Thesis for doctoral degree (Ph.D.) 2015

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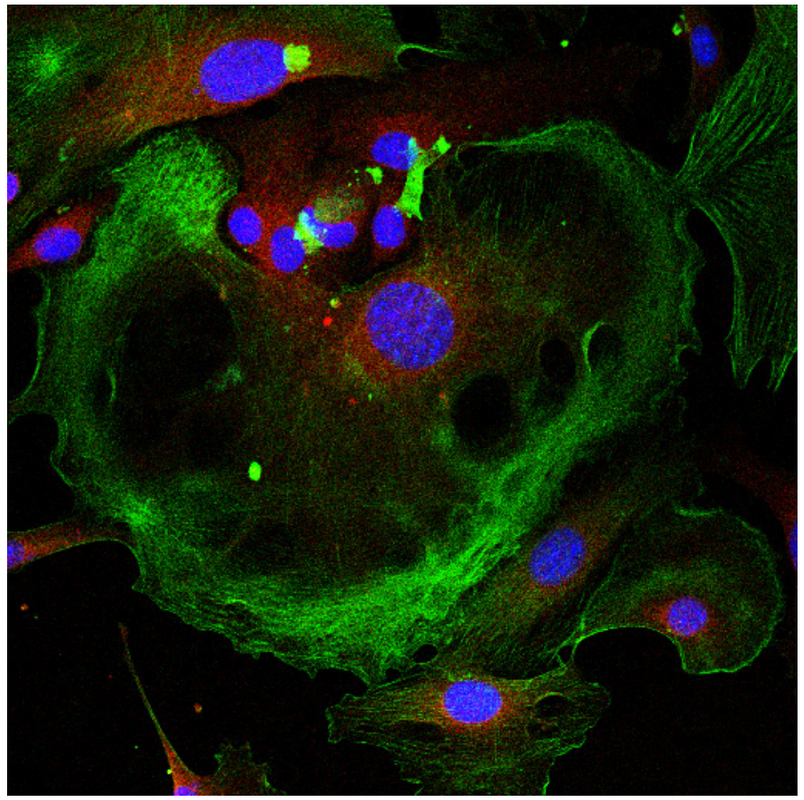

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THE ROLE OF FPR2/ALX IN THE VASCULAR WALL

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ABSTRACT

Cardiovascular diseases caused by atherosclerosis are a leading cause of mortality worldwide. Inflammation has been described as a key component in the development of atherosclerosis. Lipoxin A_4 (LXA_4) is a lipid mediator derived from arachidonic acid, which has anti-inflammatory and pro-resolution properties mediated through the FPR2/ALX receptor. This receptor is however, not specific to LXA_4 and can transduce pro-inflammation or pro-resolution effects depending on the different ligands present in the atherosclerotic *milieu.*

The aim of the present thesis was to unravel the role of FPR2/ALX signaling in the atherosclerotic vascular wall. In addition, the effects of LXA_4 were examined with 2 goals: to assess the use of LXA_4 as possible therapeutic option in mouse models of atherosclerosis, and to elucidate if LXA_4 effects were mediated through FPR2/ALX signaling. To this end, *in vivo*, *in vitro* and *ex vivo* experiments were used to evaluate the role of the FPR2/ALX receptor in human samples of atherosclerosis and in mice either expressing or lacking the murine homologue of the FPR2/ALX receptor (Fpr2).

It was discovered that macrophages, smooth muscle cells and endothelial cells in human atherosclerotic lesions expressed FPR2/ALX and it was up-regulated by pro-inflammatory stimuli in human monocytes *in vitro*. In three different mouse models, Fpr2 deletion resulted in decreased atherosclerosis. Macrophages derived from Fpr2 knock-out mice exhibited reduced inflammation and Fpr2 knock-out mice exhibited endothelial dysfunction.

Finally, treatment with the aspirin-triggered LXA_4 (ATL, a LXA_4 analogue) significantly reduced atherosclerosis, smooth muscle cell migration *in vitro* and intimal hyperplasia after carotid ligation *in vivo*. Since this effect was absent in Fpr2 knock-out mice, it supports that Fpr2 transduces anti-inflammatory signaling in response to this lipid mediator.

In summary, the results of the present thesis suggest a dual role for FPR2/ALX signaling in atherosclerosis, with pro-inflammatory property as the disease develops; whereas anti-inflammatory signaling was induced by LXA_4. In addition, it was discovered that Fpr2 signaling induced differential effects on different cell types within the atherosclerotic lesion.

In conclusion, FPR2/ALX signaling is present in major cells of the vascular wall and is crucial for atherosclerosis development. LXA_4 is signaling through FPR2/ALX and this supports the use of LXA_4 as a therapeutic option in atherosclerosis as well as restenosis.

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LIST OF PUBLICATIONS

I. **Petri MH**, Laguna-Fernández A, Gonzalez-Diez M, Paulsson-Berne G, Hansson GK and Bäck M.
The role of the FPR2/ALX receptor in atherosclerosis development and plaque stability.
Cardiovasc Res. 2015;105(1):65-74

II. **Petri MH**, Laguna-Fernandez A, Tseng CN, Hedin U, Perretti M and Bäck M.
Aspirin-triggered 15-epi-lipoxin A4 signals through FPR2/ALX in vascular smooth muscle cells and protects against intimal hyperplasia after carotid ligation.
Int J Cardiol. 2015;179:370-2

III. **Petri MH**, Thul S, Ovchinnikova O and Bäck M.
Differential regulation of monocytic expression of leukotriene and lipoxin receptors
Prostaglandins Other Lipid Mediat. 2015 (in press)

IV. **Petri MH**, Gonzalez-Diez M, Andonova T, Perretti M, Laguna-Fernandez A and Bäck M.
Genetic deletion of FPR2/ALX in mice induces endothelial dysfunction
Manuscript

V. **Petri MH**, Laguna-Fernandez A, Perretti M, Hansson G and Bäck M.
Aspirin-triggered lipoxin decreases atherosclerosis in ApoE-/- mice
Manuscript

OTHER RELATED PUBLICATIONS

I. Van Noolen L, Bäck M, Arnaud C, Rey A, **Petri MH**, Levy P, Faure P, Stanke-Labesque F.
Docosahexaenoic acid supplementation modifies fatty acid incorporation in tissues and prevents hypoxia induced-atherosclerosis progression in apolipoprotein-E deficient mice.
Prostaglandins Leukot Essent Fatty Acids. 2014;91(4):111-7.

II. Gautier-Veyret E, Arnaud C, Bäck M, Pépin JL, **Petri MH**, Baguet JP, Tamisier R, Lévy P, Stanke-Labesque F.
Intermittent hypoxia-activated cyclooxygenase pathway: role in atherosclerosis.
Eur Respir J. 2013;42(2):404-13.

III. Stanke-Labesque F, Pépin JL, de Jouvencel T, Arnaud C, Baguet JP, **Petri MH**, Tamisier R, Jourdil JF, Lévy P, Bäck M.
Leukotriene B4 pathway activation and atherosclerosis in obstructive sleep apnea.
J Lipid Res. 2012;53(9):1944-51.

IV. **Petri MH**, Tellier C, Michiels C, Ellertsen I, Dogné JM, Bäck M.
Effects of the dual TP receptor antagonist and thromboxane synthase inhibitor EV-077 on human endothelial and vascular smooth muscle cells.
Biochem Biophys Res Commun. 2013;441(2):393-8.

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
Ach	Acetylcholine
AKC	Ammonium-Chloride-Potassium
ANOVA	Analysis of variance
AP1	Activator protein 1
ApoE	Apoliprotein E
ATL	Aspirin triggered Lipoxin A_4 (15-epi-LXA_4)
BiKE	Biobank of Karolinska Endarterectomies
BM	Bone marrow
BMT	Bone marrow transplantation
BP	Blood pressure
BSA	Bovine serum albumin
CD	Cluster of differentiation
CVD	Cardiovascular disease
CO2	Carbon dioxide
COX	Cyclooxygenase
CPT	Cell Preparation Tube
CT	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
EC	Endothelial cells
ECM	Extracellular Matrix
EEL	External elastic layer
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FLAP	5 Lipoxygenase activating protein
FPR	Formyl peptide receptor
GFP	Green fluorescent protein
HETE	Hydroxyeicosatetraenoic acid
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HFD	High fat diet
IEL	Internal elastic layer
ICAM	Intercellular Adhesion Molecule
IMT	Intima-media thickness
IL	Interleukin
IFN	Interferon
KS	Krebs solution
LB	Luminal border
LDL	Low density lipoprotein
LO	Lipoxygenase
LT	Leukotriene
LX	Lipoxin
LXA_4	Lipoxin A_4
MaR	Maresin

mAEC	Mouse aortic endothelial cell
MI	Myocardial infarction
MIF	Median intensity fluorescence
Min	Minute
miR	MicroRNA
ml	Milliliter
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
mSMC	Mouse smooth muscle cell
NF-κb	Nuclear factor Kappa b
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	Optimal Cutting Temperature
ω	Omega
OVA	Ovoalbumin
oxLDL	Oxidized low density lipoprotein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCI	percutaneous coronary interventions
PD	Protectin
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
PG	Prostaglandin
PI	Propidium iodide
PPIA	Peptidylprolyl Isomerase A
PUFA	Polyunsaturated fatty acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
Rv	Resolvin
SAA	Serum amyloid A
SEM	Standard error of the mean
siRNA	Small interfering RNA
SMC	Smooth muscle cells
SNP	Sodium nitroprusside
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinases
TM	Trans-membrane
TNF	Tumor necrosis factor
TX	Thromboxane
UV	Ultra violet
VCAM	Vascular cell adhesion molecule
vWF	Von Willebrand factor

1 Introduction

1.1 The vascular wall

The vascular wall is organized in three different layers: intima, media and adventitia. The inner layer, closest to the lumen, contains the endothelial cells (ECs), which are crucial for homeostasis and cell recruitment. Once the EC is activated, adhesion molecules are expressed and induce the recruitment of leukocytes from the blood stream. These leukocytes can go to the adjacent tissues or remain within the vessel wall, mainly in the intima. The media is predominantly composed by smooth muscle cells (SMC) with contractile properties, which gives the vascular tone. The SMC population can be divided into two phenotypes: the contractile phenotype with high amounts of different actins in the cytoplasm and very limited proliferation properties; and the synthetic phenotype with less contractile ability and increased proliferation rate [1]. The outer layer of the arterial wall is the adventitia, a collagen-rich connective tissue matrix containing fibroblasts, nerve fibers, and immune cells such as mast cells, macrophages and lymphocytes.

1.2 Atherosclerosis

Cardiovascular diseases (CVD), such as ischemic heart disease and stroke, are the leading cause of mortality in western developed countries. Besides the increased mortality, around 30% of all disability in European Union is CVD-related. The costs as a result of CVD are estimated to almost 196 billion Euros spent every year [2], which illustrates the large impact CVD has in the society. The underlying cause of coronary artery and cerebrovascular disease is atherosclerosis. This chronic disease is characterized by the accumulation of lipids and inflammatory cells within the vessel wall.

1.2.1 Initiation

The early stages of atherosclerosis are characterized by the deposition of low density lipoproteins (LDL) within the intima [3], leading to EC activation and recruitment of leukocytes [4]. LDL can be oxidized by for example direct oxidation or lipoxygenase activity. The oxidized

LDL (oxLDL) is then taken up by macrophages, leading to the formation of foam cells, which are the main cells in atherosclerotic lesions [5].

The main immune cells that ingress from the bloodstream to the atherosclerotic lesions are: monocytes/macrophages [6], neutrophils [7], dendritic cells [8], T and B cells [9]. The ingress of leukocytes promotes inflammation, which alters the homeostatic state of the vascular wall components. Furthermore, the cells interact with each other, which further enhances the inflammatory process and creates a vicious circle by which the inflammatory *milieu* keeps the EC activated and recruits more cells from the blood stream.

1.2.2 Progression

Several pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α [10] and IL-6 [11] contribute to the continued immune stimulation in atherosclerotic lesions, leading to chronic inflammation. In addition to these proteins, activation of enzymes that oxidize lipids will lead to the formation of lipid mediators of inflammation, which further enhance inflammatory reactions through specific receptors [12].

Initially, the resolution of inflammation was believed to be a passive phenomenon, due to the reduction of inflammatory stimulation. Recent knowledge has however shown that specific pro-resolution mediators can act as "stop-signals" for inflammation, promoting the ending of inflammation and subsequently healing [13]. In other words, to achieve healing after inflammation is induced, an active resolution process needs to take place. It is now widely accepted that there is a failure in the resolution of vascular inflammation during plaque progression and the persistence of the inflammation [14]. For example, IL-10 and transforming growth factor (TGF)-β have been suggested as anti-inflammatory mediators, that act by limiting inflammation within atherosclerotic lesions [10]. Likewise, specific lipid mediators are also involved in the resolution of inflammation.

Along with the accumulation of immune cells and inflammatory mediators, also growth factors are produced and released within the atherosclerotic lesion. This environment will stimulate SMCs to migrate from the media to the intima [15] and increase their proliferation rate [16]. As the plaque progresses, cell death [17], and production of free radicals [18] will further increase the inflammatory process, promoting neoangiogenesis within the plaque and the formation of a necrotic core. In addition, the formation of a fibrous cap composed of SMCs and collagen will protect the plaque content to be exposed to the blood stream [19].

In advanced atherosclerotic lesions, the inflammatory process is associated with the accumulation of dead cells [20]. This accumulation can be due to either an increased apoptosis within the plaque [21] or to a lack of clearance of apoptotic cells, also known as efferocytosis [20]. The latter is a hallmark of the resolution of inflammation, and the lack of efferocytosis has been associated with increased atherosclerosis [22].

1.2.3 Unstable plaques

As mentioned above, the atherosclerotic plaque is covered by a fibrous cap consisting of SMCs and collagen. Although the trigger remains not fully understood, the onset of collagen break-down and SMC death will lead to a weakening of the fibrous cap. Several enzymes have been implicated in this process, of which the matrix metalloproteinase (MMP) family of proteases is the most studied [23]. For example, MMP-13 is the most abundant and the predominant enzyme that is associated with plaque collagen degradation in murine models of atherosclerosis [24].

Another contributing factor is the appearance of intraplaque hemorrhage [25]. In the latter process, neutrophils produce a number of proteases that further increase the extracellular matrix (ECM) degradation [7]. As a result, the fibrous cap is degraded, and the atherosclerotic lesion becomes unstable, indicating that it is prone to rupture.

1.2.4 Plaque rupture

When the fibrous cap of the atherosclerotic plaque ruptures, the inner content of the atheroma is exposed to the circulating blood. The exposure of the collagen under the EC layer

will promote platelet activation and coagulation, leading to the formation of a thrombus [26]. This clot may obstruct the lumen of the blood vessel hence leading to occlusion of blood flow depriving the brain or the heart of oxygen, leading to stroke or myocardial infarction, respectively. In addition, parts of the atheroma and/or thrombus may produce an embolism and obstruct a distal smaller artery.

1.2.5 Diagnosis

At initial stages atherosclerosis is asymptomatic and should be suspected when risk factors are found. Carotid artery atherosclerosis can be detected by means of an ultrasound. This examination can reveal if atherosclerotic plaques are present or not, and determine the degree of obstruction of the vessel. In addition, the intima-media thickness (IMT) can be measured. The IMT indicates the thickening of the arterial wall and has been associated with an increased cardiovascular risk [27, 28].

Coronary atherosclerosis can be diagnosed by angiography, in which the administration of contrast media will reveal the degree of obstruction or stenosis that the atherosclerotic lesion causes of the vascular lumen. Likewise, a transthoracic Doppler echocardiography of the left anterior descending coronary artery can give information of reduced coronary flow [29]. Also in the diagnosis of both cerebrovascular and coronary atherosclerotic lesions, computerized tomography angiography can be used, just to mention a few methods to assess atherosclerotic plaques.

In all the above-mentioned methodologies, the definition of a significant lesion relies on the degree of vascular obstruction. However, these diagnostic measures do not distinguish stable from unstable lesions. Importantly, plaques not causing either anatomical lumen obstruction or hemodynamically significant flow restriction may however be prone to rupture [29], and studies are ongoing how to obtain imaging modalities that can identify unstable atherosclerotic lesions.

1.2.6 Management

1.2.6.1 Medical treatment

The management of atherosclerotic vessel disease relies on three different approaches, namely lifestyle changes, medical treatments and invasive procedures. In addition, management of co-morbidities are important, for example dyslipidemia, diabetes, high blood pressure just to mention a few.

The medical treatment applied in the context of atherosclerosis may impact the process of disease. For example, lipid management with the aim to improve lipid profiles in terms of reducing LDL and increasing HDL, has shown convincing beneficial effects in patients with CVD [30]. Although statins have been suggested to exhibit some anti-inflammatory properties, the beneficial effects of this class of drugs are mainly due to their effects on lipids. Low dose aspirin is also used in the management of CVD to inhibit platelet aggregation [31]. Again, the effect of aspirin in CVD is mainly due to platelet inhibition rather than anti-inflammatory effects. Drugs targeting inflammation that are widely used in inflammatory diseases such as rheumatic diseases, for instance, methotrexate, colchicine, allopurinol among others has been shown to reduce CVD events in epidemiologic or animal studies, and randomized controlled trials are ongoing to evaluate the effects of specific anti-inflammatory treatments on cardiovascular risk [30].

1.2.6.2 Angioplasty and restenosis

Obstructive atherosclerotic lesions as well as vascular occlusions following plaque rupture can be treated invasively. In coronary atherosclerosis, percutaneous coronary interventions (PCI) are performed by catheter-based balloon dilatation and/or placement of a vascular mesh called stent [32]. After this process, vascular SMCs increase proliferation and migration, as well as ECM remodeling [33]. This process may result in the formation of an intra-stent intimal hyperplasia causing restenosis of the coronary vessel [34]. To overcome this problem, stents coated with anti-proliferative agents were introduced [35], which reduced the

incidence of in-stent restenosis. However, a concern about increased thrombotic events using drug-eluting stents has been raised [36]. This reinforces the importance of a better understanding of how the vascular wall reacts to injury for improved therapeutic options.

1.2.7 Mouse models of atherosclerosis

Hyperlipidemic mice, such as Apolipoprotein E ($ApoE^{-/-}$) and LDL receptor ($Ldlr^{-/-}$) knock-out mice are widely used models of atherosclerosis. Whereas $ApoE^{-/-}$ mice develop a spontaneous atherosclerosis, administration of high fat diet (HFD) is needed in $Ldlr^{-/-}$ mice. The advantages of using these mouse models are mainly four: (1) Atherosclerosis development is similar to humans. (2) The mouse genome has been described, opening the options to create directed knock out (deletion) of a specific gene, hence disrupting its expression or its activity. (3) The possibility to apply drugs that block enzymes or receptors. (4) Mice require small space and reproduce fast making it possible to handle colonies in small spaces.

1.3 Lipoxygenases

1.3.1 Lipid mediator biosynthesis

There are three main classes of lipoxygenases (LOs) in humans: 5-, 12- and 15-LO. The number identifies the carbon atom of the fatty acid that is oxidized by these enzymes. LOs may participate in the LDL oxidation during atherosclerosis initiation [37]. Importantly, LO metabolism of polyunsaturated fatty acids (PUFA) will yield a series of bioactive lipid mediators, with important function within the vascular wall. For example, 5-LO metabolism of the omega (ω)-6 PUFA arachidonic acid (AA) will yield the inflammatory mediators leukotrienes (LTs) [38]. 5-LO together with the 5-LO activating protein (FLAP) lead to formation of the unstable LTA_4. Through subsequent enzymatic steps, LTA_4 is either converted to LTB_4 or conjugated with glutathione to form the cysteinyl-LTs (CysLT, LTC_4, LTD_4, and LTE_4). These lipid mediators induce leukocyte recruitment and activation, and in addition, regulate critical functions of the vascular wall, such as endothelial permeability [40] and vascular tone [41, 42].

In contrast, AA is also the substrate for the anti-inflammatory and pro-resolving lipoxin A_4 (LXA_4) [39]. The biosynthesis of LXA_4 requires sequential dual lipoxygenation involving all

three different classes of lipoxygenases: 5-LO, 12-LO and 15-LO (Figure 1). Since these enzymes exhibit a differential cellular expression pattern, LXA_4 biosynthesis may involve trans-cellular metabolism. In brief, a donor cell transforms the PUFA into an intermediate and release it, and an acceptor cell, expressing another enzymatic step, collects this intermediate and metabolizes it into the bioactive molecule [43]. For example, the formation of LTA_4 via the 5-LO pathway takes place mainly in myeloid cells [39], and platelets that are rich in 12-LO are able to collect LTA_4 and transform it to LXA_4 [44] (Figure 1). As a consequence, although platelets cannot form LXA_4 by themselves, they are the main source of LXA_4 *in vivo* [44].

The 15-epimer of LXA_4, a more stable LXA_4 analogue with the same pro-resolving properties, can be formed by the acetylation of the cyclooxygenase 2 (COX2) enzyme by aspirin. This leads to 15(R)-HETE formation, which can be further processes by 5-LO leading to 15-epi-LXA_4 (Figure 1). Since 15-epi-LXA_4 is produced only in the presence of aspirin, it is also known as aspirin triggered lipoxin (ATL).

Likewise, LO metabolism of ω–3 PUFA, leads to the formation of lipid mediators with mainly anti-inflammatory properties, such as resolvins (Rv), protectins (PD) and maresins (MaR), which participate in the resolution of inflammation [13].

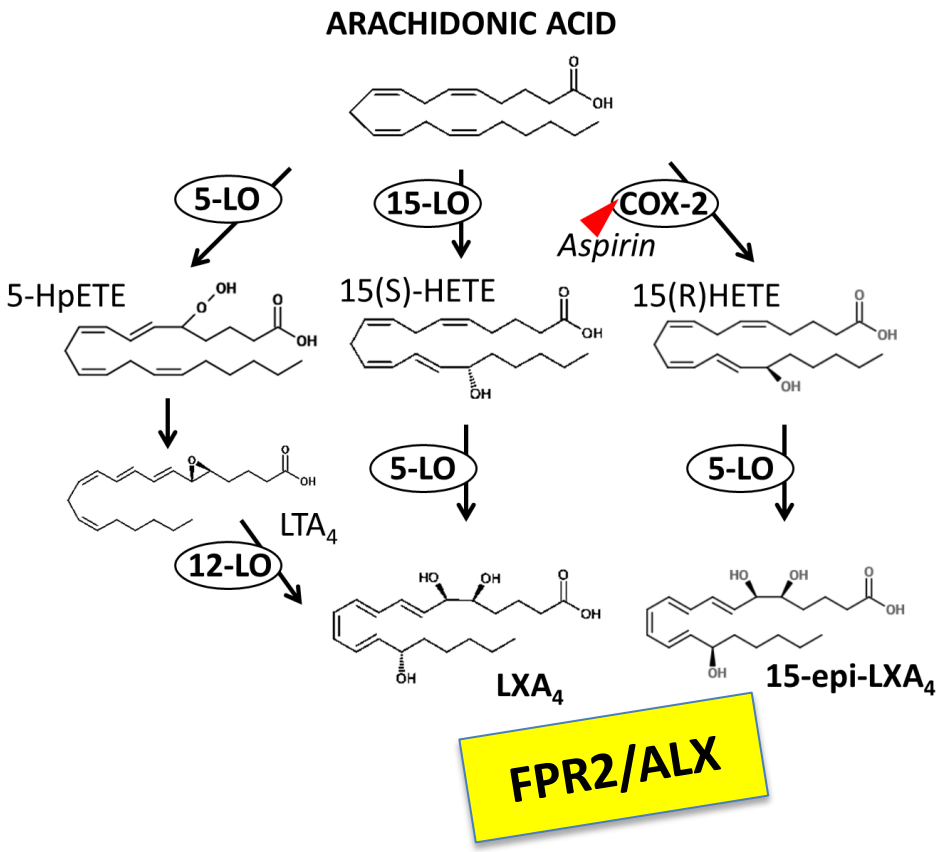

Figure 1: Lipoxin biosynthesis.
The figure represents the different enzymes that metabolize arachidonic acid into lipoxin A_4 (LXA_4) and aspirin-triggered 15-epi LXA_4 (ATL); 5-lipoxygenase (LO), 12-LO and 15-LO or cyclooxygenase 2 (COX2). Both LXA_4 and ATL are known to bind to the FPR2/ALX receptor.

The differential effects (pro-inflammation and pro-resolution) of the different LO-derived lipid mediators have led to the hypothesis that their role in inflammation can be divided into 3 phases, as depicted in Figure 2. The initiation is where the insult happens and many pro-inflammatory lipid mediators are produced. The resolution phase is when the insult is absent; a lipid switch takes place by the increase in pro-resolving lipid mediator and a reduction of pro-inflammatory mediators, which act as "stop signals". Finally, the termination phase is where the healing is concluded and the tissue goes back to homeostasis.

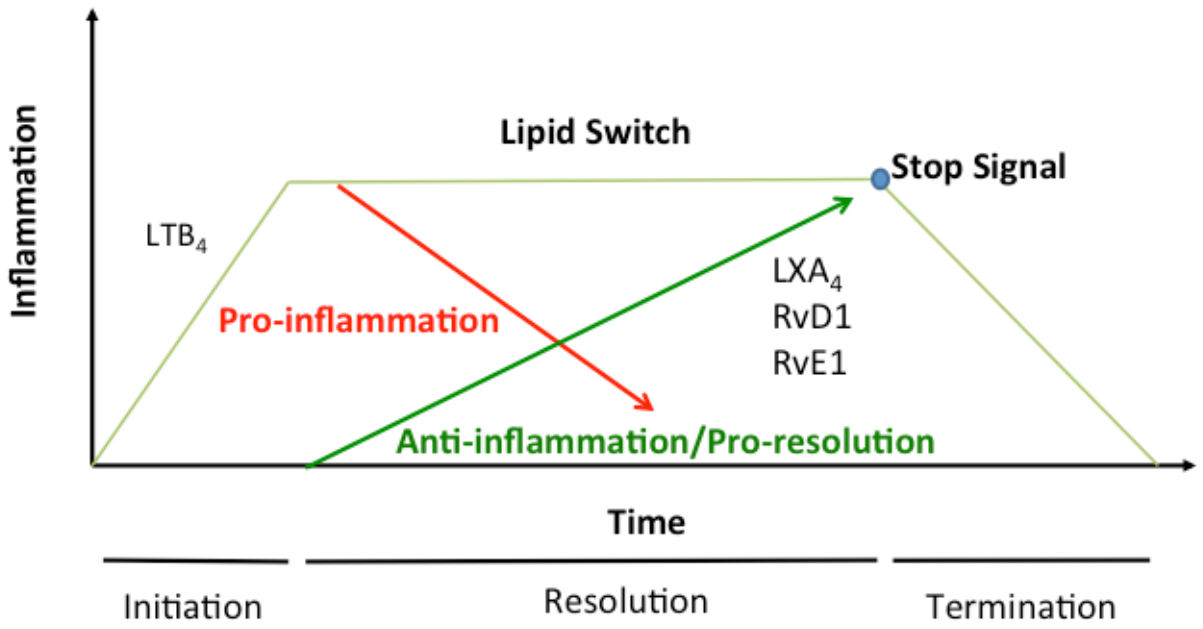

Figure 2. Resolution of inflammation
The resolution is divided in 3 phases: During initiation there is an increase in pro-inflammatory mediators, such as leukotriene B_4 (LTB_4). Subsequently, resolution starts as the levels of the pro-resolution lipid mediators increase, for example lipoxin A_4 (LXA_4), resolvin D1 and E1 (RvD1 and RvE1), and the levels of pro-inflammatory lipid mediators decrease, which is referred to as the "lipid switch". The pro-resolution lipid mediators promote a "stop signal" which leads to the third and last phase, the termination of inflammation. Adapted from Bosma-Den Boer, Nutrition & Metabolism 2012 [45]

1.3.2 Lipoxygenases in CVD

Human studies in CVD have shown associations of genetic variations in the 5-LO promoter with subclinical atherosclerosis [46], the presence of leukotriene receptors in human atherosclerotic lesions [47] and leukotrienes as biomarkers of atherosclerosis [48]. In addition, a main source of knowledge about the role of LOs in CVD has emerged from mouse models. As mentioned above, these enzymes are important for the production of lipid mediators with effects on both inflammation and resolution, which could offer an explanation as to the contradictory results obtained in different studies.

For example, $Ldlr^{-/-}$ mice heterozygous for 5-LO ($5\text{-}LO^{-/+}$) exhibited 95% inhibition of atherosclerosis [49]. However, subsequent studies failed to repeat those findings in either homozygous or heterozygous 5-LO deletion in both $ApoE^{-/-}$ and $Ldlr^{-/-}$ mice and reported neutral effects on atherosclerosis [50, 51]. It is important to take into consideration that genetic targeting of 5-LO will disrupt the formation of all 5-LO-derived lipid mediators. In contrast, BAYx1005,

which is an antagonist of the 5-LO activating protein (FLAP), inhibited LT synthesis but increased LX formation [52]. This suggests that FLAP is a specific target of LT biosynthesis, and hyperlipidemic mice treated with BAYx1005 exhibit decreased atherosclerosis [53, 54]. Nevertheless, although a subsequent study supported that FLAP is obligatory for LT, but not LX production [55], a recent study claimed LX biosynthesis to be dependent on FLAP [56].

On the other hand, the 12- and 15-LO are necessary for LXA_4 formation from AA (Figure 1), and genetic targeting of this enzyme blocked LX formation in a murine model of rheumatoid arthritis [57]. However, 12/15-LO deletion in hyperlipidemic mice has also generated contradictory results. Most studies point to protection against atherosclerosis after 12/15-LO knock-out (Table 1) which has been proposed to be due to LDL oxidation by 12/15-LO [37]. Likewise, overexpession of human 15-LO under the preproendothelin promoter, to generate a vascular wall-specific transgene, increased atherosclerosis in $Ldlr^{-/-}$ mice [58]. However, one study reported accelerated atherosclerosis in $ApoE^{-/-}$ x $12/15\text{-}LO^{-/-}$ mice, which was mimicked by bone marrow transplantation (BMT), and interestingly, pro-resolution mediators, such as LXA_4 and RvD1 were increased [59]. Also, macrophage-specific overexpression of either human 15-LO in hyperlipidemic rabbits or murine 12/15-LO in $ApoE^{-/-}$ mice is atheroprotective and associated with increased LXA_4 formation [59, 60].

Gender	Genotype	Diet	Time on diet	Aortic root	En Face	Comments	Ref
M & F	$ApoE^{-/-}$	Chow	22 w	↑	↑	-	[59]
NA	$ApoE^{-/-}$	Chow	15 w and 52 w	↓	↓	-	[61]
M & F	$Ldlr^{-/-}$	Western	3, 6, 9, 12 and 18 w	↓	↓	-	[62]
M & F	$Ldlr^{-/-}$	Chow	15 w and 32 w	NA	↓	Reduced in males and no differences in females.	[63]
M & F	$ApoE^{-/-}$	Chow+vit E	12 w	NA	↓	-	[64]
M & F	$ApoE^{-/-}$	Chow	25 w	↓	↓	Reduced in females and no differences in males	[65]

Table 1. Effect of 12/15-LO disruption in mouse models of atherosclerosis.
The table describe different studies using mice lacking 12/15-LO in atherogenic background evaluated at the aortic root and en face.
M= males, F= females; w= weeks; NA= Not available

Taken together, those studies illustrates the complexity of studing the 12/15-LO pathway in experimental atherosclerosis. Therefore, studying the receptors activated by the respective lipid mediators may be an appropriate means of obtaining insight into the role of these bioactive lipids in atherosclerosis. For example, in support of the pro-inflammatory role of LTs, either genetic or pharmacological targeting of the BLT_1 receptor for LTB_4 reduces atherosclerosis [66] and intimal hyperplasia after vascular injury [47, 67].

1.3.3 The possible role of Lipoxin A_4 in the vascular wall

As mentioned above, LXA_4 has been described as an anti-inflammatory and pro-resolving mediator in myeloid cells [12]. For example, LXA_4 down-regulates IL-1β in a human astrocytoma cell line [68] and in BV2 cells, a mouse microglia cell line [69]. LXA_4 also reduces IL-8 in human neutrophils *in vitro* [70] and MMP-9 *in vivo* in a mouse model of endometriosis [71].

In the vascular wall, LXs and ATL block vascular endothelial growth factor-stimulated angiogenesis [72] and migration of EC [73]. In addition, LXs stimulate prostacyclin [74] and nitric oxide (NO) [75] production in EC, thus promoting SMCs relaxation and subsequently, arterial dilation. Since NO also has an impact on inflammation [76], LXA_4 could play an indirect role by NO modulation. In SMCs, ATL inhibits SMCs proliferation after platelet derived growth factor (PDGF) stimulation [77], but otherwise previous studies of LX-induced effects in SMCs are scarce. Finally, it has been reported that circulating LXA_4 levels are reduced in patients with peripheral arterial disease [77], suggesting that this disease may be associated with a failure in the resolution of inflammation.

1.4 FPR2/ALX receptor

The formyl peptide receptor (FPR), subtype FPR2/ALX, was first described as a chemotaxis receptor. This seven trans-membrane (TM) G-protein coupled receptor exhibits 86% homology to the FPR1 receptor [12] and responds to bacterial peptides initiated with an N-formylmethionine [78]. Furthermore, other ligands have been described to bind FPR2/ALX, such

as different endogenous proteins and lipids [12]. Proteins such as LL-37, serum amyloid A (SAA) and formyl peptides are known to transduce pro-inflammatory responses through FPR2/ALX [78]. However, other proteins such as annexin A1 [79] or the lipid mediators, LXA_4 [80] and RvD1 [81] were described as FPR2/ALX ligands that exert anti-inflammatory effects on neutrophils and monocytes [12]. Furthermore, the binding sites may be different based on the ligand's properties. For example, whereas the sixth TM and the third external loop are crucial for peptide/protein binding, the seventh TM with adjacent regions and glycosylation are essential for LXA_4 binding [82]. This duality of the receptor has been examined in different disease models [83], and recently reviewed [84]. Figure 3 depicts some of the effects mediated by FPR2/ALX depending on the ligand. Binding assay experiments have confirmed that the highest endogenous FPR2/ALX affinity ligand is LXA_4 [85].

Currently there are FPR2/ALX antagonists on the market that work *in vivo,* but reports claims that they might not be so specific and may also bind FPR1 and FPR3 [78, 86]. The generation of knock-out mice lacking the mouse homologue of FPR2/ALX, Fpr2 has allowed the examination of the role that this receptor may play in different pathophysiological contexts. So far, studies using the knock-out of the mouse homologue of the human FPR2/ALX receptor have generated variable results depending on the disease model assessed. For example, Fpr2 knock-out mice exhibit exacerbated leukocyte recruitment after mesentery ischemia-reperfusion, paw edema and arthritis [87]. However, in a mouse model of asthma, with OVA/alum-induced allergic airway inflammation, the lack of the receptor conferred protection to the mice [88]. Additionally, in a bowel disease model, mice expressing Fpr2 presented higher inflammatory state with more intestinal crypt formation, but protecting the mice from death at later stages [89].

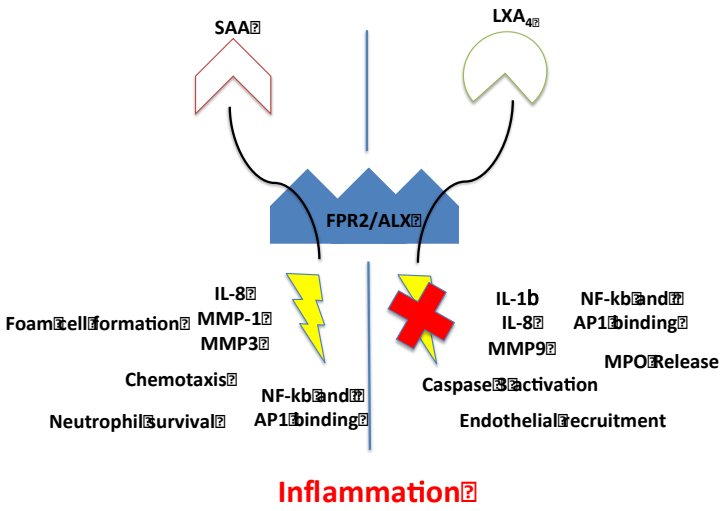

Figure 3: The duality of the FPR2/ALX receptor.
Based on what ligand binds to the receptor, differential effects will be transduced. For example, ligation of SAA will induce a pro-inflammatory response, whereas LXA_4 ligation will induce anti-inflammation and pro-resolution. The figure depicts the different pro-inflammatory actions triggered by SAA (left) and pro-resolving events triggered by LXA_4 (right).

Currently there is no knowledge about the use of LXA_4 and its analogs in atherosclerosis, but evidence supports the possible use of this lipid mediator in CVD. For instance, a genetic variation (polymorphism) at the promoter region of FPR2/ALX conferred a low expression of the receptor in neutrophils, and this was associated with higher risk of premature myocardial infarction [90]. On the other hand, another study exploring polymorphisms of FPR2/ALX receptor and coronary artery disease failed to show any association [91].

2 Aims

In general, the aim was to unravel the role of FPR2/ALX receptor signaling and its main ligand LXA_4 in the context of vascular wall pathologies such as atherosclerosis and stenosis.

- Uncover the presence of the receptor FPR2/ALX, in human atherosclerotic lesions and its expression in most common cells: macrophages, smooth muscle cells and endothelial cells.
- Explore the Fpr2 signaling in mouse models of atherosclerosis *in vivo* along with its major cells: macrophages, smooth muscle cells and endothelial cells *in vitro*.
- Assess a possible therapeutic potential of LXA_4 in atherosclerosis and stenosis.
- Elucidate if the pro-resolution effect of LXA_4 is mediated by the FPR2/ALX receptor.

3 Material and methods

3.1 Materials

All reagents were from Sigma-Aldrich unless otherwise specfied.

3.2 Experiments performed in the laboratory

3.2.1 Plasma measurements

Plasma cholesterol and triglyceride levels were determined by enzymatic assays (Randox Laboratories). ELISA was used to evaluate the plasma levels of SAA (Invitrogen) and LXA_4 (Neogen).

3.2.2 Animal genotyping

DNA was isolated from ear punches or tail biopsies using E.Z.N.A. kit (OMEGA Biotek). All animal experiments were approved by Karolinska ethical committee (#139-12).

The genotype for Fpr2, ApoE and Ldlr genes was assessed as follows:

3.2.2.1 Fpr2$^{-/-}$

Primers: F1 – tgagtgtcatgtcagaaggagcc, B11 – cggaatccagctacccaaatc and GB4 – ataaccttcgggcatggcactc. PCR conditions: 92˚C for 3 min followed by 35 cycles of 92˚C for 30s, 54˚C for 15 s, and 72˚C for 15 s. A final step of 72˚C for 10 min assured the complete elongation of the amplicons. A 2 % agarose gel revealed a 233-base pairs band for the wild-type animals or a 351-base pairs band for the knock-out animals.

3.2.2.2 ApoE$^{-/-}$

Primers: oIMR0180 (common forward) – gcctagccgagggagagccg, oIMR0181 (Wild-type reverse) – tgtgacttgggagctctgcagc and oIMR0182 (Mutant reverse) – gccgccccgactgcatct. PCR conditions: 94˚C for 3 min followed by 35 cycles of 94˚C for 30 s, 68˚C for 40 s and 72˚C for 1 min. A final step of 72˚C for 10 min assured the complete elongation of the amplicons. A 2 % agarose gel revealed a 155-base pairs band for the wild-type animals or a 245-base pairs band for the knock-out animals.

3.2.2.3 Ldlr$^{-/-}$

Primers: Ldlr common forward – aggtgagatgacaggagatc, Ldlr wild-type reverse – accccaagacgtgctcccaggatga, Ldlr mutant reverse – cgcagtgctcctcatctgacttgt. PCR conditions: 94^{o} C for 3 min followed by 35 cycles of 94˚C for 30 s, 68˚C for 40 s and 72˚C for 1 min. A final step of 72˚C for 10 min assured the complete elongation of the amplicons. A 2 % agarose gel revealed a 400-base pairs band for the wild-type animals or a 800-base pairs band for the knock-out animals.

3.2.3 RNA isolation

Multiple murine tissues homogenized in QIAZOL (Qiagen) or cultured cells lysed in RLT buffer (Qiagen) were used for the semi-automatic isolation of total RNA (Qiacube; Qiagen). RNA concentration was spectrophotometrically measured (Nanodrop 1000; Thermo Fisher Scientific) and its quality determined by capillary electrophoresis (Bioanalyzer; Agilent).

3.2.4 Real-Time PCR

Reverse-transcription reaction was developed by either Superscript-II kit using random hexamers (Life Technologies) or High Capacity RNA to cDNA kit (Life Technologies). Real-time PCR was developed on a 7900HT Real-Time PCR system (Life Technologies) using TaqMan assays (Life Technologies). Results were expressed as $2^{-\Delta CT}$ using hypoxanthine phosphoribosyl-transferase (HPRT) or peptidylprolyl isomerase A (PPIA) as housekeeping genes, for mouse and human samples respectively. Changes in gene expression due to a treatment or genotype were assessed by comparison with the expression of the experimental controls.

3.2.5 Cell isolation

Human cells were purified from buffy coats or venous blood. Murine cells were isolated after euthanizing the animals by CO_2 inhalation.

3.2.5.1 Human CD4^{+} positive cells

Peripheral blood monocyte cells (PBMC) were isolated from buffy coats or peripheral blood by means of gradient centrifugation. CD4-antibody-coated magnetic beads (Miltenyi Biotec) beads were incubated with the PBMC. The mixture was subsequently exposed to a

magnetic field that separated the $CD4^+$ cells from the rest of the cells. The $CD4^+$ cells were either incubated with RPMI media supplemented with 10% FCS for 3 days (non-activated $CD4^+$ cells) or cultured in CD3/CD28-immobilized-antibodies petri dishes for 3 days (activated $CD4^+$ cells). After this period, non-activated and activated $CD4^+$ cells and their supernatants were used to stimulate macrophages.

3.2.5.2 Human Monocytes/Macrophages

The negative cellular fraction from the $CD4^+$ selection derived from PBMC (mainly monocytes) was seeded on a petri dish for 3 h. Non-adherent cells were washed away and the attached cells were left to differentiate with 5% FCS and 5% serum obtained from the same donor for 3 days. The differentiated cells (macrophages) were exposed for 24h to the $CD4^+$ cells and supernatants described in the previous section.

3.2.5.3 Mouse Peritoneal Macrophages

Peritoneal cells were isolated from non-stimulated peritoneum of 8-10 weeks old mice with 8 ml of RPMI supplemented with antibiotics and 10% FCS. Peritoneal cells were seeded at $1x10^6$ cells/ml density in petri dishes. After 2-3 h, non-adherent cells were removed. The attached cells, $F4/80^+$ by FACS analysis (peritoneal macrophages), were used for further experimentation.

3.2.5.4 Mouse bone marrow cells

Mice lower limbs were separated from the body and kept in RPMI supplemented with antibiotics and 10% FCS. In a sterile environment, muscles and tendons were removed from femurs and tibias. After the removal of the epiphyses, the bone marrow (BM) was isolated from the bones using a 21 G needle and cell culture medium. Ammonium-Chloride-Potassium (ACK) buffer was used to lyse the erythrocytes. Finally, BM cells were washed, re-suspended in cell culture medium and counted.

3.2.5.5 Mouse smooth muscle cells

Mouse aortic smooth muscle cells (mSMC) were isolated from 4-6 weeks old mice. The aortas were dissected from adjacent tissues, cut into pieces and digested for 4 h with type I

collagenase in DMEM with 2 % FCS. FACS analyses determined that more than 90% of the cells were positive for α-actin. mSMC at passages 1-3 were used in the experiments.

3.2.5.6 Mouse endothelial cells

Murine aortic EC (mAEC) were collected from 4-6 weeks old animals. The aortas were cut into pieces and digested with type II collagenase for 45 min. After this period, the mixture of cells was incubated with magnetic beads (Invitrogen) previously coated with CD31 antibody (BD Bioscience). The $CD31^+$ cells were purified by magnetic field separation and cultured in gelatin-treated microplates in the presence of EC medium (F12\Glutamax DMEM medium supplemented with endothelial cell growth factors and heparin). FACS analyses determined that the cells exhibited an average of 92% and 94% of purity for CD31 and CD105, respectively. mAEC at passages 1-3 were used in the experiments.

3.2.6 Intracellular calcium measurements

Free cytoplasmic calcium was measured using a Zeiss Axio system (Zeiss). Freshly isolated peritoneal macrophages were exposed to the calcium indicator Fluo-4 (Molecular Probes) in Krebs solution (KS). The fluorescence emitted by Fluo-4 (480 nm) was recorded every 2 sec for 5-15 min. Single-cell changes in fluorescence were expressed in arbitrary units (A.U.). Peritoneal cells exhibiting a characteristic oscillating pattern of variations in intracellular calcium were analyzed. The average of the 10 highest values (peaks) and the average of the 10 lowest values (bases) were collected and the amplitude of the oscillation was calculated as the difference between peaks minus bases. The frequencies of oscillation were analyzed by Spectral Analysis software (Version 3.0; http://www.molneuro.mbb.ki.se/uhlen/downloadable. php). At least 15 cells per field were analyzed from each experiment.

3.2.7 Flow cytometry

The cells were fixed in 1% PFA before being used for one of the following protocols. For extracellular staining, the cells were incubated with anti-FPR2/ALX antibody (rabbit anti-human, Abcam - ab63022) followed by incubation with anti-rabbit dylight-488. For intracellular staining,

the cells were permeabilized with Cytofix/Cytoper solution (BD Bioscience), washed twice with Perm/Wash buffer (BD Bioscience) and incubated with anti-FPR2/ALX diluted in Perm/Wash buffer, followed by incubation with anti-rabbit dylight-488 in Perm/Wash buffer. Unstained cells, isotype control and secondary antibody in the absence of the primary were used as negative controls to confirm the specificity of the procedure. Median Intensity fluorescence (MIF) was recorded and analyzed in 3 independent experiments.

3.2.8 *In vitro* efferocytosis assay.

Efferocytosis assay was performed with pHrodo-marked splenocytes as previously described [92]. Briefly, spleens from $Fpr2^{+/+}$ and $Fpr2^{-/-}$ mice were collected, minced and exposed to a UV light source for 8 min to induce apoptosis. Cell apoptosis was confirmed using Annexin V and propidium iodide (PI) staining by FACS. The cells were then incubated with pHrodo for 30 min. Peritoneal macrophages were incubated with the apoptotic fluorescently-labeled splenocytes at 1:2 ratio (efferocyte:apoptotic cell). After 4 h of incubation, the non-uptaken cells were washed and the efferocytes were detached from the plate and subsequently analyzed by FACS. MIF of each sample was normalized to the uptake of $Fpr2^{+/+}$ apoptotic splenocyte by $Fpr2^{+/+}$ macrophages.

3.2.9 Cell proliferation assay

mSMC and mAEC proliferation rate was evaluated using WST-1 reagent (Chemicon) as previously described [47]. In brief, 3500 cells/well were seeded with DMEM supplemented with 10% FCS. After 48h, 200 μL of phenol red-free DMEM supplemented with FCS and 10 μL of WST-1 reagent was added to each well. After 2h of incubation, the absorbance of the formazan dye formed was measured at 440 nm using a microplate reader (Perkin Elmer).

3.2.10 Cell migration assay

The *in vitro* migration of mSMC was assessed through wound migration assay as previously described [67]. Using a sterile 1.15 mm diameter pipette tip, a scratch was created at the center of confluent $Fpr2^{+/+}$ and $Fpr2^{-/-}$ mSMC cultured in 12-wells plates. Multiple regions of the scratch were photographed every hour using the Cell-IQ system (Cenibra) for 48 h. The

percentage of wound closure over time was determined by analyzing sequential images of the same region.

3.2.11 Immunohistochemistry

Human atherosclerotic plaques and multiple mouse tissues were used for immunological detection of proteins. Cryosections were obtained from tissues previously embedded in Optimal Cutting Temperature (OCT) reagent and preserved at -80°C. Alternatively, zinc-formaldehyde fixed samples were embedded in paraffin and cut in a microtome. Sections were processed for light microscopy or immunofluorescence depending on the antigen of interest and the specifications of the antibody. Removal of paraffin and antigen retrieval were specific steps for the preparation of the paraffin samples. After those steps, all the sections followed a standard immunohistochemistry protocol.

3.2.11.1 Visible light microscopy

Human atherosclerotic plaques were stained with the following primary antibodies: FPR2/ALX (Abcam, ab63022), CD68 (BD, #556059), CD163 (Novocastra, NCL-CD163), CD31 (Dako, JC70A) and α-actin (Abcam, ab8211). On the other hand, murine tissues and cells were stained with: CD68 (Serotec, MCA1957), CD3 (Abcam, ab19639) and CD4 (Pharmingen, 553647). Secondary biotinylated antibodies (Vector Labs), Avidin-biotin complex (Vector, UK) and NovaRed (Vector, UK) were used for the visualization of the immune recognition; counterstaining was done with hematoxylin.

3.2.11.2 Fluorescence

The same primary antibodies described in the previous section were used for immunofluorescence. In this context, secondary antibodies were conjugated with Dylight-488 or -594 and 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Isotype IgG was used as negative control.

3.2.11.3 Collagen quantification

Collagen fibers were visualized by means of picrosirius red (Histolab) staining in a circularly polarized light microscope (Leica). The staining were performed on eight formalin-fixed sections collected every 100 µm from the aortic valve cusps.

3.2.12 Organ bath experiments

Aortas were quickly dissected, cut into 2-mm long rings and mounted in a Multi Wire Myograph System (DMT). In some preparations, the endothelium was removed and compared with intact rings. Aortic isometric force development in the presence of several concentrations of vasoactive substances was evaluated as previously described [93].

3.2.13 Biobank

Karolinska carotid endarterectomy biobank (BiKE) was used to determine the expression pattern of FPR2/ALX in human atherosclerosis. Gene expression microarrays hybridized with mRNA isolated from 127 atherosclerotic plaques, 10 control arteries, and from circulating PBMCs obtained from patients undergoing an endarterectomy procedure were used to measure FPR2/ALX expression and to determine its association with several cell markers in a multi-perspective transcriptomical approach. Additional plaques were used for immunological staining of FPR2/ALX and co-localization experiments. Ethical permits were approved by Karolinska ethical committee (#02-146 and #02-147).

3.3 Experiments performed in the animal facility

3.3.1 Mouse colonies

3.3.1.1 $Fpr2^{-/-}$ mice

Fpr2 deficient mice were generated as previously described [86], and genotyped according to established protocols described previously in this thesis. Of note, these knock-out mice were generated through the insertion of a gene cassette containing a GFP reporter in reverse orientation into intron 1 of Fpr2 gene, which prevented transcriptional read-through of the Fpr2 as well as Fpr3 genes [86]. Therefore, these mice have also been termed Fpr2/Fpr3 knock-out mice [94], but will be referred to as $Fpr2^{-/-}$ in the present thesis.

3.3.2 Double knock-out mice

For the purpose of studying the absence of Fpr2 in atherosclerosis, two mice models were created.

3.3.2.1 $Ldlr^{-/-}$ x $Fpr2^{-/-}$ mice

After backcrossing for at least 8 generations onto C57BL/6J background, $Fpr2^{-/-}$ mice were crossed with $Ldlr^{-/-}$ mice (Jackson Laboratory) to generate the $Ldlr^{-/-}$ x $Fpr2^{-/-}$ strain. Age-matched $Ldlr^{-/-}$ x $Fpr2^{+/+}$ littermates were used as controls. 12-weeks old males were fed on a HFD (Lantmännen, R638) for 8, 12, 16 and 20 weeks.

3.3.2.2 $ApoE^{-/-}$ x $Fpr2^{-/-}$ mice

After backcrossing for at least 8 generations onto C57BL/6J background, $Fpr2^{-/-}$ mice were crossed with $ApoE^{-/-}$ mice (Taconic) to generate the $ApoE^{-/-}$ x $Fpr2^{-/-}$ strain. Age-matched $ApoE^{-/-}$ x $Fpr2^{+/+}$ littermates was used as controls. $ApoE^{-/-}$ x $Fpr2^{-/-}$ mice were not exposed to HFD since these animals develop spontaneous atherosclerosis.

3.3.2.3 Evaluation of atherosclerosis

After euthanasia by CO_2, aortas were dissected from the surrounding tissues. Thoracic aortas were longitudinally opened, pinned, and stained with Sudan IV. The images of the aortas were captured using a Leica camera and the analyses were performed using Image J (NIH) in a blinded fashion. The area covered by the atherosclerotic lesions divided by the area of the entire aorta was calculated and compared by either genotype or treatment. Abdominal aortas were used for mRNA extraction.

The upper portion of the heart (containing the aortic root) was embedded in OCT and frozen at -80°C. 10-µm cryosections were collected covering the entire aortic valve, as previously described [95]. Serial sections were stained with Oil red-O and lesion area was determined in a blinded fashion by image analyses using Leica Qwin. The lesion area and % of lesion per cross-sectional vessel area were calculated. Immunohistochemical analysis of CD68, CD3 and CD4 was performed at the level of the largest lesion size for each sample.

3.3.3 Bone marrow transplantation

$Fpr2^{-/-}$ mice on a mixed 129SvEv background were used for cell isolation and BMT. After euthanasia by CO_2, BM cells were collected from the femurs and tibias of either $Ldlr^{+/+}$ x $Fpr2^{+/+}$ or $Ldlr^{+/+}$ x $Fpr2^{-/-}$ donor mice. Recipient $Ldlr^{-/-}$ mice at the age of 8-10 weeks were lethally irradiated (800 Rad), and subsequently injected with $5x10^6$ BM cells through tail vein. Animals were kept in intra-ventilated cages and antibiotics (sulfadiazine and trimethoprim) were administered for 3 weeks. Four weeks after BMT, mice were placed on HFD for 20 weeks. After this period, mice were sacrificed and organs were collected for the protocols described below.

3.3.4 Carotid ligation

$Fpr2^{-/-}$ and $Fpr2^{+/+}$ littermates in a mixed background (6–8 weeks old) were subjected to total common carotid artery ligation, as previously described [96]. Briefly, after anesthesia (Sevoflorane and buprenorphine), a midline incision was performed on the frontal neck from the manubrium until 1 cm near the chin. The left common carotid was identified and a 9-0 nylon suture was applied just above the carotid bifurcation. Arterial occlusion was confirmed by the visual lack of flow in the external and internal carotids. Wound was closed using 6-0 continuous suture (Vykril®). An osmotic pump containing either vehicle (15% ethanol) or ATL (10 μg/kg) was placed in the mouse back. Mice were kept in heat pads until they were fully awaked; then, they were transferred back to their cages. Buprenorphine was applied every 12 h for the next 3 days and no sign of stroke was observed in any mice. The animals were sacrificed 4 weeks after the implantation of the pump. Perfusion fixation was performed immediately after CO_2 euthanasia. Heart puncture was performed with a 25 G needle for the infusion of 4% zinc-formaldehyde in PBS under physiologic pressure. Blood was drained through an incision of the inferior vena cava and perfusion was continuous for 10-15 min. The ligated left and the non-ligated right carotid arteries were excised and post-fixated overnight with 4% PBS-buffered formaldehyde; afterwards, the arteries were transferred to 70% ethanol and embedded in paraffin.

3.3.4.1 Evaluation of carotid intima

Paraffin-embedded carotid arteries were cut in series of 8-10 sections (10 μm each) every 100 μm. The morphology of the arteries was evidenced by means of hematoxilin-eosin staining. Using Image J software, the circumference of the external elastic lamina (EEL), the internal elastic lamina (IEL), and the luminal border (LB) were measured. Areas were calculated from circumference measurements assuming a circular structure under *in vivo* conditions. Total vessel area, luminal area, total wall area, medial area and intimal area were calculated from EEL- and LB-circumference, by subtracting LB-area from EEL-area, IEL-area from EEL-area and LB-area from IEL-area, respectively.

3.4 Data analysis

Clinical parameters are expressed as median and ranges. Univariate correlations between continuous variables and FPR2/ALX mRNA levels were performed using Spearman correlation, and binary values were compared using Fisher's exact test. Mann-Whitney U test or Student t-test was used for comparisons between 2 groups depending on the population distribution. One-way or two-way analysis of variances (ANOVA) as appropriate, with Kruskall-Wallis or Bonferroni post-hoc test respectively, were used for multiple comparisons. $P<0.05$ was considered as significant. All analyses were performed using SigmaPlot version 12.5 (Systat Software Inc). Experimental data are expressed as mean ± standard error of the mean (SEM).

4 Results

4.1 FPR2/ALX expression in human atherosclerotic lesions

In Paper I, immunohistological examination of human atherosclerotic plaques revealed three major cell types expressing FPR2/ALX, namely macrophages, SMC and EC. Furthermore, mRNA levels of FPR2/ALX in human atherosclerotic plaques were significantly higher compared with healthy arteries, and were significantly correlated with mRNA levels of markers for monocytes (CD14) and macrophages (CD163) but not with mRNA levels of a neutrophil marker (CD66b). Subsequently, the clinical characteristics of the patients donating the vascular samples were added into a multivariate regression model in an attempt to establish the association of FPR2/ALX mRNA levels with clinical features of disease. This analysis revealed that FPR2/ALX mRNA levels in atherosclerotic plaques were significantly associated with age and creatinine and exhibited an inverse correlation with recent clinical manifestations, stratified according to none (0), past (>3 months; 1), or recent (<3 months; 2) clinical signs of cerebral ischemia (Paper I). Since the patients with either none or longer time to clinical signs of stroke also exhibited the highest FPR2/ALX mRNA levels, the interpretation of these data was that FPR2/ALX expression was highest in the most stable atherosclerotic lesions.

4.1.1 Macrophages

The co-localization of FPR2/ALX with a macrophage marker by immunohistochemistry suggests that the receptor is active in this myeloid cell. This was further supported by the significant correlation between the mRNA levels for FPR2/ALX and macrophage markers, besides, these findings were accompanied by a significant correlation of FPR/ALX mRNA with cytokines such as IL-1β, CXCL-1 and IL-6 (Paper I). Furthermore, in a subset of patients, peripheral blood mononuclear cells (PBMC) were also isolated and in line with the findings in plaques, the analysis of mRNA levels in circulating PBMCs revealed a similar significant positive association between FPR2/ALX and IL-1β and CXCL-1 but not for IL-6 (Paper I).

Since these findings indicated a pro-inflammatory action of monocyte/macrophage FPR2/ALX expression in the context of atherosclerosis, human cells were subsequently studied *in vitro* (Paper III). These experiments revealed that pro-inflammatory stimuli, *i.e* T-lymphocytes, their supernatants, IFN-γ, and LPS induced FPR2/ALX up-regulation in human macrophages and THP-1 cells. However, THP-1 cells exposed to LXA_4 (100 nM) for 24 h exhibited significantly reduced mRNA levels of MMP-9, IL-1β IL-8, and CXCL-1 (Paper I).

Taken together, in humans, FPR2/ALX is highly expressed in the atherosclerotic plaque, mainly on monocytes/macrophages. The receptor is up-regulated by activated $CD4^+$ cells and there is a positive correlation of mRNA of FPR2/ALX receptor with cytokines production more importantly in plaque then in circulating cells. These findings suggest that FPR2/ALX is associated with inflammation in the atherosclerosis context.

4.2 Mouse Fpr2 in the context atherosclerosis

With the information that human plaques are rich in FPR2/ALX and that it might impact plaque stability, three different murine models of atherosclerosis were used in order to determine the role of this receptor in the disease.

First, mice lacking both Fpr2 and Ldlr were generated, and evaluated for the diet-induced atherosclerosis at different time points (Paper I). Interestingly, these experiments revealed a time-dependent effect, in which $Ldlr^{-/-}$ x $Fpr2^{-/-}$ mice exhibited a delayed atherosclerosis development compared with $Ldlr^{-/-}$ x $Fpr2^{+/+}$. The results showed that after 12 weeks on HFD, mice lacking the receptor exhibited significantly smaller lesions compared with Fpr2 expressing mice (Figure 4).

Second, to specifically examine the role of myeloid cell Fpr2 in atherosclerosis, BMT was performed. These experiments pointed to pro-atherogenic signaling through myeloid Fpr2, since $Ldlr^{-/-}$ mice receiving $Fpr2^{-/-}$ BM exhibited a reduced atherosclerosis compared with those receiving $Fpr2^{+/+}$ BM (Figure 4).

The third and final mouse model of atherosclerosis generated was $ApoE^{-/-}$. Being either wild-type or knock-out for Fpr2 (Figure 4). These mice develop spontaneous atherosclerosis on

normal chow diet. Importantly, the findings in ApoE$^{-/-}$ mice corroborated those observed in HFD-fed Ldlr$^{-/-}$ mice, since mice lacking Fpr2 on chow diet exhibited significantly smaller atherosclerotic lesions compared with Fpr2 expressing mice (Figure 4).

Interestingly, when the latter ApoE$^{-/-}$ and Fpr2 double knock-out mice were exposed to ATL treatment by osmotic mini-pumps atherosclerotic lesion size was reduced, whereas in ApoE$^{-/-}$ mice lacking Fpr2, ATL treatment did not induce any significant effects. ATL treatment significantly decreased the amount of CD68 positive cells in the lesions and significantly reduced IL-6 and MMP-13 mRNA levels (Paper V). The latter effect was not only observed locally in the aorta, but also in other organs such as spleen and lung, indicating systemic anti-inflammatory effects of ATL treatment. Interestingly, no ATL-induced effects were observed in ApoE$^{-/-}$ x Fpr2$^{-/-}$ mice and ATL-treatment was not associated with changes in circulating leukocytes.

Given that the consistent findings of reduced atherosclerosis induced by Fpr2 deletion in all three models explored were in contrast to the anti-inflammatory effects of LXA_4 *in vitro* (Paper III) and the anti-atherosclerotic effects of ATL *in vivo* (Paper V), the concentration of putative Fpr2 agonists was also explored. These results showed that the circulating levels of the pro-inflammatory agonist SAA were 296 + 62 μg/mL, whereas LXA_4 levels were 34 + 3.3 ng/mL. This represent a difference around 10 000 fold difference favoring the pro-inflammatory ligand (Paper I).

Importantly, serum lipid levels and leukocyte counts were not altered by Fpr2 deletion in any of the mouse models explored (Paper I, Paper V).

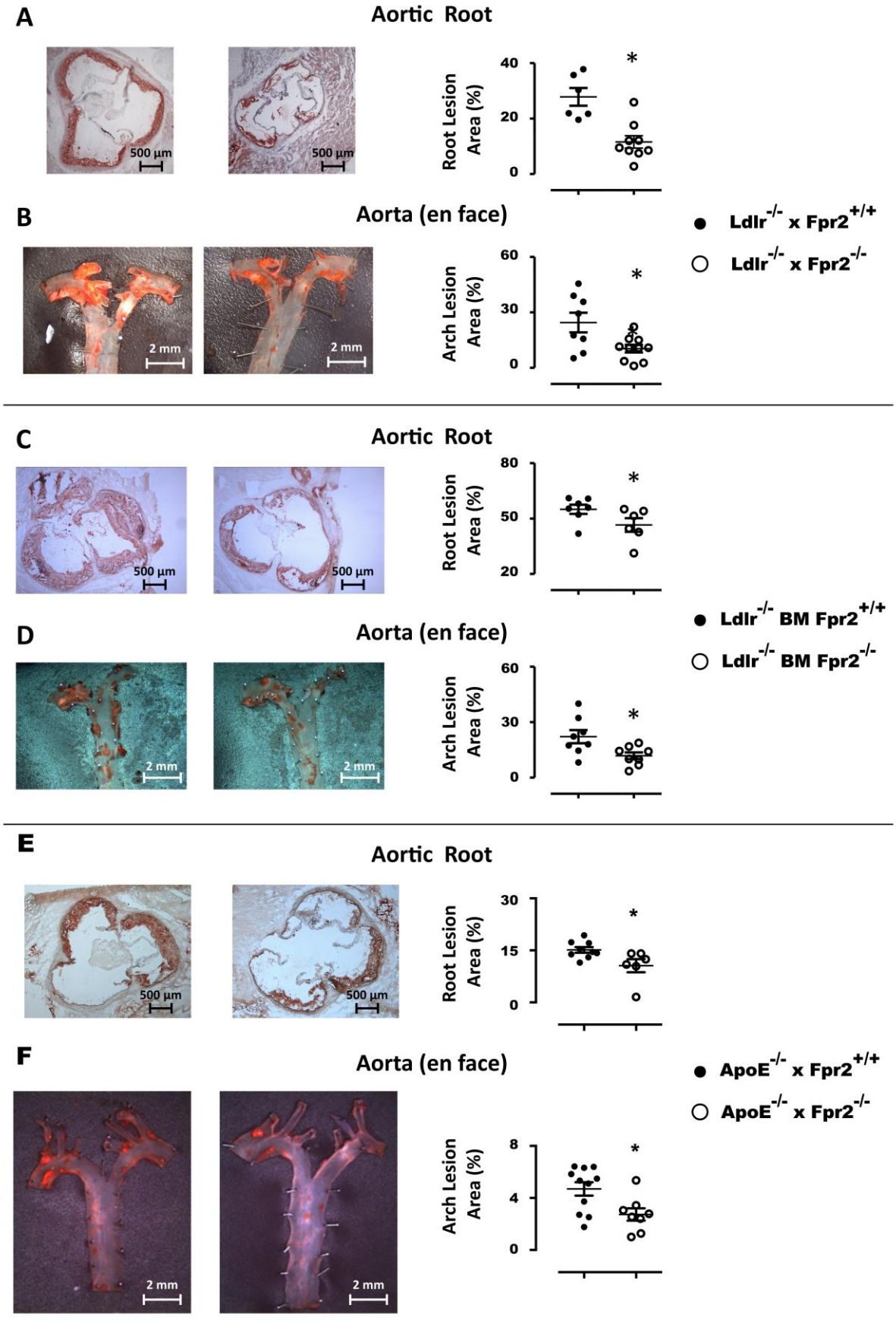

Figure 4: Atherosclerotic lesion evaluated in multiple Fpr2 deficient animal models.
Atherosclerosis lesions measured in aortic root and en face respectively in $Ldlr^{-/-}$ x $Fpr2^{-/-}$ (A and B), $Ldlr^{-/-}$ transplanted with either $Fpr2^{+/+}$ or $Fpr2^{-/-}$ BM (C and D) and $ApoE^{-/-}$ x $Fpr2^{-/-}$ (E and F).

4.2.1 Leukocytes

Given that macrophages were associated with FPR2/ALX expression in human atherosclerotic lesions (Paper 1), the effects of murine Fpr2 deletion was subsequently explored *in vitro* using resident peritoneal macrophages. Macrophages derived from $Fpr2^{-/-}$ mice exhibited less calcium oscillations compared with those derived from wild-type mice (Paper I). These macrophages also exhibited a lower efferocytosis activity when exposed to apoptotic cells (Paper I), suggesting a reduced activation of $Fpr2^{-/-}$ macrophages. This phenotype was accompanied by significantly lower mRNA levels of IL-6 and IL-1β (Paper I). These observations corroborated the results in human atherosclerotic plaques, which exhibited a significant positive correlation with these cytokines and FPR2/ALX expression (Paper I).

Since atherosclerosis progression is related with the ingress of leukocytes to the lesions, the macrophage marker CD68 was quantified in the aortic root of the studied animals. These experiments revealed a significantly decreased macrophage content in $Ldlr^{-/-}$ x $Fpr2^{-/-}$ mice and in $Ldlr^{-/-}$ mice transplanted with $Fpr2^{-/-}$ BM (Figure 5 A).

Interestingly, $ApoE^{-/-}$ mice receiving ATL treatment exhibited significantly smaller lesions and a reduction of CD68 expression in the aortic root (Figure 5 C), supporting the anti-inflammatory properties of ATL through of Fpr2 signaling.

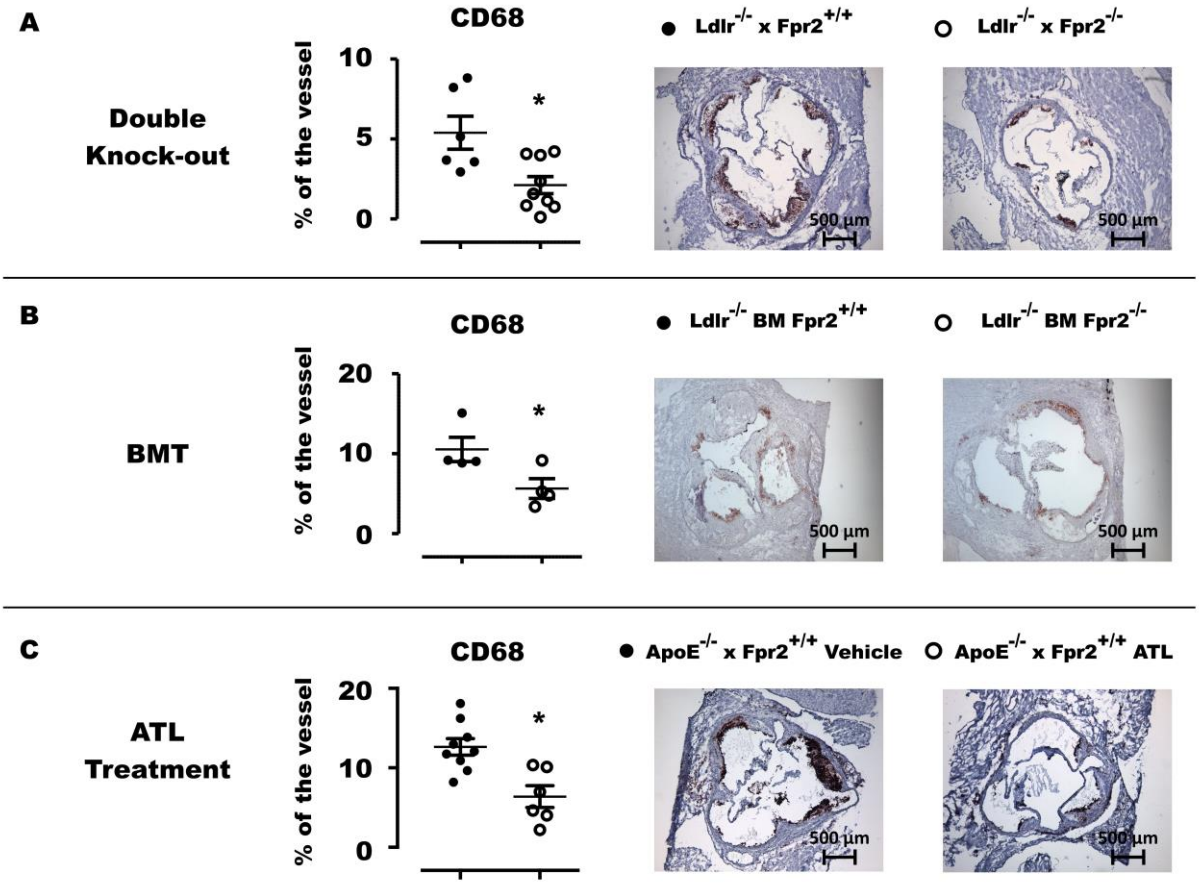

Figure 5: Macrophage presence in several Fpr2-defcient atherosclerotic models
CD68 expression in aortic roots of (A) Ldlr$^{-/-}$ x Fpr2$^{-/-}$, (B) Ldlr$^{-/-}$ with BM Fpr2$^{-/-}$ and (C) ApoE$^{-/-}$ x Fpr2$^{-/-}$ mice.

4.2.2 SMC

In addition to macrophages, cells expressing the SMC marker α-actin also exhibited positivity for FPR2/ALX in human atherosclerotic lesions (Paper I). Therefore, Fpr2 signaling in aortic mSMC derived from Fpr2$^{+/+}$ and Fpr2$^{-/-}$ mice was further explored. Fpr2 expression in SMC was determined by immunofluorescence, as shown in the cover image (Fpr2 in red, α-actin in green and DAPI in blue, Paper I). Interestingly, mSMC derived from Fpr2$^{-/-}$ mice exhibited a specific phenotype as compared to Fpr2$^{+/+}$ mSMC. First, mRNA levels of several components involved in collagen deposition and maturation were significantly lower in Fpr2$^{-/-}$ compared with wild-type mSMC (Paper I), whereas ECM degrading pathways appeared activated by means of increased MMP-13 and reduced Timp1 mRNA levels in Fpr2$^{-/-}$ mSMC (Paper I). The latter findings were supported by significantly less collagen in the atherosclerotic lesions of Ldlr$^{-/-}$ x Fpr2$^{-/-}$ compared with Ldlr$^{-/-}$ x Fpr2$^{+/+}$ mice. Interestingly, the latter finding was not observed

after BMT, supporting that structural cells, such as mSMC, and not myeloid cells mediated those changes in lesion collagen (Figure 6).

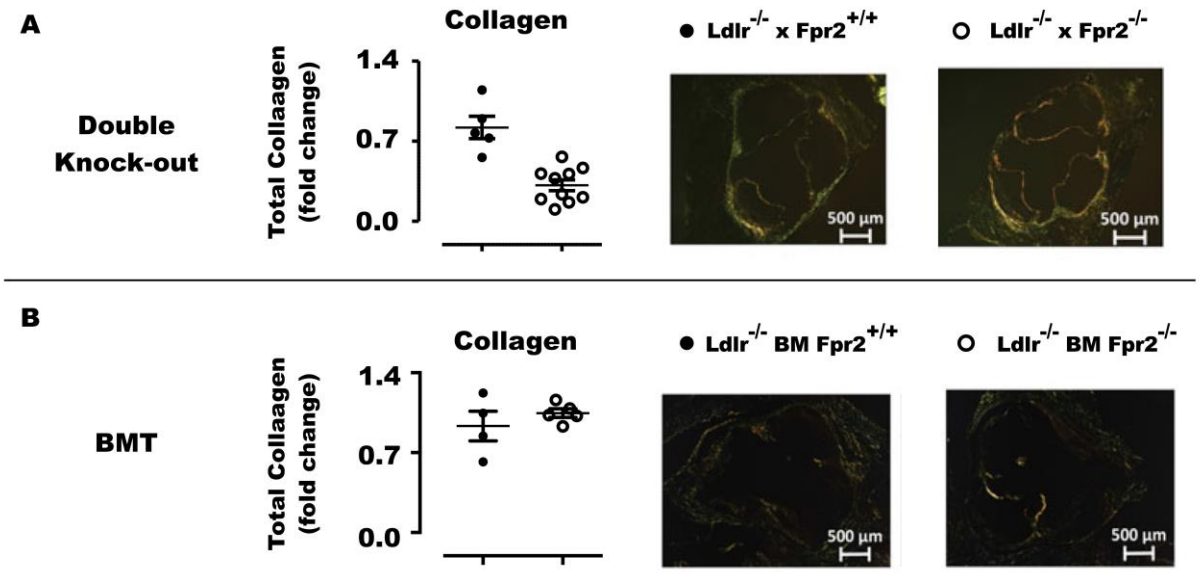

Figure 6: Total collagen of aortic root comparing Fpr2-deficient and expressing vascular wall.
The picture represents the total amount of collagen giving by picrosirius red staining under polarized light of (A) $Ldlr^{-/-}$ x $Fpr2^{-/-}$ and (B) $Ldlr^{-/-}$ transplanted with either $Fpr2^{-/-}$ or $Fpr2^{+/+}$ BM.

In addition to the differences in collagen, $Fpr2^{-/-}$ mSMC also exhibited a significantly increased proliferation compared with wild type mSMC (Figure 7 A). In a wound migration assay $Fpr2^{-/-}$ mSMC in addition exhibited increased migratory properties compared with wild type mSMC (Paper II). Using the latter assay, ATL treatment significantly reduced migration of $Fpr2^{+/+}$ SMC, whereas $Fpr2^{-/-}$ mSMC migration was unaltered after ATL treatment (Paper 2).

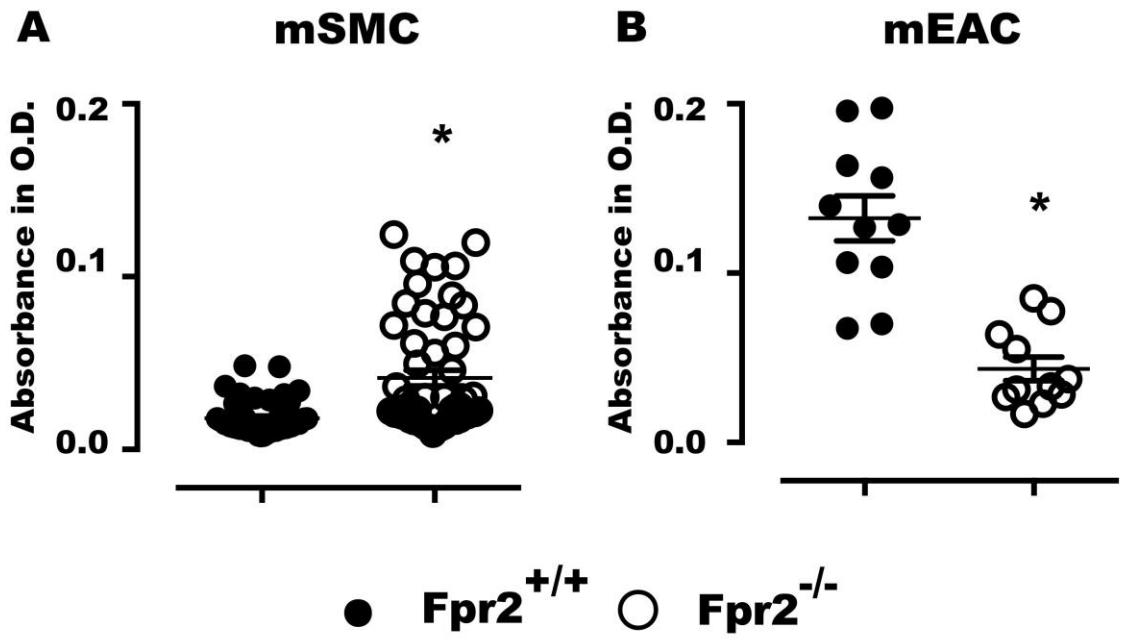

Figure 7: Proliferation rate of Fpr2-deficient cells from components of the vascular wall
Proliferation rate in absorbance (OD) of (A) mouse aortic smooth muscle cells (mSMC) and (B) mouse aortic endothelial cells (mAEC).

Given these marked phenotypic changes of Fpr2 deletion in terms of mSMC responses *in vitro*, the role of Fpr2 signaling in mSMC proliferation and migration was subsequently examined *in vivo*. Using the total carotid ligation model, a significant reduction of intimal hyperplasia after ATL treatment was revealed (Figure 8 B). Since the latter effects of ATL were observed only in wild type mice, and not in $Fpr2^{-/-}$ mice, these experiments further reinforced the importance of ATL signaling by means of Fpr2 in the SMC response to vascular injury.

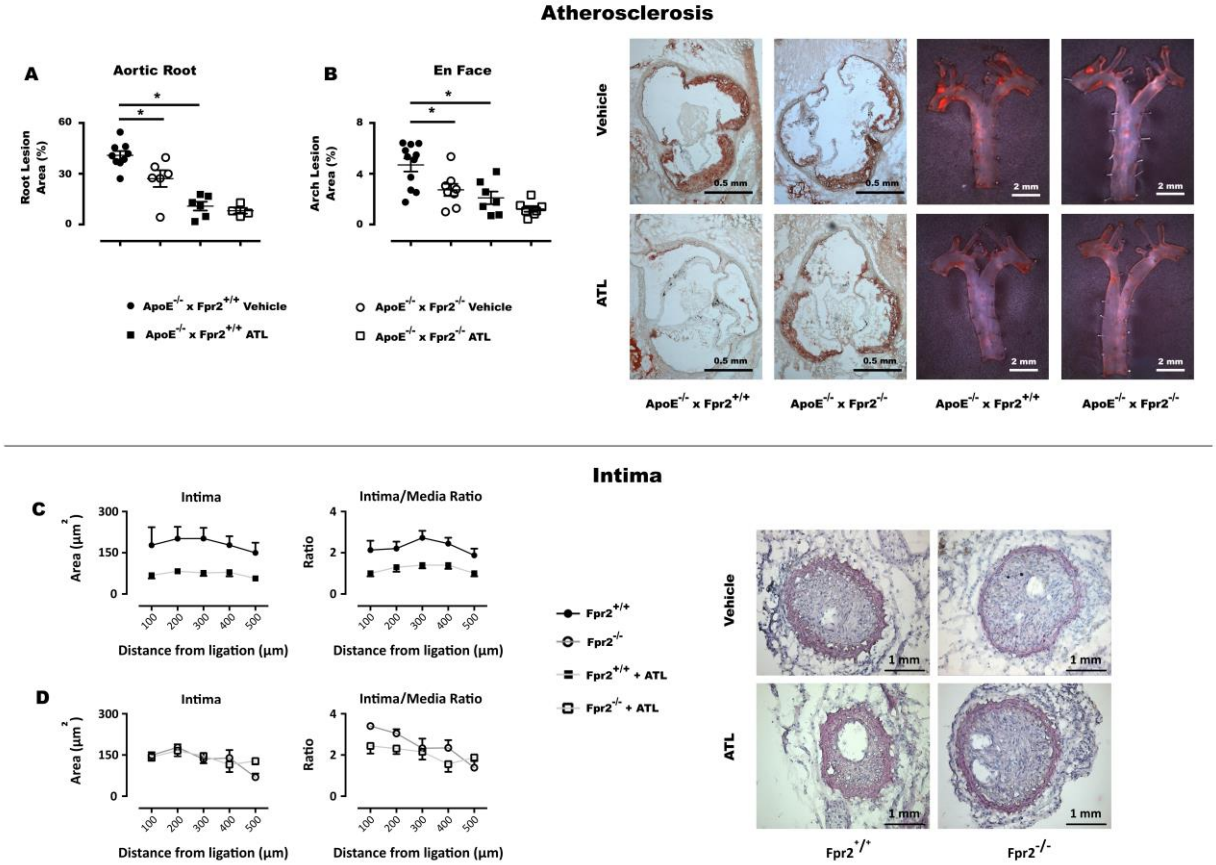

Figure 8: ATL effects on atherosclerosis and intima formation after caroti ligation

Atherosclerosis lesions of ApoE$^{-/-}$ x Fpr2$^{+/+}$ and ApoE$^{-/-}$ x Fpr2$^{-/-}$ treated either with vehicle (15% ethanol) or aspirin-triggered lipoxin (ATL) at aortic root (A) and en face (B). Intima formation of Fpr2$^{+/+}$ (C) and Fpr2$^{-/-}$ (D) treated with either vehicle or ATL after total carotid ligation. On the right side representative figures are shown.

4.2.3 Endothelial cells

The third cell type, which was identified as expressing Fpr2 in human atherosclerotic lesions, was the EC (Paper I). These observations prompted for the exploration of Fpr2 signaling in EC function and activation. To this end, aortic ECs were isolated from mice wild type and Fpr2$^{-/-}$ mice and examined *in vitro*. First, Fpr2$^{-/-}$ mAEC proliferated significantly slower compared with Fpr2$^{+/+}$ mAEC (Figure 8 B). Second, mRNA levels of the adhesion molecules VCAM-1 and ICAM-1 were significantly higher in Fpr2$^{-/-}$ mAEC compared with Fpr2$^{+/+}$, whereas NOS-3 mRNA levels were significantly lower (Paper IV). Since the latter enzyme is of importance for vascular relaxation, the notion that Fpr2 signaling confers better endothelial function was explored in organ bath experiments.

Endothelium-intact murine aortic rings derived from Fpr2$^{-/-}$ mice exhibited significantly decreased acetylcholine-induced relaxations compared with those derived from Fpr2$^{+/+}$ mice

(Paper IV). In contrast, no significant difference between the genotypes was observed when the endothelium were removed from the aortic rings

To finally assess the role of EC Fpr2 signaling *in vivo*, physiological angiogenesis was explored in neonatal hearts. In histological analysis, hearts derived from $Fpr2^{-/-}$ mice exhibited significantly lower vessel counts positive for α-actin (Paper IV) compared with $Fpr2^{+/+}$ mice, which corroborates the *in vitro* finding on decreased proliferation in $Fpr2^{-/-}$ mAECs (Figure 7 B).

5 Discussion

The results of the present thesis identified three important target cells for LX in atherosclerosis, based on the co-localization of FPR2/ALX with macrophages, SMC and EC in human atherosclerotic lesions. The signaling though this receptor in each of these cell types was in addition explored and revealed to be complex, with both pro- and anti-inflammatory responses being identified. *In vivo* models of atherosclerosis, intimal hyperplasia and neoangiogenesis confirmed the pivotal role of Fpr2 signaling in pathophysiological processes of disease. Finally, the results obtained indicate a potential benefit of ATL treatment in CVD.

5.1 FPR2/ALX in human atherosclerotic lesions

Morphological studies of human atherosclerotic lesions have previously demonstrated the expression of several receptors for lipid mediators on different cells types, such as for example the BLT and CysLT receptors for LTs [47, 97], Such studies may be crucial for the possibility of identifying potential targets in atherosclerosis to be moved forward from observational patient studies to mouse models and the exploration of their pathophysiological role of those receptors' signaling in the atherosclerotic process. Paper I showed that key components of atherosclerosis such as macrophages, SMC and EC expressed FPR2/ALX. This is supported by other works describing the FPR2/ALX receptor in primary macrophages [98] and fresh isolated neutrophils [90], confirming the presence of this receptor in myeloid cells. In terms of vascular structural cells, EC, more specifically HUVECs are known to respond to FPR2/ALX ligands [99] and express the receptor [72]. Finally, previous to this thesis FPR2/ALX signaling was reported only in SMCs isolated from saphenous veins [77] and Paper I was the first report on arterial SMC expressing the FPR2/ALX.

In human atherosclerotic lesions, FPR2/ALX expression was significantly higher compared with healthy vessels and exhibited a positive correlation with markers for monocytes and macrophages (Paper I). These findings were further supported by the results in Paper III showing a significant up-regulation of FPR2/ALX mRNA after pro-inflammatory stimuli. These

results suggest that the high level of FPR2/ALX mRNA levels in atherosclerotic lesion compared with healthy arteries discovered in Paper I, could be due to leukocyte recruitment and monocyte/macrophage presence in the plaque.

It is known that $CD4^+$ cells are decisive for plaque development in human atherosclerosis [100, 101] and in mouse models [102]. Based on the observation that FPR2/ALX was up-regulated in atherosclerotic lesions (Paper I), which are rich in $CD4^+$ cells, the hypothesis was raised that activated $CD4^+$ could promote FPR2/ALX expression in macrophages/monocytes. The results of Paper III indicated that activated $CD4^+$ cells released a soluble factor, which induced an up-regulation of FPR2/ALX in macrophages/monocytes, with a pronounced response at the mRNA level, but to a lesser extent, albeit significant, at the protein level. Previous studies have revealed that IFN-γ up-regulates FPR2/ALX mRNA in microglia from mice [103], in human enterocytes [104] and macrophages [105], supporting that a pro-inflammatory *milieu* induces FPR2/ALX up-regulation.

Whereas LXA_4 reduced cyto- and chemokine levels in human monocytes *in vitro*, the mRNA levels of FPR2/ALX in both atherosclerotic lesions derived from patients undergoing carotid endarterectomy, and in circulating leukocytes from a subset of these patients, were positively associated with cyto- and chemokine mRNA levels (Paper I). These findings support the notion that FPR2/ALX signaling may be associated with both pro- and anti-inflammatory signaling. Interestingly, LXA_4 concentrations decrease with age and in peripheral artery disease compared with healthy subjects [77]. These observations support the failure in the resolution of inflammation in atherosclerosis and that this possibly could be due to lack of ligand rather than lack of the receptor, and that FPR2/ALX is activated by pro-inflammatory ligands in the absence of LXA_4. Finally, high expression of FPR2/ALX mRNA in human atherosclerotic lesions was independently associated with features of more stable plaques in a multivariate analysis (Paper I).

Taken together, the observational studies of human atherosclerotic lesions and cells in the present thesis (Paper I and III) suggest that, although LXA_4 induced anti-inflammatory responses

in monocytes *in vitro*, a pro-inflammatory response appeared to dominate and be transduced through FPR2/ALX in human atherosclerotic lesions. In contrast, the results suggested that FPR2/ALX was associated with a more stable plaque phenotype.

One important limitation of the above associations using mRNA levels of FPR2/ALX is that the receptor mRNA levels may not always reflect its protein levels. This notion was initially reported in human macrophages, in which two different promoter regions were identified, which control the regulation of FPR2/ALX expression, and that one of the promoters yielded a longer mRNA, which did not translate into protein in macrophages [105]. Those findings were further extended in Paper III of the present thesis showing that the increase in FPR2/ALX protein levels after stimulation of human monocytes were less pronounced compared with what was observed for mRNA levels. In addition, an intracellular localization of FPR2/ALX was detected in human monocytes (Paper III), which also raises the question whether the intracellular receptor proteins are functional. One possibility could be that the intracellular receptor pool either translocates to the cell surface upon activation or represents a result of receptor internalization following agonist stimulation [106]. Interestingly, similar intracellular localizations have been reported for other G-protein coupled receptors, such as CysLT receptor [97] and angiotensin receptor [107], but the exact role of the intracellular FPR2/ALX in monocytes remains to be established. Other limitations to be considered for this part of the study are that control arteries were not from carotids, the cross-sectional design of the cohort making associations of mRNA levels to prognosis or outcome of patient not possible, and the stratification of stable/unstable plaques based on clinical signs rather than morphological plaque analysis.

5.2 The role of Fpr2 in murine models of atherosclerosis

To explore the causal role of FPR2/ALX in atherosclerosis, the effects of genetic deletion of the receptor, and the effects of LXA_4 was subsequently evaluated in the thesis project. To this end, mouse models representing different approaches were generated and used to define the role of the murine FPR2/ALX homologue (Fpr2) in the development of atherosclerosis. Genetic

deletion of Fpr2 reduced atherosclerosis in both $Ldlr^{-/-}$ mice fed a HFD (Paper I) and in $ApoE^{-/-}$ on a chow diet (Paper V). The initial study showed that in the development of atherosclerosis, Fpr2 deletion protected against atherosclerosis development in the initial phase of the disease, whereas no difference in terms of plaque burden were observed at later stages of disease (Paper I). This finding is in agreement with one recent study, published after our original report, that Fpr2 expression is reduced in advanced plaque compared with early stages [108]. This could be one possible explanation why later stages (after 16 and 20 weeks on HFD) exhibited no differences in terms of atherosclerosis (Paper I).

Since the mice generated are global knock-out, it was important to discriminate if the observations were due to structural cells or myeloid cells. A BMT was performed and revealed that after 20 weeks on HFD, Fpr2 promoted atherosclerosis in same fashion as in the $Ldlr^{-/-}$ x $Fpr2^{-/-}$ mice (Paper I). These finding hence confirmed that leukocytes expressing Fpr2 were determinants of plaque development.

To further extend the studies of the role of Fpr2 in atherosclerosis, another model was used, namely $ApoE^{-/-}$ mice with and without Fpr2 deletion. This study again showed that Fpr2 expressing hyperlipidemic mice presented pro-inflammatory properties with more atherosclerosis compared with mice lacking the receptor (Paper V). These results were in line with the observations in $Ldlr^{-/-}$ mice (Paper I), but in contrast, another study showed that Fpr2 conferred protection at initial stages of the disease [109].

The protection against atherosclerosis by Fpr2 deletion was not related to changes in serum lipids (Paper I and Paper V), and suggests that pro-inflammatory rather than anti-inflammatory responses are transduced through Fpr2 in atherosclerosis. Measuring serum levels of two main ligands for Fpr2 showed remarkable differences in ligand concentrations, the concentration of SAA being around 10,000 fold higher compared with LXA_4 (Paper I). These results suggest that Fpr2 transduced a pro-inflammatory response since in the atherosclerotic context the pro-inflammatory ligands are more abundant. Of note, pro-inflammatory FPR2/ALX ligands are

associated with atherosclerosis. For example such as LL-37 has been reported in human lesions [110, 111] and knock-out of the mouse homologue for LL-37 (mCRAMP) reduces atherosclerosis in ApoE$^{-/-}$ mice [112]. Besides, SAA promotes atherosclerosis *in vivo* [113] and foam cell formation *in vitro* Fpr2 dependently [114, 115].

The interpretation of our data is hence that during the development of atherosclerosis, Fpr2 signaling is mandatory and driven by the abundant pro-inflammatory ligands such as SAA. However, SAA increases as the disease develops [116], and another study using very young ApoE$^{-/-}$ mice on HFD showed that Fpr2 signaling was protective during initial atherosclerosis development [109]. The later experimental protocol (early time point and HFD in ApoE$^{-/-}$ mice), was not evaluated in the present project. Additionally, it should also be noted that the mouse used in the current thesis (Paper I and V), lacks both Fpr2 and Fpr3, whereas those used in the other study lacked only Fpr2 [109].

In summary, extensive studies of Fpr2 in mouse models of atherosclerosis in the current thesis suggest that Fpr2 is activated by pro-inflammatory ligands resulting in signaling that accelerates atherosclerosis.

5.2.1 Leukocytes

As described above, macrophages in human atherosclerotic lesions expressed FPR2/ALX (Paper I), monocyte/macrophages up-regulated FPR2/ALX expression after inflammatory stimulation, and THP-1 cells exposed to LXA_4 reduced their levels of pro-inflammatory mediators (Paper III). In mice, Fpr2 signaling was associated with increased atherosclerotic plaque formation, which was mimicked by BMT, supporting the role of Fpr2 as a pro-inflammatory receptor in atherosclerosis.

The use of cells with genetic deletion of Fpr2 offers several advantages compared with other methods for targeting Fpr2 signaling *in vitro* and *in vivo*: (1) The use of FPR2/ALX antagonists has been widely used in many studies. The results with these compounds must be interpreted with care, these antagonists have a high affinity towards FPR2, but t-Boc-FLFLF has

been reported to also binds to FPR1 [117, 118] and WRWWWW also binds to FPR3 [119] receptors. (2) siRNA might not be very efficient by the fact that the receptor is recycled after internalization upon ligand binding [120] and if internalization is inhibited apoptosis mechanisms are triggered [106]. (3) Different myeloid cells exhibit different Fpr2 expression, being highly expressed in neutrophils [121], to a lesser extent in monocytes and down-regulated as differentiation to macrophages occurs [98].

Since the observation that Fpr2 knock-out mice exhibited less atherosclerosis compared to wild-type, which was reproduced by BMT (Paper I) strongly suggested that BM cells could impact lesion, and therefore the effects of Fpr2 deletion in leukocytes were further explored.

Aortic roots of Ldlr and Fpr2 double knock-out mice exhibited a reduced amount of CD68-postive cells compared with $Ldlr^{-/-}$ x $Fpr2^{+/+}$ (Paper I). In addition, the levels of pro-inflammatory mediators were reduced in $Fpr2^{-/-}$ macrophages and those cells also exhibited lower intracellular calcium oscillations compared with wild-type macrophages (Paper I). Taken together, these results indicate that Fpr2 may be involved in both leukocyte recruitment and activation in the context of atherosclerosis, and further reinforces the major pro-inflammatory component of Fpr2 signaling in atherosclerosis.

5.2.2 SMC

In addition to leukocytes, immunohistological stainings identified FPR2/ALX expression in SMCs of human atherosclerotic lesions and Fpr2 expression in murine aortic SMCs (Paper I). Interestingly, $Fpr2^{-/-}$ SMCs exhibited a specific spontaneous phenotype *ex vivo*, with an increased proliferation (Paper II), and mRNA expression patterns indicative of reduced collagen production and maturation, and increased collagen break-down (Paper I). The latter notion was supported by measuring the total collagen in atherosclerotic lesions *in vivo*. Aortic root lesions of $Ldlr^{-/-}$ x $Fpr2^{-/-}$ contained less collagen compared with lesions in $Ldlr^{-/-}$ x $Fpr2^{+/+}$ mice (Paper I). Importantly, transplantation of $Fpr2^{-/-}$ BM to $Ldlr^{-/-}$ mice did not mimic this response, suggesting that vessel wall Fpr2 expression is pivotal for changes in atherosclerotic lesion collagen content.

Since decreased collagen content in atherosclerotic lesions is a hallmark of unstable plaques [122], these results suggest that although Fpr2 may confer increased atherosclerosis, signaling through this receptor appears to induce a more stable plaque phenotype (Paper I). Those results are indeed interesting given the observations in human disease; that Fpr2 expression increased with atherosclerosis and was inversely correlated with clinical signs of cerebral ischemia in our biobank study (Paper I). Taken together, the translational interpretation of the human observational (Paper I) and murine interventional studies of the present thesis (Paper I and V) is hence that Fpr2 signaling may be beneficial in terms of stimulating SMC to stabilize atherosclerotic lesions.

The relationship with inflammation and SMC motility has been explored for other lipid mediator receptors such as BLT_1. For example, LTB_4 increases chemotaxis that is absent in $BLT_1^{-/-}$ cells [66], and by BLT_1 antagonist, showing less SMC migration after LTB_4 challenge [47]. Therefore, we subsequently explored if ATL was able to exert its anti-inflammatory effect through Fpr2 by reducing SMC migration. Whereas one previous study described that ATL inhibited the proliferation of venous SMC in response to PDGF [77], the receptor involved in LX-induced responses in SMCs had not been explored. Using a wound assay, it was revealed that SMCs lacking Fpr2 exhibited increased migratory properties compared with wild type cells. Treatment with ATL significantly reduced SMC migration in wild-type but not in Fpr2 knock-out SMCs, hence providing the first evidence for ATL signaling by means of Fpr2 in SMCs (Paper II).

To explore the pathophysiological role of Fpr2 signaling *in vivo*, a carotid ligation model of intimal hyperplasia was established in the present thesis project. Despite the obvious SMC phenotype *in vitro*, those *in vivo* experiments did not reveal any significant differences in terms of neo-intima formation associated with genotype (Paper II). The reason for the genotype difference observed *in vitro* was not observed *in vivo* could be due to the fact that in neo-intima formation models, the migration and proliferation of SMC occur mainly at the first 2 weeks [17]

and in the present project, mice were sacrificed 4 weeks after carotid ligation. It should also be mentioned that compensatory mechanisms for the lack of Fpr2 receptor in this model could not be excluded.

In contrast to the lack of a spontaneous phenotype, wild-type and Fpr2 knock-out mice exhibited an important difference in the response to ATL treatment. Whereas ATL reduced intimal hyperplasia after carotid ligation in wild-type mice, this Fpr2 ligand was ineffective in altering the response to ligation in Fpr2 knock-out mice (Paper II). These observations may have therapeutic implications, which will be discussed below, and also provide compelling evidence of ATL signaling by means of Fpr2 in the response to vascular injury.

In summary, the present thesis identified FPR2/ALX expression on SMCs in human atherosclerotic lesions, and revealed that a major role of ATL signaling through Fpr2 in SMC migration *in vitro* and intimal hyperplasia formation *in vivo*. In addition, Fpr2 was associated with pathways of increased collagen production and maturation, and decreased collagen break-down, which may drive the atherosclerotic lesions towards a more stable phenotype as suggested from both human observational studies (Paper I) and murine atherosclerosis models (Paper I and V).

5.2.3 Endothelial cells

EC represented the third FPR2/ALX expressing cell type identified in human atherosclerotic lesions in the present thesis (Paper I). In the initiation of the atherosclerotic lesion EC recruit leukocytes to the vascular wall and regulate vascular tone. Early phases of atherosclerosis are characterized by an endothelial dysfunction, which can be detected as reduced endothelium-dependent vascular relaxations both *in vivo* and *in vitro* [29, 123, 124]. Organ bath experiments revealed that Fpr2 knock-out mice exhibited reduced endothelium-dependent relations in response to ACh (Paper IV), suggesting that these mice have an endothelial dysfunction. Although previous reports that have shown vasoactive effects of potential FPR2/ALX agonists [125, 126], the receptor involved in the responses were not explored in those

studies. The implications for these findings *in vivo* has also been supported by studies in rats, in which intra-venous LXA_4 causes a reduction of blood pressure in short term (~4 min), while blocking the LXA_4 production or adding FPR2/ALX antagonists abolished this effect [125]. In support of a major role for Fpr2 in regulating endothelial function, $Fpr2^{-/-}$ EC exhibited lower levels of mRNA of NOS-3, the endothelial type of nitric oxide synthase (Paper IV).

Since the focus of this thesis is atherosclerosis, which is an arterial disease, the task to isolate aortic EC from mice was taken. It was crucial to isolate EC from mouse aorta based on that other sources of EC such as lung or kidney, very rich in capillaries, would have a contamination of venous EC. Once primary murine aortic EC cultures were established, two experiments were performed; (1) mRNA isolation and (2) evaluation of EC phenotype (Paper IV).

For ECs to be able to recruit leukocytes, the expression of cellular adhesion molecules are important. In Paper IV, VCAM-1 and ICAM-1 were increased in $Fpr2^{-/-}$ EC, suggesting that Fpr2 deletion promoted endothelial activation. These results are hence in sharp contrast to the observations of a lower inflammatory state in $Fpr2^{-/-}$ macrophages (Paper I, *cf. supra*), and support that EC Fpr2 signaling would potentially reduce cell recruitment towards the inflammation site. Interestingly, previous studies have shown that Fpr2-induced endothelial adhesion was dependent on an integrin conformational change on leukocytes, which allowed monocyte integrin binding to EC VCAM-1 and ICAM-1 [109]. The higher levels of ICAM-1 and VCAM-1 in ECs derived from $Fpr2^{-/-}$ mice compared with those derived from wild-type mice (Paper IV) point to differential effects of Fpr2 signaling on monocytes and EC. This suggests that the same receptor may promote inflammation by leukocyte recruitment and activation, by means of direct effects on monocyte integrins, whereas Fpr2 activation on EC may limit inflammation by down-regulation of adhesion molecules. By this way, reduced recruitment of inflammatory cells to a site of high Fpr2 agonist concentrations. These results are in agreement with previous results with the same mice, in which intra-vital microscopy after 30 min of ischemia showed that

$Fpr2^{-/-}$ recruited more cells after reperfusion suggesting higher endothelial activation compared to $Fpr2^{+/+}$ mice [86, 94]. However, further studies are needed to determine the relative role of different Fpr2 expression cell types in different inflammatory conditions.

The enhanced EC activation after genetic targeting of Fpr2 in Paper IV is in line with the studies of SMC (Paper I and Paper II), in which Fpr2 signaling reduced SMC migration and proliferation *in vitro*, and conferred protective effects on the development of intimal hyperplasia in response to carotid ligation *in vivo*. Taken together, whereas Fpr2 may mediate both pro- and anti-inflammation in leukocytes, Fpr2 signaling may be predominantly anti-inflammatory in structural cells of the vascular wall, *i.e.* SMCs and ECs (Paper II and Paper IV).

As a subsequent step, EC proliferation was evaluated and showed to be impaired in Fpr2 knock-out EC. There are however some important methodological considerations to take into account: (1) The time from sacrifice until the final yield of the cells (around 6 h) and the incubation with collagenases may put EC in the process of de-differentiation and the cells might not express the same levels of EC markers as in their original state. (2) EC were studied in culture dishes without shear stress, which may alter EC responses [29] and it cannot be excluded that Fpr2 expression/signaling could be flow dependent and that important information may be missed under static conditions.

One crucial observation of the present work is the different proliferation profile observed by Fpr2 signaling in EC (Paper IV) compared to SMC (Paper II). Differential effects on SMC and EC have been described as LTB_4 promotes SMC proliferation [47] whereas induces EC apoptosis [127] for example. No previous reports have explored specifically the Fpr2 signaling on EC proliferation, but LXA_4 reduced VEGF-induced EC proliferation and migration [72, 99], supporting that LXA_4-Fpr2 signaling would modulate EC proliferation.

Finally it was sought if EC Fpr2 could play a physiological role during angiogenesis. It is known that from birth until the 3-4 week, mice gain at least 3-4 times their weight. This will demand that EC would proliferate in higher rate, and generate more vessels to compensate such

growth [128]. The quantification of vessels in 3 weeks old mice is an established model of neoangiogenesis [128, 129], which was established to study the role of Fpr2 signaling in angiogenesis. Interestingly, the number of vessels was reduced in $Fpr2^{-/-}$ mice compared with $Fpr2^{+/+}$ mice. These *in vivo* results confirmed the observation *in vitro* that EC lacking Fpr2 exhibited a lower proliferation. This lower amount of neonatal cardiac vessel number did not induce any apparent changes in development, since no differences in terms of size or weight of the mice were associated with the genotype (Papers I, II and IV; and [86]).

Taken together, this project discovered the presence of the FPR2/ALX receptor in EC in human atherosclerotic plaques (Paper I). Additionally, Paper IV revealed that lacking Fpr2 signaling in EC was associated with impairment of proliferation, endothelium dysfunction and a decrease in the vascular bed in neonatal hearts (Paper IV).

5.3 ATL as a possible therapeutic option for CVD

Given that LXA_4 and its analogs are efficient as anti-inflammatory and pro-resolution agents, these properties were challenged in this thesis *in vitro* and *in vivo*. First, THP-1 cells exposed to LXA_4 exhibited reduced levels of pro-inflammatory mediators such as IL-8, IL-1β, CXCL-16 and MMP-9 (Paper III). The present thesis in addition showed that vascular structural cells are able to express FPR2/ALX (Paper I) and respond to ATL (Paper II and V). The beneficial effects observed when ATL was administered *in vitro* and *in vivo* raise the notion of ATL as a possible treatment for atherosclerosis and its complications.

To further support the concept of using ATL to reduce inflammation, the atherosclerosis model with $ApoE^{-/-}$ mice expressing and lacking the Fpr2 receptor were treated with ATL. The results supported that ATL is able to retard plaque development only in Fpr2 expressing mice, along with less macrophages infiltrated to the plaque and lower levels of IL-6 and MMP-13 in the aorta, spleen and lung of ATL-treated mice (Paper V). These results are in accordance with other studies, which explored Fpr2 signaling with different anti-inflammatory/pro-resolving ligands such as Annexin A1. For instance, nanoparticles conjugated with a fragment (Ac2-26) of

Annexin A1 were able to increase collagen and reduce atherosclerosis plaque size [108]. Additionally, Annexin A1 treatment reduced atherosclerosis in $Ldlr^{-/-}$ mice [130]. Interestingly, the treatment with this FPR2/ALX ligand induced no effect after 6 weeks of treatment along with HFD, whereas the treatment was effective to reduce plaque size without SMC and macrophages changes after 12 weeks of HFD [130]. The therapeutic effect of Fpr2 signaling by pro-resolution ligands might have an important role in structural cells. Finally, recent evidence support that LXA_4-Fpr2 signaling is crucial in reduce leukocyte recruitment in a stroke model [131]. Taken together with those previous studies, the results of the present thesis indicate that Fpr2 activation by pro-inflammatory ligands may drive the atherosclerosis process but that the treatment with anti-inflammatory and pro-resolution Fpr2 ligands may overcome these pro-inflammatory properties and exert beneficial effects. These observations open up for ATL as a therapeutic option to reduce CVD.

Vascular injury, such as percutaneous coronary interventions, may lead to intimal hyperplasia and restenosis of the vessel. Previous experimental studies have suggested therapeutic options of inhibiting pro-inflammatory lipid mediator signaling to prevent intimal hyperplasia [47, 67]. The results of Paper II were the first to show that ATL reduced intimal hyperplasia *in vivo*, and that the effects of ATL were abolished in Fpr2 knock-out mice. Subsequently, similar results were reported for other pro-resolution lipid mediators [132]. The beneficial effects of ATL treatment on intimal hyperplasia could potentially be mediated both through direct effects on SMC proliferation and migration (Paper II), but it cannot be excluded that also reduced leukocyte recruitment (Paper V) and activation (Paper III) were involved in this response. Importantly, the *in vitro* studies presented in this thesis indicated that Fpr2 deletion increased EC proliferation (Paper IV). A decreased SMC proliferation and increased EC proliferation would enhance the therapeutic effects of ATL treatment in vascular injury, both inhibiting intimal hyperplasia and favoring re-endotheliazation after for example coronary angioplasty with stent implantation.

6 Conclusions

1. In human atherosclerotic lesions, FPR2/ALX is highly expressed and is present on the main components of the plaque, such as macrophages, SMC and EC.
2. Fpr2 signaling promotes atherosclerosis development, as demonstrated in 3 different mouse models.
3. In mouse macrophages, Fpr2 signaling induces a higher inflammatory phenotype.
4. In mSMC, the lack of Fpr2 signaling induces a higher proliferation and migratory state.
5. The mSMC migration is reduced by ATL treatment and is Fpr2 dependent.
6. In mAEC, the deficiency of Fpr2 signaling shows features of endothelial dysfunction and lower proliferation rate.
7. LXA_4 acts as anti-inflammatory mediator in THP-1 cells *in vitro*.
8. The anti-inflammatory effect of ATL is Fpr2 dependent.
9. ATL may be a therapeutic option by reducing atherosclerotic lesions and intima formation in vascular injury.

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8 References

1. Chaabane, C., M. Coen, and M.L. Bochaton-Piallat, *Smooth muscle cell phenotypic switch: implications for foam cell formation.* Curr Opin Lipidol, 2014. **25**(5): p. 374-9.
2. Pinsky, J.L., et al., *The Framingham Disability Study: relationship of various coronary heart disease manifestations to disability in older persons living in the community.* Am J Public Health, 1990. **80**(11): p. 1363-7.
3. Nordestgaard, B.G., R. Wootton, and B. Lewis, *Selective retention of VLDL, IDL, and LDL in the arterial intima of genetically hyperlipidemic rabbits in vivo. Molecular size as a determinant of fractional loss from the intima-inner media.* Arterioscler Thromb Vasc Biol, 1995. **15**(4): p. 534-42.
4. Yurdagul, A., Jr., et al., *alpha5beta1 integrin signaling mediates oxidized low-density lipoprotein-induced inflammation and early atherosclerosis.* Arterioscler Thromb Vasc Biol, 2014. **34**(7): p. 1362-73.
5. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.
6. Libby, P., M. Nahrendorf, and F.K. Swirski, *Monocyte heterogeneity in cardiovascular disease.* Semin Immunopathol, 2013. **35**(5): p. 553-62.
7. Doring, Y., et al., *Neutrophils in Atherosclerosis: From Mice to Man.* Arterioscler Thromb Vasc Biol, 2014.
8. Christ, A., et al., *Dendritic cells in cardiovascular diseases: epiphenomenon, contributor, or therapeutic opportunity.* Circulation, 2013. **128**(24): p. 2603-13.
9. Ammirati, E., et al., *The Role of T and B Cells in Human Atherosclerosis and Atherothrombosis.* Clin Exp Immunol, 2014.
10. Wojdasiewicz, P., L.A. Poniatowski, and D. Szukiewicz, *The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis.* Mediators Inflamm, 2014. **2014**: p. 561459.
11. Schuett, H., et al., *How much is too much? Interleukin-6 and its signalling in atherosclerosis.* Thromb Haemost, 2009. **102**(2): p. 215-22.
12. Bäck, M., et al., *Update on leukotriene, lipoxin and oxoeicosanoid receptors: IUPHAR Review 7.* Br J Pharmacol, 2014. **171**(15): p. 3551-74.
13. Serhan, C.N., *Pro-resolving lipid mediators are leads for resolution physiology.* Nature, 2014. **510**(7503): p. 92-101.
14. Viola, J. and O. Soehnlein, *Atherosclerosis - A matter of unresolved inflammation.* Semin Immunol, 2015. **27**(3): p. 184-93.
15. Chiu, J.J. and S. Chien, *Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives.* Physiol Rev, 2011. **91**(1): p. 327-87.
16. Myit, S., et al., *Different growth properties of neointimal and medial smooth muscle cells in response to growth factors.* J Vasc Res, 2003. **40**(2): p. 97-104.
17. Kumar, A. and V. Lindner, *Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow.* Arterioscler Thromb Vasc Biol, 1997. **17**(10): p. 2238-44.
18. Guzik, B., et al., *Mechanisms of increased vascular superoxide production in human varicose veins.* Pol Arch Med Wewn, 2011. **121**(9): p. 279-86.
19. Bochaton-Piallat, M.L. and G. Gabbiani, *Smooth muscle cell: a key cell for plaque vulnerability regulation?* Circ Res, 2006. **98**(4): p. 448-9.
20. Thorp, E., et al., *Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesions of apoe-/- mice.* Arterioscler Thromb Vasc Biol, 2008. **28**(8): p. 1421-8.
21. Desmouliere, A., et al., *Apoptosis during wound healing, fibrocontractive diseases and vascular wall injury.* Int J Biochem Cell Biol, 1997. **29**(1): p. 19-30.

22. Van Vre, E.A., et al., *Apoptotic cell death and efferocytosis in atherosclerosis.* Arterioscler Thromb Vasc Biol, 2012. **32**(4): p. 887-93.
23. Back, M., D.F. Ketelhuth, and S. Agewall, *Matrix metalloproteinases in atherothrombosis.* Prog Cardiovasc Dis, 2010. **52**(5): p. 410-28.
24. Quillard, T., et al., *Matrix metalloproteinase-13 predominates over matrix metalloproteinase-8 as the functional interstitial collagenase in mouse atheromata.* Arterioscler Thromb Vasc Biol, 2014. **34**(6): p. 1179-86.
25. Michel, J.B., et al., *Intraplaque haemorrhages as the trigger of plaque vulnerability.* Eur Heart J, 2011. **32**(16): p. 1977-85, 1985a, 1985b, 1985c.
26. van der Wal, A.C. and A.E. Becker, *Atherosclerotic plaque rupture--pathologic basis of plaque stability and instability.* Cardiovasc Res, 1999. **41**(2): p. 334-44.
27. Shore, A.C., et al., *Measures of atherosclerotic burden are associated with clinically manifest cardiovascular disease in type 2 diabetes: a European cross-sectional study.* J Intern Med, 2015. **278**(3): p. 291-302.
28. Naik, V., R.S. Gamad, and P.P. Bansod, *Carotid artery segmentation in ultrasound images and measurement of intima-media thickness.* Biomed Res Int, 2013. **2013**: p. 801962.
29. Kwak, B.R., et al., *Biomechanical factors in atherosclerosis: mechanisms and clinical implications.* Eur Heart J, 2014. **35**(43): p. 3013-20, 3020a-3020d.
30. Back, M. and G.K. Hansson, *Anti-inflammatory therapies for atherosclerosis.* Nat Rev Cardiol, 2015. **12**(4): p. 199-211.
31. Silva, M.V., et al., *Platelet antiaggregants in primary and secondary prevention of atherothrombotic events.* Arq Bras Cardiol, 2013. **100**(6): p. e78-84.
32. Estevez-Loureiro, R., et al., *Timely reperfusion for ST-segment elevation myocardial infarction: Effect of direct transfer to primary angioplasty on time delays and clinical outcomes.* World J Cardiol, 2014. **6**(6): p. 424-33.
33. Thyberg, J., et al., *Phenotypic modulation of smooth muscle cells during the formation of neointimal thickenings in the rat carotid artery after balloon injury: an electron-microscopic and stereological study.* Cell Tissue Res, 1995. **281**(3): p. 421-33.
34. Simard, T., et al., *The evolution of coronary stents: a brief review.* Can J Cardiol, 2014. **30**(1): p. 35-45.
35. Holmes, D.R., Jr., et al., *Analysis of 1-year clinical outcomes in the SIRIUS trial: a randomized trial of a sirolimus-eluting stent versus a standard stent in patients at high risk for coronary restenosis.* Circulation, 2004. **109**(5): p. 634-40.
36. Patel, M.J., et al., *Current status and future prospects of drug eluting stents for restenosis.* Acta Pharm, 2012. **62**(4): p. 473-96.
37. Yla-Herttuala, S., et al., *Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein.* J Clin Invest, 1995. **95**(6): p. 2692-8.
38. Capra, V., et al., *Impact of vascular thromboxane prostanoid receptor activation on hemostasis, thrombosis, oxidative stress, and inflammation.* J Thromb Haemost, 2014. **12**(2): p. 126-37.
39. Samuelsson, B., et al., *Leukotrienes and lipoxins: structures, biosynthesis, and biological effects.* Science, 1987. **237**(4819): p. 1171-1176.
40. Dahlen, S.E., et al., *Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response.* Proc Natl Acad Sci U S A, 1981. **78**(6): p. 3887-91.
41. Secrest, R.J. and B.M. Chapnick, *Endothelial-dependent relaxation induced by leukotrienes C4, D4, and E4 in isolated canine arteries.* Circ Res, 1988. **62**(5): p. 983-91.
42. Back, M., et al., *Endothelium-dependent vascular responses induced by leukotriene B4.* Prostaglandins Other Lipid Mediat, 2007. **83**(3): p. 209-12.

43. Peters-Golden, M. and W.R. Henderson, Jr., *Leukotrienes.* N Engl J Med, 2007. **357**(18): p. 1841-54.
44. Serhan, C.N. and K.A. Sheppard, *Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro.* J Clin Invest, 1990. **85**(3): p. 772-80.
45. Bosma-den Boer, M.M., M.L. van Wetten, and L. Pruimboom, *Chronic inflammatory diseases are stimulated by current lifestyle: how diet, stress levels and medication prevent our body from recovering.* Nutr Metab (Lond), 2012. **9**(1): p. 32.
46. Dwyer, J.H., et al., *Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis.* N Engl J Med, 2004. **350**(1): p. 29-37.
47. Bäck, M., et al., *Leukotriene B4 signaling through NF-kappaB-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia.* Proc Natl Acad Sci U S A, 2005. **102**(48): p. 17501-6.
48. van den Borne, P., et al., *Leukotriene B4 levels in human atherosclerotic plaques and abdominal aortic aneurysms.* PLoS One, 2014. **9**(1): p. e86522.
49. Mehrabian, M., et al., *Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice.* Circ Res, 2002. **91**(2): p. 120-6.
50. Zhao, L., et al., *The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm.* Nat Med, 2004. **10**(9): p. 966-73.
51. Cao, R.Y., et al., *Genetic and pharmacological inhibition of the 5-lipoxygenase/leukotriene pathway in atherosclerotic lesion development in ApoE deficient mice.* Atherosclerosis, 2009. **203**(2): p. 395-400.
52. Titos, E., et al., *Inhibition of 5-lipoxygenase-activating protein abrogates experimental liver injury: role of Kupffer cells.* J Leukoc Biol, 2005. **78**(4): p. 871-8.
53. Jawien, J., et al., *Inhibition of five lipoxygenase activating protein (FLAP) by MK-886 decreases atherosclerosis in apoE/LDLR-double knockout mice.* Eur J Clin Invest, 2006. **36**(3): p. 141-6.
54. Back, M., et al., *5-Lipoxygenase-activating protein: a potential link between innate and adaptive immunity in atherosclerosis and adipose tissue inflammation.* Circ Res, 2007. **100**(7): p. 946-9.
55. Fredman, G., et al., *Resolvin D1 limits 5-lipoxygenase nuclear localization and leukotriene B4 synthesis by inhibiting a calcium-activated kinase pathway.* Proc Natl Acad Sci U S A, 2014. **111**(40): p. 14530-5.
56. Lehmann, C., et al., *Lipoxin and resolvin biosynthesis is dependent on 5-lipoxygenase activating protein.* FASEB J, 2015.
57. Kronke, G., et al., *12/15-lipoxygenase counteracts inflammation and tissue damage in arthritis.* J Immunol, 2009. **183**(5): p. 3383-9.
58. Harats, D., et al., *Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice.* Arterioscler Thromb Vasc Biol, 2000. **20**(9): p. 2100-5.
59. Merched, A.J., et al., *Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators.* FASEB J, 2008. **22**(10): p. 3595-606.
60. Shen, J., et al., *Macrophage-mediated 15-lipoxygenase expression protects against atherosclerosis development.* J Clin Invest, 1996. **98**(10): p. 2201-8.
61. Cyrus, T., et al., *Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice.* J Clin Invest, 1999. **103**(11): p. 1597-604.
62. George, J., et al., *12/15-Lipoxygenase gene disruption attenuates atherogenesis in LDL receptor-deficient mice.* Circulation, 2001. **104**(14): p. 1646-50.
63. Zhao, L., et al., *Selective interleukin-12 synthesis defect in 12/15-lipoxygenase-deficient macrophages associated with reduced atherosclerosis in a mouse model of familial hypercholesterolemia.* J Biol Chem, 2002. **277**(38): p. 35350-6.

64. Zhao, L., et al., *12/15-Lipoxygenase gene disruption and vitamin E administration diminish atherosclerosis and oxidative stress in apolipoprotein E deficient mice through a final common pathway.* Prostaglandins Other Lipid Mediat, 2005. **78**(1-4): p. 185-93.
65. Poeckel, D., et al., *Dual 12/15- and 5-lipoxygenase deficiency in macrophages alters arachidonic acid metabolism and attenuates peritonitis and atherosclerosis in ApoE knock-out mice.* J Biol Chem, 2009. **284**(31): p. 21077-89.
66. Heller, E.A., et al., *Inhibition of atherogenesis in BLT1-deficient mice reveals a role for LTB4 and BLT1 in smooth muscle cell recruitment.* Circulation, 2005. **112**(4): p. 578-86.
67. Hlawaty, H., et al., *Leukotriene receptor antagonism and the prevention of extracellular matrix degradation during atherosclerosis and in-stent stenosis.* Arterioscler Thromb Vasc Biol, 2009. **29**(4): p. 518-24.
68. Decker, Y., G. McBean, and C. Godson, *Lipoxin A4 inhibits IL-1beta-induced IL-8 and ICAM-1 expression in 1321N1 human astrocytoma cells.* Am J Physiol Cell Physiol, 2009. **296**(6): p. C1420-7.
69. Wu, J., et al., *Lipoxin A4 inhibits the production of proinflammatory cytokines induced by beta-amyloid in vitro and in vivo.* Biochem Biophys Res Commun, 2011. **408**(3): p. 382-7.
70. He, R., H. Sang, and R.D. Ye, *Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R.* Blood, 2003. **101**(4): p. 1572-81.
71. Xu, Z., et al., *Lipoxin A4 inhibits the development of endometriosis in mice: the role of anti-inflammation and anti-angiogenesis.* Am J Reprod Immunol, 2012. **67**(6): p. 491-7.
72. Baker, N., et al., *Lipoxin A4: anti-inflammatory and anti-angiogenic impact on endothelial cells.* J Immunol, 2009. **182**(6): p. 3819-26.
73. Fierro, I.M., J.L. Kutok, and C.N. Serhan, *Novel lipid mediator regulators of endothelial cell proliferation and migration: aspirin-triggered-15R-lipoxin A(4) and lipoxin A(4).* J Pharmacol Exp Ther, 2002. **300**(2): p. 385-92.
74. Brezinski, M.E., et al., *Lipoxins stimulate prostacyclin generation by human endothelial cells.* FEBS Lett, 1989. **245**(1-2): p. 167-72.
75. Paul-Clark, M.J., et al., *15-epi-lipoxin A4-mediated induction of nitric oxide explains how aspirin inhibits acute inflammation.* J Exp Med, 2004. **200**(1): p. 69-78.
76. Guzik, T.J., R. Korbut, and T. Adamek-Guzik, *Nitric oxide and superoxide in inflammation and immune regulation.* J Physiol Pharmacol, 2003. **54**(4): p. 469-87.
77. Ho, K.J., et al., *Aspirin-triggered lipoxin and resolvin E1 modulate vascular smooth muscle phenotype and correlate with peripheral atherosclerosis.* Am J Pathol, 2010. **177**(4): p. 2116-23.
78. Ye, R.D., et al., *International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family.* Pharmacol Rev, 2009. **61**(2): p. 119-61.
79. Hayhoe, R.P., et al., *Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: indication of distinct receptor involvement.* Blood, 2006. **107**(5): p. 2123-30.
80. Chiang, N., et al., *The lipoxin receptor ALX: potent ligand-specific and stereoselective actions in vivo.* Pharmacol Rev, 2006. **58**(3): p. 463-87.
81. Krishnamoorthy, S., et al., *Resolvin D1 receptor stereoselectivity and regulation of inflammation and proresolving microRNAs.* Am J Pathol, 2012. **180**(5): p. 2018-27.
82. Le, Y., et al., *Identification of functional domains in the formyl peptide receptor-like 1 for agonist-induced cell chemotaxis.* FEBS J, 2005. **272**(3): p. 769-78.
83. Bozinovski, S., D. Anthony, and R. Vlahos, *Targeting pro-resolution pathways to combat chronic inflammation in COPD.* J Thorac Dis, 2014. **6**(11): p. 1548-56.
84. Cattaneo, F., M. Parisi, and R. Ammendola, *Distinct signaling cascades elicited by different formyl peptide receptor 2 (FPR2) agonists.* Int J Mol Sci, 2013. **14**(4): p. 7193-230.

85. Fiore, S., et al., *Lipoxin recognition sites. Specific binding of labeled lipoxin A4 with human neutrophils.* J Biol Chem, 1992. **267**(23): p. 16168-76.
86. Dufton, N., et al., *Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation.* J Immunol, 2010. **184**(5): p. 2611-9.
87. Dufton, N., et al., *Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation.* Journal of immunology, 2010. **184**(5): p. 2611-9.
88. Chen, K., et al., *A critical role for the g protein-coupled receptor mFPR2 in airway inflammation and immune responses.* J Immunol, 2010. **184**(7): p. 3331-5.
89. Chen, K., et al., *Formylpeptide receptor-2 contributes to colonic epithelial homeostasis, inflammation, and tumorigenesis.* J Clin Invest, 2013. **123**(4): p. 1694-704.
90. Simiele, F., et al., *Transcriptional regulation of the human FPR2/ALX gene: evidence of a heritable genetic variant that impairs promoter activity.* FASEB J, 2012. **26**(3): p. 1323-33.
91. Waechter, V., et al., *No evidence for the involvement of the lipoxin A4 receptor (FPR2/ALX) gene in the susceptibility to coronary artery disease.* Clin Chem Lab Med, 2012. **50**(1): p. 177-9.
92. Miksa, M., et al., *A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester.* J Immunol Methods, 2009. **342**(1-2): p. 71-7.
93. Boels, P.J., et al., *Maturation of the response to bradykinin in resistance and conduit pulmonary arteries.* Cardiovasc Res, 1999. **44**(2): p. 416-28.
94. Brancaleone, V., et al., *A vasculo-protective circuit centered on lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 operative in murine microcirculation.* Blood, 2013. **122**(4): p. 608-17.
95. Nicoletti, A., et al., *Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice.* J Clin Invest, 1998. **102**(5): p. 910-8.
96. Kumar, A., et al., *Remodeling and neointimal formation in the carotid artery of normal and P-selectin-deficient mice.* Circulation, 1997. **96**(12): p. 4333-42.
97. Eaton, A., et al., *Cysteinyl leukotriene signaling through perinuclear CysLT(1) receptors on vascular smooth muscle cells transduces nuclear calcium signaling and alterations of gene expression.* J Mol Med (Berl), 2012. **90**(10): p. 1223-31.
98. Waechter, V., et al., *Characterization of the promoter and the transcriptional regulation of the lipoxin A4 receptor (FPR2/ALX) gene in human monocytes and macrophages.* J Immunol, 2012. **188**(4): p. 1856-67.
99. Cezar-de-Mello, P.F., et al., *ATL-1, an analogue of aspirin-triggered lipoxin A4, is a potent inhibitor of several steps in angiogenesis induced by vascular endothelial growth factor.* Br J Pharmacol, 2008. **153**(5): p. 956-65.
100. Guzik, T.J., et al., *Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction.* J Exp Med, 2007. **204**(10): p. 2449-60.
101. Hermansson, A., et al., *Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis.* J Exp Med, 2010. **207**(5): p. 1081-93.
102. Zhou, X., et al., *Adoptive transfer of CD4+ T cells reactive to modified low-density lipoprotein aggravates atherosclerosis.* Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 864-70.
103. Chen, K., et al., *Induction of the formyl peptide receptor 2 in microglia by IFN-gamma and synergy with CD40 ligand.* Journal of immunology, 2007. **178**(3): p. 1759-66.
104. Gronert, K., et al., *Identification of a human enterocyte lipoxin A4 receptor that is regulated by interleukin (IL)-13 and interferon gamma and inhibits tumor necrosis factor alpha-induced IL-8 release.* J Exp Med, 1998. **187**(8): p. 1285-94.

105. Waechter, V., et al., *Characterization of the promoter and the transcriptional regulation of the lipoxin A4 receptor (FPR2/ALX) gene in human monocytes and macrophages.* Journal of immunology, 2012. **188**(4): p. 1856-67.
106. Thompson, D., et al., *Identification of a Novel Recycling Sequence in the C-tail of FPR2/ALX Receptor: ASSOCIATION WITH CELL PROTECTION FROM APOPTOSIS.* J Biol Chem, 2014. **289**(52): p. 36166-78.
107. Tadevosyan, A., et al., *Photoreleasable ligands to study intracrine angiotensin II signalling.* J Physiol, 2015. **593**(3): p. 521-39.
108. Fredman, G., et al., *Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice.* Sci Transl Med, 2015. **7**(275): p. 275ra20.
109. Drechsler, M., et al., *Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment.* Circ Res, 2015. **116**(5): p. 827-35.
110. Ciornei, C.D., et al., *Human antimicrobial peptide LL-37 is present in atherosclerotic plaques and induces death of vascular smooth muscle cells: a laboratory study.* BMC Cardiovasc Disord, 2006. **6**: p. 49.
111. Edfeldt, K., et al., *Involvement of the antimicrobial peptide LL-37 in human atherosclerosis.* Arterioscler Thromb Vasc Biol, 2006. **26**(7): p. 1551-7.
112. Doring, Y., et al., *Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice.* Circ Res, 2012. **110**(8): p. 1052-6.
113. Krishack, P.A., et al., *Serum Amyloid A Facilitates Early Lesion Development in Ldlr-/- Mice.* J Am Heart Assoc, 2015. **4**(7).
114. Lee, H.Y., et al., *Role of formyl peptide receptor 2 on the serum amyloid A-induced macrophage foam cell formation.* Biochem Biophys Res Commun, 2013. **433**(2): p. 255-9.
115. Lee, H.Y., et al., *Oxidized low-density lipoprotein-induced foam cell formation is mediated by formyl peptide receptor 2.* Biochem Biophys Res Commun, 2014. **443**(3): p. 1003-7.
116. Johnson, B.D., et al., *Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE).* Circulation, 2004. **109**(6): p. 726-32.
117. Wenzel-Seifert, K., L. Grunbaum, and R. Seifert, *Differential inhibition of human neutrophil activation by cyclosporins A, D, and H. Cyclosporin H is a potent and effective inhibitor of formyl peptide-induced superoxide formation.* J Immunol, 1991. **147**(6): p. 1940-6.
118. Wenzel-Seifert, K. and R. Seifert, *Cyclosporin H is a potent and selective formyl peptide receptor antagonist. Comparison with N-t-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L- leucyl-L-phenylalanine and cyclosporins A, B, C, D, and E.* J Immunol, 1993. **150**(10): p. 4591-9.
119. Shin, E.H., et al., *Trp-Arg-Trp-Trp-Trp-Trp antagonizes formyl peptide receptor like 2-mediated signaling.* Biochem Biophys Res Commun, 2006. **341**(4): p. 1317-22.
120. Maderna, P., et al., *FPR2/ALX receptor expression and internalization are critical for lipoxin A4 and annexin-derived peptide-stimulated phagocytosis.* FASEB J, 2010. **24**(11): p. 4240-9.
121. Liu, M., et al., *Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes.* Sci Rep, 2012. **2**: p. 786.
122. Hansson, G.K., P. Libby, and I. Tabas, *Inflammation and plaque vulnerability.* J Intern Med, 2015.
123. Rafnsson, A. and M. Bäck, *Urinary leukotriene E4 is associated with renal function but not with endothelial function in type 2 diabetes.* Dis Markers, 2013. **35**(5): p. 475-80.

124. Dodd-o, J.M., et al., *Endothelium-independent relaxation of aortic rings by the nitric oxide synthase inhibitor diphenyleneiodonium.* Br J Pharmacol, 1997. **120**(5): p. 857-64.
125. von der Weid, P.Y., et al., *Aspirin-triggered, cyclooxygenase-2-dependent lipoxin synthesis modulates vascular tone.* Circulation, 2004. **110**(10): p. 1320-5.
126. Berkestedt, I., A. Nelson, and M. Bodelsson, *Endogenous antimicrobial peptide LL-37 induces human vasodilatation.* Br J Anaesth, 2008. **100**(6): p. 803-9.
127. Tian, W., et al., *Blocking macrophage leukotriene b4 prevents endothelial injury and reverses pulmonary hypertension.* Sci Transl Med, 2013. **5**(200): p. 200ra117.
128. Tian, X., et al., *Vessel formation. De novo formation of a distinct coronary vascular population in neonatal heart.* Science, 2014. **345**(6192): p. 90-4.
129. Tomanek, R.J., et al., *Vascular endothelial growth factor and basic fibroblast growth factor differentially modulate early postnatal coronary angiogenesis.* Circ Res, 2001. **88**(11): p. 1135-41.
130. Kusters, D.H., et al., *Pharmacological Treatment with Annexin A1 Reduces Atherosclerotic Plaque Burden in LDLR-/- Mice on Western Type Diet.* PLoS One, 2015. **10**(6): p. e0130484.
131. Smith, H.K., et al., *Targeting formyl peptide receptor 2 reduces leukocyte-endothelial interactions in a murine model of stroke.* FASEB J, 2015.
132. Akagi, D., et al., *Systemic delivery of proresolving lipid mediators resolvin D2 and maresin 1 attenuates intimal hyperplasia in mice.* FASEB J, 2015.