The role of FPR2/ALX in the vascular wall

Marcelo Heron Petri
THE ROLE OF FPR2/ALX IN THE VASCULAR WALL

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet
offentligen försvaras i föreläsningssalen CMM, L8:00
Fredagen den 16:e oktober 2015, Kl. 09:00

Av

Marcelo Heron Petri

Huvudhandledare:
Docent Magnus Bäck
Karolinska Institutet
Institutionen för medicin, Solna

Bihandledare:
Andres Laguna-Fernandez, PhD
Karolinska Institutet
Institutionen för medicin, Solna

Fakultetsopponent:
Professor Tomasz Guzik
University of Glasgow
Institute of Cardiovascular and Medical Sciences

Betygsämnd:
Docent Isabel Goncalves
Lunds Universitet, Malmö
Experimentell kardiovaskulär forskning

Professor Hans-Ekik Claesson
Karolinska Institutet
Institutionen för medicin, Solna

Docent Huamei Forsman
Göteborgs Universitet
Reumatologi och inflammationsforskning

Stockholm 2015
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 A₄ (LXA₄) 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 LXA₄ 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 LXA₄ were examined with 2 goals: to assess the use of LXA₄ as possible therapeutic option in mouse models of atherosclerosis, and to elucidate if LXA₄ 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 LXA₄ (ATL, a LXA₄ 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 LXA₄. 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. LXA₄ is signaling through FPR2/ALX and this supports the use of LXA₄ as a therapeutic option in atherosclerosis as well as restenosis.
THE ROLE OF FPR2/ALX IN THE VASCULAR WALL

Marcelo Heron Petri

Stockholm 2015
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 A₄ (LXA₄) 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 LXA₄ 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 LXA₄ were examined with 2 goals: to assess the use of LXA₄ as possible therapeutic option in mouse models of atherosclerosis, and to elucidate if LXA₄ 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 LXA₄ (ATL, a LXA₄ 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 LXA₄. 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. LXA₄ is signaling through FPR2/ALX and this supports the use of LXA₄ as a therapeutic option in atherosclerosis as well as restenosis.
LIST OF PUBLICATIONS

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

Docosahexaenoic acid supplementation modifies fatty acid incorporation in tissues and prevents hypoxia induced-atherosclerosis progression in apolipoprotein-E deficient mice.

Intermittent hypoxia-activated cyclooxygenase pathway: role in atherosclerosis.

Leukotriene B4 pathway activation and atherosclerosis in obstructive sleep apnea.

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.
CONTENTS

1 Introduction .................................................................................................................. 1
  1.1 The vascular wall....................................................................................................... 1
  1.2 Atherosclerosis ........................................................................................................ 1
     1.2.1 Initiation ............................................................................................................. 1
     1.2.2 Progression ......................................................................................................... 2
     1.2.3 Unstable plaques ............................................................................................... 3
     1.2.4 Plaque rupture .................................................................................................... 3
     1.2.5 Diagnosis .......................................................................................................... 4
     1.2.6 Management ....................................................................................................... 5
     1.2.7 Mouse models of atherosclerosis ................................................................. 6
  1.3 Lipoxygenases ........................................................................................................... 6
     1.3.1 Lipid mediator biosynthesis ............................................................................. 6
     1.3.2 Lipoxygenases in CVD .................................................................................... 9
     1.3.3 The possible role of Lipoxin A4 in the vascular wall ................................... 11
  1.4 FPR2/ALX receptor ................................................................................................. 11
  2 Aims ............................................................................................................................. 14
  3 Material and methods ................................................................................................. 15
     3.1 Materials ................................................................................................................ 15
     3.2 Experiments performed in the laboratory .......................................................... 15
        3.2.1 Plasma measurements ...................................................................................... 15
        3.2.2 Animal genotyping .......................................................................................... 15
        3.2.3 RNA isolation .................................................................................................. 16
        3.2.4 Real-Time PCR ............................................................................................... 16
        3.2.5 Cell isolation ..................................................................................................... 16
        3.2.6 Intracellular calcium measurements ............................................................. 18
        3.2.7 Flow cytometry ............................................................................................... 18
        3.2.8 In vitro effectorcytosis assay ........................................................................... 19
        3.2.9 Cell proliferation assay ..................................................................................... 19
        3.2.10 Cell migration assay ....................................................................................... 19
        3.2.11 Immunohistochemistry ................................................................................. 20
        3.2.12 Organ bath experiments ............................................................................... 21
        3.2.13 Biobank ............................................................................................................ 21
  3.3 Experiments performed in the animal facility .......................................................... 21
     3.3.1 Mouse colonies .................................................................................................. 21
     3.3.2 Double knock-out mice ..................................................................................... 22
     3.3.3 Bone marrow transplantation .......................................................................... 23
     3.3.4 Carotid ligation ................................................................................................ 23
  3.4 Data analysis ............................................................................................................. 24
  4 Results .......................................................................................................................... 25
     4.1 FPR2/ALX expression in human atherosclerotic lesions .................................... 25
        4.1.1 Macrophages ...................................................................................................... 25
     4.2 Mouse Fpr2 in the context atherosclerosis ......................................................... 26
        4.2.1 Leukocytes ........................................................................................................ 29
        4.2.2 SMC .................................................................................................................. 30
        4.2.3 Endothelial cells ............................................................................................... 33
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AKC</td>
<td>Ammonium-Chloride-Potassium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ATL</td>
<td>Aspirin triggered Lipoxin A&lt;sub&gt;4&lt;/sub&gt; (15-epi-LXA&lt;sub&gt;4&lt;/sub&gt;)</td>
</tr>
<tr>
<td>BiKE</td>
<td>Biobank of Karolinska Endarterectomies</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CPT</td>
<td>Cell Preparation Tube</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EEL</td>
<td>External elastic layer</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FLAP</td>
<td>5 Lipoxygenase activating protein</td>
</tr>
<tr>
<td>FPR</td>
<td>Formyl peptide receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>IEL</td>
<td>Internal elastic layer</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>IMT</td>
<td>Intima-media thickness</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>KS</td>
<td>Krebs solution</td>
</tr>
<tr>
<td>LB</td>
<td>Luminal border</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LO</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>LX</td>
<td>Lipoxin</td>
</tr>
<tr>
<td>LXA&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Lipoxin A&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>MaR</td>
<td>Maresin</td>
</tr>
</tbody>
</table>
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-kb 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 bloodstream.

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 bloodstream [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 intrastent 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 Lipooxygenases

1.3.1 Lipid mediator biosynthesis

There are three main classes of lipooxygenases (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 LTA4. Through subsequent enzymatic steps, LTA4 is either converted to LTB4 or conjugated with glutathione to form the cysteinyl-LTs (CysLT, LTC4, LTD4, and LTE4). 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 A4 (LXA4) [39]. The biosynthesis of LXA4 requires sequential dual lipooxygenation 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, LXA₄ biosynthesis may involve transcellular 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 LTA₄ via the 5-LO pathway takes place mainly in myeloid cells [39], and platelets that are rich in 12-LO are able to collect LTA₄ and transform it to LXA₄ [44] (Figure 1). As a consequence, although platelets cannot form LXA₄ by themselves, they are the main source of LXA₄ in vivo [44].

The 15-epimer of LXA₄, a more stable LXA₄ 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-LXA₄ (Figure 1). Since 15-epi-LXA₄ 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 A₄ (LXA₄) and aspirin-triggered 15-epi LXA₄ (ATL); 5-lipoxygenase (LO), 12-LO and 15-LO or cyclooxygenase 2 (COX2). Both LXA₄ 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 B₄ (LTB₄). Subsequently, resolution starts as the levels of the pro-resolution lipid mediators increase, for example lipoxin A₄ (LXA₄), 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-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 LXA₄ 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, overexpression 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-LO⁻/⁻ mice, which was mimicked by bone marrow transplantation (BMT), and interestingly, pro-resolution mediators, such as LXA₄ 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 LXA₄ formation [59, 60].

![Table 1](#)

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype</th>
<th>Diet</th>
<th>Time on diet</th>
<th>Aortic root</th>
<th>En Face</th>
<th>Comments</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>M &amp; F</td>
<td>ApoE⁻/⁻</td>
<td>Chow</td>
<td>22 w</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>[59]</td>
</tr>
<tr>
<td>NA</td>
<td>ApoE⁻/⁻</td>
<td>Chow</td>
<td>15 w and 52 w</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>[61]</td>
</tr>
<tr>
<td>M &amp; F</td>
<td>Ldlr⁻/⁻</td>
<td>Western</td>
<td>3, 6, 9, 12 and 18 w</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>[62]</td>
</tr>
<tr>
<td>M &amp; F</td>
<td>Ldlr⁻/⁻</td>
<td>Chow</td>
<td>15 w and 32 w</td>
<td>NA</td>
<td>↓</td>
<td>Reduced in males and no differences in females.</td>
<td>[63]</td>
</tr>
<tr>
<td>M &amp; F</td>
<td>ApoE⁻/⁻</td>
<td>Chow+vit E</td>
<td>12 w</td>
<td>NA</td>
<td>↓</td>
<td>-</td>
<td>[64]</td>
</tr>
<tr>
<td>M &amp; F</td>
<td>ApoE⁻/⁻</td>
<td>Chow</td>
<td>25 w</td>
<td>↓</td>
<td>↓</td>
<td>Reduced in females and no differences in males.</td>
<td>[65]</td>
</tr>
</tbody>
</table>

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 studying 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 BLT1 receptor for LTB4 reduces atherosclerosis [66] and intimal hyperplasia after vascular injury [47, 67].

### 1.3.3 The possible role of Lipoxin A4 in the vascular wall

As mentioned above, LXA4 has been described as an anti-inflammatory and pro-resolving mediator in myeloid cells [12]. For example, LXA4 down-regulates IL-1β in a human astrocytoma cell line [68] and in BV2 cells, a mouse microglia cell line [69]. LXA4 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], LXA4 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 LXA4 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, LXA₄ [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 LXA₄ 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 LXA₄ [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 LXA₄ 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 LXA₄ (right).

Currently there is no knowledge about the use of LXA₄ 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 LXA₄ 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 LXA₄ in atherosclerosis and stenosis.
- Elucidate if the pro-resolution effect of LXA₄ is mediated by the FPR2/ALX receptor.
3 Material and methods

3.1 Materials

All reagents were from Sigma-Aldrich unless otherwise specified.

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 LXA₄ (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 – tgagtgtcatgtcagaaggcc, B11 – cggaatccagctacccaaatc and GB4 – ataacctcgggcatgacctc. 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: oLMR0180 (common forward) – gcctagccgaggagccg, oLMR0181 (Wild-type reverse) – tgtgacttcaggtcagc and oLMR0182 (Mutant reverse) – gccgcagcgtcactc. 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 \textit{Ldlr\textsuperscript{+/--}}

Primers: Ldlr common forward – aggtgagatgacaggagatc, Ldlr wild-type reverse – accccaagagtgcctccaggatga, Ldlr mutant reverse – cgcaagtgcctctcatgtgt. 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 400-base pairs band for the wild-type animals or a 800-base pairs band for the knockout 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\textsubscript{2} inhalation.

3.2.5.1 Human CD4\textsuperscript{+} 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.
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 CO₂, 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 5\(\times\)10\(^6\) 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 \(\mu\)g/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 LXA₄ (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 \(\mu\)g/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<sup>−/−</sup> x Fpr2<sup>+/+</sup> (A and B), Ldlr<sup>−/−</sup> transplanted with either Fpr2<sup>+/+</sup> or Fpr2<sup>−/−</sup> BM (C and D) and ApoE<sup>−/−</sup> x Fpr2<sup>−/−</sup> (E and F).
4.2.1 Leukocytes

Given that macrophages were associated with FPR2/ALX expression in human atherosclerotic lesions (Paper I), 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-deficient atherosclerotic models
CD68 expression in aortic roots of (A) Ldlr<sup>−/−</sup> x Fpr2<sup>+/+</sup>, (B) Ldlr<sup>−/−</sup> with BM Fpr2<sup>−/−</sup> and (C) ApoE<sup>−/−</sup> x Fpr2<sup>−/−</sup> 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<sup>+/+</sup> and Fpr2<sup>−/−</sup> 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<sup>−/−</sup> mice exhibited a specific phenotype as compared to Fpr2<sup>+/+</sup> mSMC. First, mRNA levels of several components involved in collagen deposition and maturation were significantly lower in Fpr2<sup>−/−</sup> 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<sup>−/−</sup> mSMC (Paper I). The latter findings were supported by significantly less collagen in the atherosclerotic lesions of Ldlr<sup>−/−</sup> x Fpr2<sup>−/−</sup> compared with Ldlr<sup>−/−</sup> x Fpr2<sup>+/+</sup> 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).
Given these marked phenotypic changes of Fpr2 deletion in terms of mSMC responses \textit{in vitro}, the role of Fpr2 signaling in mSMC proliferation and migration was subsequently examined \textit{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\textsuperscript{+/+} mice, these experiments further reinforced the importance of ATL signaling by means of Fpr2 in the SMC response to vascular injury.
The role of FPR2/ALX in the vascular wall

Figure 8: ATL effects on atherosclerosis and intima formation after carotid 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 LXA4 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, LXA4 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 LXA4. 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 LXA4 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 LXA₄ 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 bind 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-positive 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 \textit{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 \textit{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 BLT1. For example, LTB4 increases chemotaxis that is absent in BLT1−/− cells [66], and by BLT1 antagonist, showing less SMC migration after LTB4 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 \textit{in vitro} and intimal hyperplasia formation \textit{in vivo}. In addition, Fpr2 was associated with pathways of increased collagen production and maturation, and decreased collagen breakdown, 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).

\subsection{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 \textit{in vivo} and \textit{in vitro} \cite{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 \cite{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 LXA₄ causes a reduction of blood pressure in short term (~4 min), while blocking the LXA₄ 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 LTB4 promotes SMC proliferation [47] whereas induces EC apoptosis [127] for example. No previous reports have explored specifically the Fpr2 signaling on EC proliferation, but LXA4 reduced VEGF-induced EC proliferation and migration [72, 99], supporting that LXA4-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 LXA₄ 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 LXA₄ 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<sup>−/−</sup> 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<sub>4</sub>-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-endothelialazation 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. LXA₄ 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.
7 Acknowledgements

I would like to thank to everyone that have been supporting me from the beginning until the end of this journey. The only fact that you are reading this, makes clear the support of people around me made it possible.

If you are not mentioned in this text, I am really sorry, but you know that during this time as a PhD student definitely I have thanked you in person for the support. Besides this, I would like to give a special attention to:

Magnus Bäck, my main supervisor. Since we met in France, it has been an honor to work with you. The support from the personal point of view has very important for me and I am thankful for it. The discussions and talks about science and projects has been and still is very inspiring. The enthusiasm that you talk about science and results/findings is very contagious, no wonder why we work so much! From the bottom of my heart, thank you so much for everything; Along with Magnus, I would like to thank Sandrine. Meeting you back in 2007 was essential for this journey, as a consequence, this thesis is a result of all the support from France and in Sweden. You are a prove of a Brazilian saying: behind a big man, there is a big woman

Andrés Laguna-Fernandez, my co-supervisor. I believe that this text will be short if I try to express what I really think about you. I have told you many times, has been a pleasure working with you. I know that you are a fine man with a focused goal in life! it was very good for me to have you as co-supervisor since I also admired your way of doing things, the organization and how to make science were very important for my formation. Thank you!

Göran Hansson. Thank you for all the time and support during this time. Your suggestions on how to approach the results and strategies on how to handling data were very good on my formation. It has been an honor to work on your lab!

To my wife Laura Estephany. You have no idea how much of this thesis is yours. The patience when I was working late, the times you brought dinner to the lab, the weekends that we had to be in the lab. Thank you so much for all the support! I love you I am really glad to have you on my side. My in laws, Esperanza and Alfonso, thank you for make Laura so perfect as she is.

My parents! My mother Cleopatra Rosana Silva and my father Valdemiro Paulo Petri, but it would not be possible without the help of my second parents, Adolar Pieske and Angela Barbosa. You were the pillar and the strength that support me through the steps before this. I am very grateful to have you as family!

Ann Hellström. You have no idea how much thankful I am. You helped me since I got here, with many administrative stuff. You know how the system works, and was always free for questions and fixing problems. Thank you for all your time!

All the Translational Cardiology group that has been and still are around. Miguel Carracedo, has been very nice to work with you. Your motivation is contagious. Oscar, although we have short time working together, you are always up for learning, this will take you
very far, keep doing your best. **Gonzalo**, you are young and full of energy, thank you for all the support so far. **Jasmin**, it was very nice to meet you, and taking the decision to make medicine was a big step. I wish all the best and if you want to watch some scary movie, fanny and I would love to catch some scary movie together at our place. **Teodora**, you are a very courageous woman. I admire you for it. I know that the master will be a little step on your big future, All the best! **Silke**, since you came to visit the lab, I felt a good vibe from you. You are a focused woman and have been great work and collaborate with you. I wish all the best on your marriage! **Ingvill**, It was an honor to work with you during the summer projects and having you and **Magnus** on my wedding. I am sure your life as MD will be as rich as your live in research has been! **Allison Eaton, Sangeetha, Edit, Kari and Martina** you have not been so much in the lab, but I wish all the best!

**Linda**, the master of histology! Thank you for all the support! All the best with the baby! **Anneli**, Thank you with all the help and troubleshooting on qPCR issues and **Ingrid**, you are the “lab guru”! Thank you for all the help with cell culture issues and genotyping! You are the three crowns of the lab! The pillars that gave us all the support that we needed, from ordering to troubleshooting. Thank you so much with all the help. It was very good to work around such professional people.

The Göran’s group. **Gabrielle**, thank you for all the talks about Swedish culture and apples. **Daniel K**, you know that I admire you, you are an awesome scientist and I am honored to be able to work with you. **Anton**, during my PhD you have actually transformed yourself, and I am not kidding. Your dedication in the lab and on the gym expires the people around you! **Martin**, my fellow MD, you are the most relaxed guy I ever met. Seems like the stress in research is just a Fairy tales. Keep up the good work. It has been very good to share the lab time with you! Hope to see you in clinic anytime soon. **Maria Klement**, Thank you for the support and the culture/musical Swedish references such as Kent for instance. **Sophie** and **Zhong-Qun**, thank you for the comments about innate immunity and SMC! **Kostas**, my dear Greek friend, your calm and nice way of taking things in the work impresses me. I wish I was that hard working with this tranquility. **Maria Jose**, it was a pleasure to work with you one time, and was even more delightful to see you around again for more time. Thank you for the musical/cultural talks is nice to share musical tastes and get some feedback. I know you have a bright future in the lab. Keep working hard and all the best! **Roland**, your tips from Austria were very good. You are a nice guy with always a good mood. I am sure that your future as researcher will be bright. **Jonh**, my dear French friend, I can see your evolution since you came to the lab. From drinking vodka to bear, is a bit evolution. Thank you sharing the outside lab life with me! **Glykeria**, Keep up the good work! You enthusiasm will take you very far. **Robert, Cheryl** and **Yajuan** although you are not in the lab anymore, thank you for your support.

The people that are around in the KI that also shared this journey: **Björn**, thank you for the talks about endothelial cells on the corridor! And with **Maripaz**, thank you for the good times outside the lab, that help me recharge the battery! I still need to take the wine tasting course! **Helena**, my dear Portuguese lady, your good mood made you the best Portuguese girl of the floor! **Lynn** and **Alice**, sharing the flat made the friendship very strong, besides the scientific talks were as excited as the personal experience. Getting to know Austria and Vienna were wonderful and impossible without meeting you. **Maria Gonzalez Diez and David**, it has been very nice to meet and share our lives outside the lab with you, collaborating with you has been very motivated and I wish the best of luck in your carriers. **Aleen**, we start the PhD together, and
have been nice to share the journey with you. KC, you are the man! Every time a reagent was missing you were always there to give a hand and help out. Thank you! Vanessa, I was very happy to share the time in the lab with a German girl that always have chocolates. I wish all the best with the baby! Angela, Thanks for the talks in the corridors. Lars, my dear German friend/collaborator, thank you for the support during the projects and the talks about the NFL. I hope the 49ers would go far this year, but not higher than the Houston Texans of course! All the best! Hong and all the Lars group, it has been very nice to work with you. Always with a positive mind going forward. Thank you! Tony and Dude, always nice to meet so happy people sharing the Brazilian background. It was a pleasure having meeting you, thank you! Ewa, Fariba, Apostolos, Laura Brion, Paula Ciscoto, Rodrigo, Ulf Hedín, Jack, Joy Roy Mette, Malin, Silvia and Ljubica, I hope I did not forget anyone, but if I did I am sorry. Some of you

I would like to thank all the animal technicians that support the many breeding and animal handling. Kicky, Malin Esp, Sandra Olsson, Sandra Mezei, Jessica Lundgren, Ann-Christine Eklöf, Anna Lena, Selam and Leo, just to mention a few, there was many more that helped that I can’t name all of them. I just wanted to express my gratitude for all the support, you were very important for this book and the well been of the animals! Thank you!

People outside the lab that helps this achievement: Jean Baptiste Michel and Olivier Meilhac, Thank you for the opportunity to get into the cardiology research, I started learning from the best. Also Monica Vazquez Mercado, my former supervisor back in Mexico during the Master’s Degree. Thank you for the time and the support in the initial phase of my carrier. I guess a good start finishes with a good ending.

My personal friends in Sweden that also helped on the tough times during the thesis. Christian Ibarra and Claudia, Karla and Kenneth, Dulce and Alessandro, Gary and Jayma, Alejandra and Erik. Also in Brazil Julio, Guto and the whole Mafra family, Daise Lara and family, Alfredo Rosa, Carlinhos and family, Beto, Rosane, Suely and Moni, my god parents, Mauren and Karl. My uncles and cousins Julius and family, Zeca and family, Valdir and Family, Arsenio and Family, Emilia and family. Just to mention a few good friends. Thank you for the time we have spent together.

Finally I would like to thank to Danilo Gentili along with everyone on The Noite program. Along with Rafinha Bastos, you were important on my mornings to give me extra energy and humour to start my day.

Special thanks for Yayo, Oscu and Pina from Tabu (AKA Black Jacks) that always relaxed me with awesome music! For last, all the bands that were with me during the experiments, Ramones, Ultrade A Rigor, Pearl Jam, Ira!, Charlie Brown Jr, Pixies, Bush, Blues Etilicos, Blink 182, The clash, Engenheiros do Hawaii, Lenny Kravitz, Ozzy Osbourne, Nirvana, Pennywise, Titãs and Rancid just to mention a few that were relaxing me and motivating me over long hours in the lab and with the animals. If any of you could see what you have helped me accomplish, this would be one of them!
8 References


