviteit en biologische relevantie score. In **hoofdstuk 6** laten we zien dat CECR1 mRNA en eiwit tot expressie komen in humane atherosclerotische laesies en dat CECR1 actief is in deze atherosclerotische laesies aangezien de locatie van CECR1 mRNA en eiwit overeen komt met hoge concentraties inosine en lage concentraties adenosine. Dit suggereert dat CECR1 een rol speelt bij de ontwikkeling van atherosclerose. Patiënten met een verlies van functie mutatie in CECR1 hebben vaak chronische of terugkerende systemische ontstekingsreacties en hebben een verhoogd risico op een ischemische of hemorragische beroerte en andere vaat gerelateerde complicaties zoals hypertensie en vasculitis[102,103]. Daarnaast is CECR1 deficiëntie geassocieerd met een verlaagde hoeveelheid anti-inflammatoire M2 macrofagen en een toename van pro-inflammatoire cytokinen[102]. De literatuur suggereert dat de vasculitis in patiënten met een CECR1 deficiëntie veroorzaakt wordt door chronische neutrofiel activatie[103], daarom hebben wij eerst gekeken of CECR1 serum concentraties geassocieerd zijn met de aanwezige hoeveelheid neutrofielen in het bloed. In lijn der verwachting vonden wij een negatieve correlatie tussen CECR1 serum concentraties en de hoeveelheid circulerende neutrofielen, wat suggereert dat CECR1 inderdaad de circulerende neutrofielen beïnvloed. Aangezien systemische ontstekingsreacties bijdragen aan de ontwikkeling van atherosclerose[104], CECR1 adenosine omzet in inosine[101] en neutrofielen alle vier de adenosine receptoren tot expressie brengen[105], stelden wij de hypothese dat CECR1 de ontwikkeling van atherosclerose vermindert door adenosine concentraties en daardoor neutrofiel activatie te verlagen. Onze *in vitro* studies hebben laten zien dat adenosine inderdaad zorgt voor neutrofiel activatie door middel van NETosis (een vorm van celdood waarbij de inhoud van de cel, waaronder DNA, naar buiten wordt gegooid) in neutrofielen van gezonde individuen en dat CECR1 dit tegen kan gaan. Dit komt overeen met een studie van Carmona-Rivera *et al.,* waarin de onderzoekers hebben laten zien dat adenosine NETosis induceert in neutrofielen van CECR1 deficiënte patiënten en dat dit geremd kan worden door CECR1[106]. Vervolgens hebben we laten zien dat het geconditioneerde medium van de door adenosine gestimuleerde neutrofielen THP-1 monocyt activatie induceert door de *MCP-1* en *IL-1β* mRNA expressie te verhogen. *MCP-1* en *IL-1β* mRNA expressie van THP-1 monocyten die gestimuleerd zijn met medium afkomstig van met CECR1 of met de combinatie van adenosine en CECR1 behandelde neutrofielen was niet veranderd ten opzichte van de controlegroep. Ten slotte hebben we laten zien dat circulerende CECR1 concentraties verhoogd zijn in patiënten met een myocard infarct of coronaire hartziekten en dat hoge circulerende CECR1 concentraties geassocieerd zijn met een lagere overlevingskans in patiënten met coronaire hartziekten. Deze laatste bevinding suggereert dat CECR1 bijdraagt aan de ontwikkeling van atherosclerose, wat tegenovergesteld is aan het beschermende effect dat CECR1 lijkt uit te oefenen door adenosine geïnduceerde neutrofiel activatie te remmen. Er zijn verschillende verklaringen te bedenken voor deze schijnbare tegenstellingen. Ten eerste zouden deze

tegengestelde effecten veroorzaakt kunnen worden door een verschil in concentratie van adenosine. Lage adenosine concentraties signaleren voornamelijk door de A_1 en A_3 adenosine receptoren en stimuleren bijvoorbeeld neutrofiel chemotaxis (verplaatsing ten gevolge van een concentratiegradiënt) en fagocytose (vernietiging van schadelijke bestanddelen door middel van insluiting) terwijl hoge adenosine concentraties, welke voornamelijk via de A_{2A} en A_{2B} adenosine receptoren signaleren, de verplaatsing van neutrofielen en het afgeven van granulen (blaasjes in de cel) juist tegen gaan[105]. Ten tweede zijn neutrofielen niet de enige cellen in het menselijk lichaam die reageren op adenosine of CECR1. Ondanks dat adenosine een ziekte verergerend effect lijkt te hebben op neutrofielen, heeft men eerder laten zien dat adenosine juist een beschermend effect kan hebben op endotheelcellen. Adenosine reduceert namelijk de door trombine geïnduceerde expressie van VCAM-1, ICAM-1 en E-selectine op de celmembraan en de expressie van pro-inflammatoire cytokinen[107]. Als de beschermende effecten van adenosine op endotheelcellen zwaarder wegen dan de ziekte verergerende effecten van adenosine op neutrofielen kan het totale effect van verlaging van adenosine concentraties dus ziekte- en atherosclerose verergerend zijn. Ten slotte hoeft de aanwezigheid van een eiwit in een atherosclerotische plaque, zoals al eerder genoemd, niet te betekenen dat het eiwit atherosclerose verergert. CECR1 zou namelijk, net als IL-10, wat tot expressie komt in gevorderde atherosclerotische laesies en ontstekingsremmende effecten heeft[108], de lokale ontstekingsreactie kunnen reguleren. CECR1 zou dus een compensatiemechanisme kunnen zijn dat de ontstekingsreactie probeert te verminderen en alleen tot expressie komt bij de aanwezigheid van een ontstekingsreactie. Concluderend, ondanks dat CECR1 de door adenosine geactiveerde neutrofiel activatie tegen gaat, wat een beschermend effect impliceert, zijn hoge CECR1 serum concentraties juist geassocieerd met een verminderde overlevingskans bij patiënten met coronaire hartziekten. Verder onderzoek zal uit moeten wijzen wat de oorzaak is van deze schijnbare tegenstellingen en is noodzakelijk voordat CECR1 daadwerkelijk overwogen kan worden als kandidaat doelwit in de bestrijding van hart- en vaatziekten.

REFLECTIE VAN ONZE AANPAK

Eén van de sterke kanten van onze benadering is het toekennen van een score op basis van genetisch bewijs, aangezien de aanwezigheid van een genetische link tussen het doelwit en de ziekte van interesse sterk bijdraagt aan de slagingskans van de studie[75]. Eén van de recente succesverhalen van het toepassen van genetica in de geneesmiddelenontwikkeling is de ontdekking van een mutatie in het humane PCSK9 gen. Deze mutatie, waarbij het PCSK9 eiwit beter gaat functioneren, zorgt voor hypercholesterolemie, terwijl mutaties die zorgen voor een verminderde functie van het eiwit juist zorgen voor een afname in LDL-cholesterol en voor een vermindering van coronair vaatlijden[109]. Deze observaties in mensen hebben geleid tot de ontwikkeling van een nieuw muismodel voor atherosclerose, waarbij atherosclerose ontwikkeling wordt geïnduceerd door een adenovirus gemedieerde overexpressie van PCSK9 in combinatie met een hoog-vet dieet[110]. Het sterke effect van deze mutatie werd in dit muismodel benadrukt doordat één enkele injectie met dit

adenovirus voldoende is om atherosclerose in een wildtype muis te induceren[110], terwijl de meeste genen waarvan men vermoedt dat ze betrokken zijn bij atherosclerose een minder groot effect hebben en onderzocht moeten worden in muizen die naast het gen van interesse ook een mutatie moeten hebben in een ander gen dat een beschermende werking heeft tegen atherosclerose, zoals Apolipoprotein E (ApoE) of de LDL receptor[110]. Echter, het maken van een zogenoemde dubbele knock-out muis is lastig en kost veel tijd, vandaar dat de creatie van een atherosclerotisch muismodel door één enkele injectie met een PCSK9 adenovirus al het eerste succes was van de ontdekking van de PCSK9 mutatie[110]. Verdere successen zijn geboekt met de ontwikkeling van PCSK9 remmers. Toediening van evolocumab of alirocumab, monoclonale antilichamen tegen humaan PCSK9, bovenop toediening van statines leidde niet alleen tot lagere plasma cholesterol levels in het ApoE3*Leiden.CETP muis model, maar ook tot een grote afname in de grootte en ernst van de atherosclerotische leasies[111,112]. Een recentere studie in het ApoE3*Leiden.CETP muismodel liet zien dat het beschermende effect tegen atherosclerose ook bereikt kan worden door toediening van een vaccinatie tegen PCSK9, wat mogelijk de dure PCSK9 antilichaamtherapie, welke veel vaker toegediend moet worden, zou kunnen vervangen[113]. De impact van de ontdekking van deze specifieke *PCSK9* mutatie wordt verder benadrukt door de snelle introductie van PCSK9 remmende medicijnen, welke minder dan 12 jaar na de initiële ontdekking van deze specifieke mutatie volgde[114]. Ondanks dat de effecten van zowel evolocumab en alirocumab op de incidentie van hart- en vaatziekten op het moment van introductie nog onderzocht moest worden in lange klinische trials, werden de geneesmiddelen al wel goedgekeurd vanwege het cholesterol verlagende effect van deze medicijnen in mensen[115,116]. Recentelijk is er meer bekend geworden over het effect van evolocumab en alirocumab op cardiovasculaire eindpunten. Evolocumab vermindert het risico op een primair eindpunt (samengesteld eindpunt bestaande uit cardiovasculaire sterfte, myocardinfarct, beroerte, ziekenhuisopname voor instabiele angina pectoris of coronaire revascularisatie) van 11.3% naar 9.8% en op een secundair eindpunt (samengesteld eindpunt bestaande uit cardiovasculaire sterfte, myocardinfarct of beroerte) van 7.4% naar 5.9%[117] en alirocumab vermindert het risico op een primair eindpunt (samengesteld eindpunt bestaande uit sterfte door coronaire hartziekten, niet-fataal myocardinfarct, ischemische beroerte of instabiele angina pectoris waarvoor ziekenhuisopname noodzakelijk is) van 11.1% naar 9.5% en op totale sterft van 4.1% naar 3.5%[118].

Ondanks dat de score voor haalbaarheid (feasibility), welke gebaseerd was op de aanwezige kennis, biobanken, technieken en *in vitro* en *in vivo* modellen die wij als consortium in huis hadden, ons heeft geholpen om een snelle start te maken met de geselecteerde doelwitten, zou dit een zwakte van onze aanpak kunnen zijn, aangezien we hiermee potentieel interessante kandidaat doelwitten buitengesloten zouden kunnen hebben. Zo zou onze sterke achtergrond op het gebied van endotheelcellen ervoor gezorgd kunnen hebben dat kandidaat doelwitten die voornamelijk effect hebben op endotheelcellen, makkelijker geselecteerd zijn dan kandidaat doelwitten die voornamelijk effect uitoefenen op andere celtypen die betrokken zijn bij de ontwikkeling van atherosclerose, zoals gladde spiercellen[68] en macrofagen[119].

Daarnaast is het belangrijk om te realiseren dat alle technieken en modellen die beschreven zijn in dit proefschrift allemaal hun eigen sterke en zwakke punten hebben. Humane biobanken bijvoorbeeld, zijn onmisbaar voor het doen van associatiestudies tussen het doelwit en de ziekte van interesse in mensen aangezien het totale ziekteproces vaak moeilijk na te bootsen is in cel-, weefsel-, of diermodellen[120]. Het nadeel van humane biobanken is echter dat deze niet gebruikt kunnen worden voor het screenen naar nieuwe kandidaat geneesmiddelen en de identificatie van een stof die het grootste potentieel heeft om ontwikkeld te worden tot mogelijk geneesmiddel. Voor deze doeleinden is het gebruik van cel modellen weer veel geschikter[121]. Een tussenliggend model dat gebruikt kan worden voor geneesmiddelenontwikkeling is het diermodel, dit model bevat (een gedeelte van) de complexiteit van het ziekteproces dat waargenomen wordt in mensen en biedt tegelijkertijd de mogelijkheid om de veiligheid en de effectiviteit van geneesmiddelen te testen[122]. Op het gebied van atherosclerose zijn muismodellen het meest gebruikte diermodel om onderzoek te doen naar de pathologie en mogelijke behandelmethoden van atherosclerose[123]. Ondanks dat deze muismodellen van grote toegevoegde waarde zijn in het onderzoek naar nieuwe geneesmiddelen, is het belangrijk om erbij stil te staan dat muizen verschillen van mensen en dat deze ook verschillen in de pathologie van atherosclerose. Ten eerste wordt atherosclerose in mensen veroorzaakt door een mix van verschillende factoren (zoals genetische vatbaarheid, verhoogd cholesterol, diabetes, hypertensie en roken) terwijl muismodellen vaak veel minder gecompliceerd zijn[124]. Een tweede verschil is het tijdsbestek waarin atherosclerose zich ontwikkeld. In mensen duurt het tientallen jaren voordat een atherosclerotische laesie gevormd is, terwijl dit in muizen slechts enkele maanden duurt[124]. Ten derde verschilt de locatie van de laesies. Ondanks dat laesies zich, zowel in mensen als muizen, voornamelijk ontwikkelen op plekken met een lage of oscillerende shear stress, komen de laesies in muizen het meest voor in het kleppengebied van de aorta, terwijl dit segment vaak vrij is van laesies in mensen[123,124]. Het laatste en misschien wel meest belangrijke verschil is dat het scheuren van de laesie en de daarop volgende trombose, wat leidt tot klinische manifestaties door afsluiting van het vat in mensen, nauwelijks voorkomt in muismodellen[123,124]. Dus, ondanks dat muismodellen onmisbaar zijn in het doen van onderzoek naar atherosclerose, moet men zich ervan bewust zijn dat observaties die gedaan worden in deze modellen niet altijd direct te transleren zijn naar de humane situatie.

TOEKOMST PERSPECTIEF

Ontstekingsremmende therapieën

De drie eiwitten die beschreven zijn in dit proefschrift hebben met elkaar gemeen dat ze betrokken zijn in het ontstekingsproces. Ondanks dat er momenteel geen medicijnen zijn tegen hart- en vaatziekten die voornamelijk het immuunsysteem beïnvloeden, hebben recente studies wel laten zien dat dit in de toekomst mogelijk een belangrijke klasse van medicatie kan zijn tegen hart- en vaatziekten. Een aantal ontstekingsremmende therapieën die onderzocht zijn in atherosclerotische muis- en konijnmodellen hebben laten zien dat zij beschermen tegen het ontstaan van atherosclerose[125]. Daarnaast hebben meerdere van

deze ontstekingsremmende therapieën ook een beschermend effect in mensen aangezien ze geassocieerd zijn met minder hartinfarcten en lagere concentraties van ontstekingseiwitten[125]. Bij twee van deze ontstekingsremmende therapieën, colchicine en canakinumab, is in klinische studies zelfs aangetoond dat ze zorgen voor minder cardiovasculaire complicaties en sterfte. Colchicine, een medicijn dat wordt voorgeschreven bij jicht en familiaire Mediterrane koorts, was de eerste ontstekingsremmende therapie die in een klinische studie getest werd als medicijn tegen hart- en vaatziekten (in de kleine LoDoCo trial). In een kleine studie leidde behandeling van patiënten met coronaire hartziekten met colchicine bovenop de bestaande therapieën tot reductie van het aantal cardiovasculaire complicaties met 72%[126]. Een nadeel van deze therapie is dat 11% van de met colchicine behandelde patiënten vroegtijdig met de behandeling moest stoppen vanwege darmproblemen en nog eens 5% vanwege een scala aan andere mogelijke bijeffecten. Toch blijft colchicine vanwege de hoge effectiviteit en gunstige prijs een mogelijk toekomstige therapie voor secundaire preventie van hart- en vaatziekten[126]. Momenteel wordt er dan ook een klinische studie uitgevoerd met een grotere groep patiënten waarin de gevonden positieve effecten van colchicine verder onderzocht worden[126]. Recentelijk zijn ook de resultaten van de CANTOS trial gepubliceerd. In deze klinische studie werden patiënten, die in het verleden een hartinfarct hebben gehad, elke drie maanden behandeld met canakinumab, een monoclonaal antilichaam tegen het cytokine IL-1β[127]. Canakinumab leidde tot een reductie van cardiovasculaire complicaties en sterfte met 20%[127]. Een groot nadeel van canakinumab is de toegenomen kans op (overlijden door) infecties en sepsis[127], wat Novartis heeft doen beslissen om canakinumab niet op de markt te brengen als medicijn tegen hart- en vaatziekten[128]. Niet alle geteste ontstekingsremmende therapieën hebben gunstige effecten op het voorkomen van hart- en vaatziekten laten zien. De CIRT trial bijvoorbeeld, waarin het effect van een lage dosering methotrexaat, een medicijn dat voorgeschreven wordt in andere ontstekingsziektes zoals reumatoïde artritis, artritis psoriatica en juveniele idiopathische artritis, op secundaire preventie van hart- en vaatziekten werd onderzocht is vroegtijdig gestopt omdat methotrexaat de hoeveelheid ontstekingsremmende eiwitten en de incidentie van cardiovasculaire complicaties niet verlaagde[129]. Ondanks het uitblijven van een gunstig effect op hart- en vaatziekten in de CIRT trial en het toegenomen risico op infecties, een bijeffect dat niet te voorkomen is bij ontstekingsremmende therapieën, laten de genoemde studies zien dat het remmen van het immuunsysteem mogelijk een veelbelovende strategie is om patiënten met hart- en vaatziekten in de toekomst te behandelen.

Geneesmiddelenontwikkeling

De voornaamste redenen dat mogelijke geneesmiddelen niet door de eerste fasen van het proces van geneesmiddelenontwikkeling komen zijn problemen rondom de veiligheid van het onderzochte geneesmiddel[49]. Desondanks blijft orgaan-specifieke toxiciteit ook na goedkeuring van een geneesmiddel een groot probleem, aangezien 90% van alle geneesmiddelen die uit de markt genomen worden terug getrokken worden vanwege veiligheidsoverwegingen[130,131]. Van de 19 geneesmiddelen die uit de Europese markt teruggetrokken werden tussen 2002 en 2011 zijn de meeste terug getrokken vanwege

cardiovasculaire problemen (9 van de 19) of vanwege leverproblemen (4 van de 19)[132]. Om de hoeveelheid tijd en geld die besteed worden aan het ontwikkelen van geneesmiddelen met ernstige toxische bijeffecten zo veel mogelijk te beperken worden potentiële kandidaat geneesmiddelen eerst getest in *in vitro* studies. De werkzame stof van het geneesmiddel of het geneesmiddel zelf wordt dan getest op een monolaag van op plastic gekweekte cellen waarbij de 3-dimensionale interacties tussen cellen, die *in vivo* erg belangrijk zijn, ontbreken[133]. De ontwikkelingen van de afgelopen jaren hebben ervoor gezorgd dat het tegenwoordig mogelijk is om 3-dimensionale weefsels te creëren, welke de *in vivo* situatie veel beter nabootsen[133]. Meerdere studies hebben al laten zien dat deze 3-dimensionale weefsels een beter model zijn om toxische effecten te onderzoeken dan conventionele testen. Eén van deze studies heeft de toxische effecten van 8 klinische geneesmiddelen (waarvan 6 mogelijk toxische effecten hebben op het hart) onderzocht op 3-dimensionaal gekweekt hartweefsel. De toxische effecten die gevonden werden in deze studie waren in lijn zijn met de gerapporteerde klinische observaties, waaruit geconcludeerd kan worden dat deze 3-dimensionale hartweefsels een betere voorspellende waarde hebben met betrekking tot de toxische effecten dan de huidige testen[134]. In een andere studie werd 3-dimensionaal leverweefsel gebruikt om de toxische effecten van 123 geneesmiddelen op leverweefsel te testen. Bij 70 van deze 123 geneesmiddelen was gerapporteerd dat ze hepatotoxische effecten hebben[135]. Het 3-dimensionale weefselmodel identificeerde 69% van de gerapporteerde hepatotoxische geneesmiddelen als hepatotoxisch, een uitkomst die beter is dan uitkomsten van de reeds bestaande *in vitro* modellen[135]. Het gebruik van deze modellen in geneesmiddelenontwikkeling zou in de toekomst kunnen bijdragen aan een betere effectiviteit en veiligheid van ontwikkelde geneesmiddelen.

Momenteel zijn kleine moleculen en eiwitten de twee grootste klassen van door de FDA goedgekeurde geneesmiddelen, deze twee klassen hebben echter beide hun beperkingen. Zo schat men dat kleine moleculen slechts aan 2-5% van alle humane eiwitten kunnen binden en het gebruik van eiwitten wordt gelimiteerd door de grootte en de instabiliteit van de eiwitten[136]. Binnen het CarTarDis consortium zijn verschillende mogelijke kandidaat doelwitten niet verder onderzocht vanwege de lage druggability score die zij toegekend hebben gekregen omdat ze niet beïnvloed konden worden door kleine moleculen of door eiwitten. Echter, de vooruitgang op het gebied van therapeutische RNA afgifte heeft het mogelijk gemaakt om de eiwitexpressie af en toe te laten nemen en zelfs het DNA aan te passen, waardoor de therapeutische mogelijkheden enorm zijn toegenomen[136]. Een mooi voorbeeld van een op RNA gebaseerde therapie in hart- en vaatziekten is het geneesmiddel mipomersen[137], wat door de FDA werd goedgekeurd in 2013 om patiënten met familiaire hypercholesterolemie te behandelen[138]. Mipomersen is een antisens oligonucleotide dat aan apolipoproteine B mRNA bindt en zo synthese van het apolipoproteine B eiwit tegen gaat[137]. Dit resulteert in een lagere hoeveelheid van atherogene lipoproteïnen[137]. Andere voorbeelden van op RNA gebaseerde geneesmiddelen tegen hart- en vaatziekten die momenteel onderzocht worden zijn inclisiran, een RNAi dat PCSK9 eiwit synthese remt en volanesorsen, wat aan apolipoproteine C3 mRNA bindt[137]. Beide geneesmiddelen hebben veelbelovende resultaten laten zien in fase II klinische studies wat verder onderzoek naar

andere mogelijke doelwitten, welke nog niet beïnvloed konden worden voor het op RNA gebaseerde geneesmiddelen tijdperk, zou kunnen bevorderen[137].

Toepassing van gepersonaliseerde geneeskunde in hart- en vaatziekten

Een andere manier om de veiligheid en de effectiviteit van zowel bestaande als nieuwe geneesmiddelen te verbeteren is het toepassen van gepersonaliseerde geneeskunde bij de behandeling van hart- en vaatziekten[139]. Gepersonaliseerde geneeskunde werd voor het eerst toegepast bij borstkankerpatiënten. Met behulp van genetische testen werden borstkankerpatiënten met een HER2-overexpressie geïdentificeerd en vervolgens succesvol behandeld met monoclonale antilichamen die specifiek gericht zijn tegen HER2[140]. Een ander voorbeeld van succesvol toegepaste gepersonaliseerde geneeskunde in de strijd tegen kanker is imatinib. Imatinib is specifiek gericht tegen het spontaan ontstane Bcr-Abl1 fusie-eiwit, wat veel actiever is dan het ongewijzigde Abl eiwit en bijdraagt aan ongereguleerde celgroei en de ontwikkeling van kanker, en wordt toegediend aan chronische myeloïde leukemie patiënten, die vrijwel allemaal een mutatie hebben die leidt tot het ontstaan van dit fusie-eiwit[140]. Maar ook voor de behandeling van patiënten met taaislijmziekte wordt gepersonaliseerde geneeskunde toegepast. Een deel van deze patiënten heeft namelijk een specifieke klasse III mutatie en deze groep patiënten kan effectief behandeld worden met ivacaftor[140]. Door de specifieke groep patiënten te identificeren die baat hebben bij het krijgen van een specifieke behandeling en door deze behandeling niet te geven aan patiënten die hier geen baat bij hebben, neemt de effectiviteit van een behandeling toe[139]. Daarnaast worden de ziektekosten hierdoor beperkt omdat patiënten die geen baat hebben bij behandeling met Ivacaftor, dit dure medicijn niet voorgeschreven zullen krijgen[141]. Gepersonaliseerde geneeskunde kan ook gebruikt worden om patiënten te identificeren die een hoog risico hebben op het krijgen van ernstige bijeffecten. Door deze groep de therapie te onthouden kan de veiligheid van bepaalde therapieën vergroot worden[139]. Bij de behandeling van hart- en vaatziekten wordt gepersonaliseerde geneeskunde nog niet toegepast. Echter zijn er wel verschillende toekomstige toepassingen te bedenken. Zo zou de eerder genoemde antilichaamtherapie tegen PCSK9 toegepast kunnen worden in patiënten met een verhoogde PCSK9 expressie, dit zou vooral een uitkomst zijn als het onderzoekers niet lukt om de PCSK9 vaccinatie succesvol verder te ontwikkelen[109,140]. Een andere mogelijke toepassing van gepersonaliseerde geneeskunde in hart- en vaatziekten zou de op genotype gebaseerde dosering van warfarine kunnen zijn. Dit medicijn is een antistollingsmiddel dat voorgeschreven wordt om trombose te voorkomen, maar het leidt bij overdosering juist tot bloedingen. Het is daarom belangrijk om voor elke patiënt zo snel mogelijk de juiste dosering te bepalen[142]. Uit de EU-PACT Warfarine trial is gebleken dat CYP2C9 en VKORC1 genotypering van patiënten het risico op bloedingen en de tijd om tot de juiste warfarine dosis te komen vermindert[142]. Ook in de eerdergenoemde CANTOS trial is gekeken of responders van non-responders onderscheiden konden worden. De onderzoekers hebben in deze studie laten zien dat de hoeveelheid high-sensitive C-reactief proteïne (hsCRP) de effectiviteit van IL-1β gerichte therapie kan voorspellen aangezien patiënten met een hogere hsCRP beginwaarde en een grotere reductie van hsCRP minder cardiovasculaire complicaties hebben dan patiënten met een geringere hsCRP reductie.

Deze waarneming zou ertoe kunnen leiden dat de hsCRP reductie in de toekomst gebruikt wordt als maatstaf om te bepalen of behandeling met IL-1β gerichte therapie voortgezet of gestopt moet worden[143]. Daarnaast zou gepersonaliseerde geneeskunde op het gebied van hart- en vaatziekten toegepast kunnen worden om patiënten met een verhoogd risico op het ontstaan van bijeffecten te identificeren. Een mogelijke toepassing hiervan zou kunnen zijn om patiënten met een genetische of immunologische vaatbaarheid voor het krijgen van door statine geïnduceerde myopathie (spierklachten) op voorhand te identificeren[144]. Vooral omdat deze groep patiënten vaak stopt met het innemen van hun medicatie[144], zou het gunstig zijn om deze patiënten, voor het ontstaan van klachten, te identificeren en deze patiënten te behandelen met andere cholesterol verlagende medicijnen.

Therapietrouw en preventie

Naast het ontwikkelen van nieuwe geneesmiddelen en het verbeteren van het geneesmiddelenontwikkelingsproces, is het ook belangrijk om bestaande therapieën te verbeteren. Een belangrijk verbeterpunt is therapietrouw. Een meta-analyse bestaande uit 11 studies van patiënten die primaire preventie therapie kregen en 9 studies van patiënten die secundaire preventie therapie kregen heeft laten zien dat slechts 57% van de patiënten trouw was aan voorgeschreven medicijnen tegen hart- en vaatziekten over een periode van twee jaar[145]. Aangezien combinatietherapie (meestal aspirine in combinatie met bloeddruk- en cholesterolverlagende medicijnen) de kans op hart- en vaatziekten gerelateerde complicaties met ongeveer 80% verlaagt, zouden alleen in de Verenigde Staten jaarlijks tot 130.000 overlijdensgevallen voorkomen kunnen worden door betere therapietrouw[145]. Eén van de oplossingen die is voorgedragen om therapietrouw te verbeteren is de invoering van de polypil, waarin de werkzame stof van verschillende geneesmiddelen gecombineerd worden in één enkele pil[146]. Hierdoor zouden er dagelijks veel minder verschillende pillen geslikt hoeven worden en aangezien patiënten trouwer zijn aan geneesmiddelen die minder frequent ingenomen hoeven te worden[147], zou dit mogelijk kunnen leiden tot een betere therapietrouw[146]. Verschillende klinische studies hebben inderdaad bevestigd dat patiënten trouwer zijn aan de polypil (therapietrouw van 70-86%), dan aan hun huidige therapieën (46-65%) na een behandelperiode van 12-18 maanden[146,148–150]. Het effect van de toegenomen therapietrouw was ook terug te zien aan de verlaagde bloeddruk en cholesterol waarden die waargenomen werden in patiënten die de polypil voorgeschreven kregen[146]. Een andere toekomstige mogelijkheid om de therapietrouw te verbeteren is de invoering van via het internet geleverde gezondheidszorg, wat ook wel eHealth genoemd wordt. Vooral naar het gebruik van mhealth (toegang tot gezondheidszorg via de smartphone) wordt momenteel veel onderzoek gedaan[151]. De eerste studies die hebben gekeken naar de invloed van mHealth op therapietrouw hebben veelbelovende resultaten laten zien[151], toekomstige studies moeten echter uitwijzen of deze resultaten bevestigd kunnen worden en wat de lange termijn effecten van het gebruik van mHealth zijn.

Naast een positieve invloed op therapietrouw van geneesmiddelen kunnen eHealth interventies ook een positief effect hebben op trouw aan een gezondere levensstijl. Studies hebben namelijk laten zien dat eHealth interventies kunnen leiden tot een lagere systolische en diastolische bloeddruk in hypertensie patiënten[152] en in ieder geval op korte termijn kan

leiden tot gewichtsverlies in individuen met overgewicht of obesitas[153]. Ondanks de positieve bevindingen in initiële eHealth en mHealth studies moeten lopende en toekomstige studies uitwijzen of deze bevindingen bevestigd kunnen worden.

Additionele eHealth mogelijkheden

Naast het leveren van informatie en het motiveren van patiënten om trouw te blijven aan hun medicatie en een gezondere levensstijl, kan eHealth ook gebruikt worden voor het verzamelen van data, waardoor onderzoekers en artsen toegang krijgen tot zogenoemde real-world data. Relatief simpele apparaten zoals smartwatches kunnen fysiologische parameters zoals lichamelijke activiteit en slaap meten en opslaan terwijl iets geavanceerdere sensoren fysiologische parameters zoals hartslag, bloeddruk en ademhaling kunnen meten[154,155]. Deze data kan onder andere gebruikt worden om de juiste doelgroep te identificeren, nieuwe behandelmethoden te ontwikkelen of de efficiëntie van een behandeling in een specifieke doelgroep te voorspellen[156]. Lv *et al.,* hebben recentelijk een gepersonaliseerd gezondheidszorg model ontwikkeld dat gepersonaliseerde feedback geeft aan hypertensie patiënten gebaseerd op data van de patiënt zelf zoals thuis gemeten bloeddruk, gewicht en levensstijlfactoren. Dit model zorgde voor een significantie afname in bloeddruk van deze patiënten[157]. Daarnaast hebben Muhlestein *et al.,* laten zien dat de ECG functie van een smartphone ontzettend goed overeen komt met de huidige gouden standaard 12-afleidingen ECG[158], wat de hoop op een succesvolle implementatie van eHealth voor in elk geval enkele fysiologische parameters verder toe doet nemen. Het gebruik van eHealth om gepersonaliseerde geneeskunde te verbeteren lijkt ook positieve effecten te hebben in andere geneeskundige gebieden zoals mentale gezondheid waar het continu monitoren van slaap, stemming en eetlust gebruikt kan worden voor het maken van klinische beslissingen[159]. Maar ook in ontwikkelingslanden kan het toepassen van eHealth de gezondheidszorg verbeteren aangezien de gezondheidszorg daar vooral in afgelegen gebieden een stuk minder toegankelijk is en deze kosteneffectief uitgebreid zou kunnen worden naar afgelegen gebieden door gebruik te maken van eHealth[159,160]. Echter, ondanks dat implementatie van eHealth erg veelbelovend lijkt te zijn, zal de tijd uit moeten wijzen of eHealth ook een daadwerkelijke impact heeft op de toekomstige gezondheidszorg.

CONCLUSIE

Het hoge risico op overlijden door hart- en vaatziekten vraagt om ontwikkeling van nieuwe therapieën tegen hart- en vaatziekten. Echter, de hoge kosten en het hoge aantal studies dat voortijdig afgebroken moet worden maken zorgvuldige overwegingen om in nieuwe therapieën te investeren noodzakelijk. In dit proefschrift is de vijf-factor-strategie die wij gebruikt hebben om potentiële doelwitten in hart- en vaatziekten te identificeren kort beschreven, evenals enkele validatiestudies die zijn uitgevoerd om meer te weten te komen over de rol van drie potentiële doelwitten in hart- en vaatziekten. Naast het uitvoerig verzamelen van informatie omtrent een bepaald doelwit om medicijnontwikkeling te verbeteren kunnen de huidige verbeteringen op het gebied van cel- en weefselkweek, die

toxische effecten veel beter voorspellen dan conventionele testen, ook bijdragen aan het verbeteren van het medicijnontwikkelingsproces. Verder zou de effectiviteit en de veiligheid van toekomstige therapieën op het gebied van hart- en vaatziekten verbeterd kunnen worden door het toepassen van gepersonaliseerde geneeskunde. Dit wordt al succesvol toegepast in andere ziektes zoals kanker en taaislijmziekte. Echter, ondanks dat er verschillende opties zijn om gepersonaliseerde geneeskunde toe te passen bij patiënten met hart- en vaatziekten zal de toekomst uit moeten wijzen of dit ook daadwerkelijk leidt tot een verbetering van de gezondheidszorg voor patiënten met hart- en vaatziekten.

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8

APPENDIX

List of publications

Dankwoord

Curriculum vitae

LIST OF PUBLICATIONS

Feyen DA, Gaetani R, Deddens J, **van Keulen D**, van Opbergen C, Poldervaart M, Alblas J, Chamuleau S, van Laake LW, Doevendans PA, Sluijter JP. *Gelatin Microspheres as Vehicle for Cardiac Progenitor Cells Delivery to the Myocardium*. Adv Healthc Mater. 2016 May; 5(9): 1071-9.

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van Keulen D, Pouwer MG, Pasterkamp G, van Gool AJ, Sollewijn Gelpke MD, Princen HMG, Tempel D. *Inflammatory cytokine oncostatin M induces endothelial activation in macro- and microvascular endothelial cells and in APOE*3Leiden.CETP mice*. PLoS One. 2018 Oct 1; 13(10): e0204911.

van Keulen D, Pouwer MG, Emilsson V, Perisic Matic L, Pieterman EJ, Hedin U, Gudnason V, Jennings LL, Holmstrøm K, Schnack Nielsen B, Pasterkamp G, Lindeman JHN, van Gool AJ, Sollewijn Gelpke MD, Princen HMG, Tempel D. *Oncostatin M reduces atherosclerosis development in APOE*3Leiden.CETP mice and is associated with increased survival probability in humans***.*** PLoS One. 2019 Aug 28; 14(8): e0221477.

IN PREPARATION

van Keulen D, van Koeverden ID, Boltjes A, Princen HMG, van Gool AJ, de Borst GJ, Asselbergs FW, Tempel D, Pasterkamp G, van der Laan SW. *Common variants in OSMR contribute to carotid plaque vulnerability.*

van Keulen D, Aldi S, Ait-Belkacem R, Emilsson V, Stauber J, Perisic Matic L, Borgman A, Hedin U, Gudnason V, Jennings LL, Holmstrøm K, Schnack Nielsen B, Pasterkamp G, van Gool AJ, Princen HMG, Tempel D. *CECR1 diminishes adenosine induced neutrophil activity and is associated with decreased survival probability in humans.*

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CURRICULUM VITAE

Daniëlle van Keulen was born on the 16th of January 1991 in Utrecht, the Netherlands. She grew up in Wijk bij Duurstede with her parents and three younger sisters. After finishing secondary school at Revius Lyceum Doorn, she continued her studies in Utrecht. There she did her Bachelor in Biomedical Sciences, followed by the master Regenerative Medicine & Technology. She conducted the first internship of her master in the Experimental Cardiology lab at the University Medical Center Utrecht under supervision of Dries Feyen and professor Joost Sluijter where she investigated the possibility to use gelatin microspheres as a vehicle to deliver cardiomyocyte progenitor cells to the infarcted myocardium. For her second internship, she went to the lab of professor Ina Gruh at Medizinische Hochschule Hannover in Germany, where she studied the impact of the geometry of bioartificial cardiac tissues on its contractile forces. When she came back, she started her PhD at the Experimental Cardiology department at the University Medical Center Utrecht in collaboration with Quorics B.V. and TNO Leiden on the validation of novel druggable targets in cardiovascular disease. The results of her work are summarized in this thesis. In March 2019 she started working as a scientist at SkylineDx, where she hopes to improve patients' lives by contributing to the development of novel diagnostic tests.