Innate Immunity in Human Atherosclerosis and Myocardial Infarction: Role of CARD8 and NLRP3
I dedicate this thesis to my parents
Paramel Solomon Varghese & Maggi Varghese
"All I am and I hope to be, I owe to you".

All this also comes from the Lord Almighty,
whose plan is wonderful, whose wisdom is magnificent.

......... Isaiah 28:29
Innate Immunity in Human Atherosclerosis and Myocardial Infarction: Role of CARD8 and NLRP3
Cover picture:
Left: Immunostaining of NLRP3 protein (red) in the human atherosclerotic lesion; Right: Immunostaining of CARD8 protein (green) in the Human Umbilical Vein Endothelial Cells (F actin stained in red, nucleus in blue).

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Title: Innate Immunity in Human Atherosclerosis and Myocardial Infarction: Role of CARD8 and NLRP3.
Publisher: Örebro University (2017)
www.publications.oru.se

Print: Örebro University, Repro 12/2016

ISSN 1652-4063
Abstract


Atherosclerosis is complex inflammatory disease of the arterial wall with progressive accumulation of lipids and narrowing of the vessel. Increasing evidence suggest that inflammation plays an important role in plaque stability and often accelerate cardiovascular events such as myocardial infarction (MI). Among the vast number of inflammatory cytokines, IL-1β is known to be a key modulator in vessel wall inflammation and acceleration of the atherosclerotic process. The biologically active IL-1β is regulated by a multiprotein complex known as the NLRP3 inflammasome complex. In this thesis, we have focused on polymorphisms in the NLRP3 and CARD8 genes and their possible association to atherosclerosis and/or MI. We have also investigated the expression of inflammasome components NLRP3 and CARD8 in atherosclerosis and the role of genetic variants for the expression of these genes. The expression of NLRP3, CARD8, ASC, caspase-1, IL-1β, and IL-18 were found significantly upregulated in atherosclerotic lesions compared to normal arteries. Human carotid plaques not only express the NLRP3 inflammasome, but also release IL-1β upon exposure to lipopolysaccharide (LPS), adenosine triphosphate (ATP) and cholesterol crystals, which suggest NLRP3 inflammasome activation in human atherosclerotic lesions. Also, CARD8 was found to be important in the regulation of several inflammatory markers in endothelial cells, like RANTES, IP10 and ICAM-1. We further assessed the potential association of a CARD8 polymorphism and polymorphisms located downstream of the NLRP3 gene to the risk of MI in two independent Swedish cohorts. The CARD8 variant exhibited no association to risk of MI in either of the two cohorts. Some of the minor alleles of NLRP3 variants were associated with increased IL-1β levels and to NLRP3 mRNA levels in peripheral blood mononuclear cells (PBMC). Taken together, the present thesis shows that NLRP3 inflammasome activation and increased expression of CARD8 in the atherosclerotic plaque might be possible contributors to the enhanced inflammatory response and leukocyte infiltration in the pathophysiology of atherosclerosis.

Keywords: Atherosclerosis, Inflammasome, NLRP3, CARD8, Myocardial infarction, Endothelial cells, Polymorphism, IL-1β, Cytokines, Innate immunity.

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Populärvetenskaplig sammanfattning

Åderförftettning (ateroskleros) är en komplex sjukdom som drabbar blodkärlsväggen genom en ackumulering av fetter och inflammatoriska celler i kärlet i form av s k plack. Tidiga tecken på sjukdomen kan hittas redan hos barn, men symtom på sjukdomen visar sig först långt senare i livet. Plackbildningen har då resulterat i en förträngning av kärlet, vilket kan leda till en hjärtinfarkt eller stroke om placket lossnar.

På senare år har det visat sig att inflammation spelar en viktig roll för utveckling av plack och en av de viktigaste molekylerna för inflammation i kärlet är molekylen interleukin-1β, vilket bildas av den s k NLRP3 inflammasomen. Fokus för denna avhandling har därför varit att undersöka NLRP3 inflammasomen och CARD8, och deras betydelse för ateroskleros och hjärtinfarkt.

Avhandlingen visar att flera av NLRP3 inflammasomens viktiga komponenter finns i stor mängd i plack jämfört med friska kärl. Dessutom har genetiska riskmarkörer hittats i en region som styr NLRP3 inflammasomen, och som är associerade med förhöjda nivåer av interleukin-1β. Avhandlingen visar också att CARD8 kan reglera inflammationen i kärlet, men att genetiska riskmarkörer i denna gen inte spelar någon roll för utvecklingen av hjärtinfarkt.

Sammanfattningsvis visar denna avhandling att NLRP3 inflammasomen och CARD8 kan ha betydelse för inflammationen i blodkärl och därmed kan ha betydelse för utveckling av ateroskleros. Resultaten kan i framtiden leda till utveckling av nya direktriktade mediciner mot ateroskleros och därmed förhindra hjärtinfarkt.
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IV. Geena Paramel Varghese, Anna Göthlin-Eremo, Liza Ljungberg, Allan Sirsjö, Karin Fransén. CARD8, a protein of innate immunity regulates the release of inflammatory cytokines in human endothelial cells. (Manuscript)
Additional studies

Studies not included in this thesis:


List of Abbreviations

ASC  Apoptosis-associated speck like protein containing a CARD  
ATP  Adenosine tri phosphate  
BiKE  Biobank of Karolinska Endarterectomies  
CAPS  Cryopyrin associated periodic fever syndrome  
CARD  Caspase activation and recruitment domain  
CINCA  Chronic infantile neurological cutaneous articular syndrome  
CRP  C-reactive protein  
CVD  Cardiovascular disease  
DAMP  Danger/Damage associated molecular pattern  
FCAS  Familial cold autoinflammatory disease  
FIA  The First-ever myocardial Infarction study in AC-county  
IL-1β  Interleukin-1 beta  
LRR  Leucine rich repeats  
MCP-1  Monocyte chemoattractant protein-1  
MWS  Muckle-Wells syndrome  
NACHT  NAIP (neuronal apoptosis inhibitor protein), CIITA (MHC class II transcription activator), HET-E (plant het product of vegetative incompatibility), and TP-1(telomerase associated protein)  
NLR  Nucleotide binding domain and leucine rich repeat containing gene family of receptor  
NOMID  Neonatal onset multiple inflammatory syndrome  
NLRP3  NLR family, pyrin-containing domain 3  
PAMP  Pathogen associated molecular pattern  
PBMC  Peripheral blood monocyctic cell  
PRR  Pattern recognition receptor  
PYD  Pyrin domain  
SCARF  Stockholm Coronary Atherosclerosis Risk Factor  
SNP  Single nucleotide polymorphism  
TLR  Toll like receptor  
MI  Myocardial infarction  
RANTES  Regulated on Activation Normal T Cell Expressed and Secreted  
IP10  FN-inducible protein-10
Innate immunity in human atherosclerosis and myocardial infarction: Role of CARD8 and NLRP3.

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>Oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>SR-A1</td>
<td>Scavenger receptor-A1</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor apoptosis repeat</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s Disease</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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Introduction

Cardiovascular disease (CVD) is the most common cause of death worldwide, accounting for 17.3 million deaths globally in 2013, and atherosclerosis is one of the common underlying causes of CVD\(^1\). Atherosclerosis is an inflammatory disease of large and medium sized arteries\(^2,3\) and is characterized by accumulation of lipids in the arterial wall that is accelerated by risk factors, such as hypertension, diabetes, smoking, genetics, age and dyslipidemia\(^10\). This leads to the narrowing of the arterial lumen to various degree, followed by increased risk for cardiovascular events, including myocardial infarction (MI).

The term MI is defined as sudden cardiac death of myocardium, usually due to thrombotic occlusion of a coronary artery caused by rupture of an atherosclerotic plaque\(^4\). In the past decades, the clinical definition of myocardial infarction has undergone enormous revisions to improve the clinical presentation and to differentiate the pathophysiological condition including atherosclerosis in association to MI \(^5-7\). The ischemic injury caused by atherosclerosis may further accelerate atherosclerosis by boosting the monocyte production from spleen, providing a new insight in the mechanism of atherogenesis\(^8\).

Over the past century, our view on the pathophysiology of atherosclerosis was primarily focused on the complex association between hypercholesterolemia and atherosclerosis, and secondarily to endothelial dysfunction, growth factors and vascular cell proliferation. In the last two decades it has become more evident that inflammation most likely plays an important role in the development and progression of atherosclerosis \(^9\).

The Arterial Vessel Wall

A healthy human artery comprises of three different layers, the intima, the media and the adventitia\(^10\). The innermost layer, the intima, consists of a monolayer of endothelial cells followed by a proteoglycan layer in the subendothelial space. The endothelial cells serve as a physiological barrier to prevent the platelets, leukocytes and coagulation factors in the blood to come in contact with the proteoglycan layer and the pro thrombotic molecules of the intima. In addition, endothelium exerts several vasoprotective effects and fibrinolytic property to regulate the vascular homeostasis\(^11\). The
proteoglycan layer of the intima is abundant in non-fibrous connective tissues and contains widely spaced smooth muscle cells (SMC), both of synthetic and contractile phenotype, and isolated macrophages. An additional musculoelastic layer underlying the proteoglycans is clearly visible in segments, with adaptive thickening of the intima, comprising of elastic fibers, collagen and mostly contractile SMCs arranged in closed layer\textsuperscript{12}. Underneath the sub endothelial layer, the internal elastic lamina represents a flexible barrier between the intimal endothelium and SMCs of the media. The layer plays a major role in modulating the migration of SMC migration from media to the intima\textsuperscript{13} and is usually absent at vascular transition, such as bifurcations.

Below the intima is the media, which is a complex network consisting mainly of SMCs, collagen fibrils and elastin\textsuperscript{14}. This layer is delimited by the internal and external elastic lamina which imparts mechanical properties to the arterial wall by easing the arterial contraction and dilation. The outermost layer, the adventitia, is a dense collagenous structure comprising of collagen fibrils, elastin fibers and fibroblasts together with some SMCs\textsuperscript{14}. The layer is infiltrated with nerve fibers and also nourishes the external tissues of the vessel via \textit{vasa vasorum}.

\textbf{Atherosclerosis}

Atherosclerosis, the slow progressive disease of large and medium sized arteries is the underlying cause of ischemic heart disease. In 1833, the German-born French pathologist Jean Lobstein (1777-1835) introduced the term Arteriosclerosis from the two Greek words ‘\textit{athere}’, meaning gruel, for the porridge-like consistency of the plaque and ‘\textit{skleros}’, which signifies hardening of the arterial wall due to tissue remodeling. Later, in 1904, the German pathologist Felix Marchand (1846-1928) proposed the term ‘Atherosclerosis’ to emphasize the accumulation of fatty substrate that he observed in the hardened artery\textsuperscript{15}. In 1985, the work by Brown and Goldstein on the regulation of cholesterol metabolism and its involvement in the pathophysiology of atherosclerosis earned them the Nobel Prize in Physiology or Medicine\textsuperscript{16}. The inflammatory nature of the atherosclerotic lesion was proposed in the 20\textsuperscript{th} century, the major paradigm shift happened in the 21\textsuperscript{th} century focusing the complex interplay of the lipids and the immune cells to impart inflammation in the arterial wall\textsuperscript{17-19}. 
Development and Progression of Atherosclerosis

The development of atherosclerosis can be categorized into three different stages; the initiation of atherosclerosis followed by progression to an advanced complex plaque and plaque rupture.20

The uptake of LDL to the arterial intima, infiltration of leukocyte, formation of foam cells are the characteristic features during the initiation of atherosclerosis.

The initiation of atherosclerosis is a combinatory effect of different factors, such as turbulent blood flow along with other risk factors such as hypertension, obesity, lipids, smoking, age, and family history of atherosclerosis21, which leads to endothelial dysfunction. Arteries tend to have an intimal thickenings at the sites of branching in the arterial tree as a physiological adaptive response to the low shear stress and increased wall tension22. These atherosclerotic sites are prone to retention of extracellular lipid droplets

Figure 1A: Schematic representation of the initiation of atherosclerosis. The uptake of LDL to the arterial intima, infiltration of leukocyte, formation of foam cells are the characteristic features during the initiation of atherosclerosis.
Endothelial dysfunction / activation is one of the earliest events in atherosclerosis\textsuperscript{11}. A dysfunctional endothelium allows lipid accumulation, such as low density lipoprotein (LDL) (i.e. formation of fatty streaks, see below) in the vessel wall as well as adherence of leukocytes to the activated endothelium\textsuperscript{25}. Leukocytes, mainly monocytes, migrate into the intima and differentiate into macrophages and produce reactive oxygen species, pro-inflammatory cytokines, such as MCP-1, IL-1β and TNFα, and initiate the inflammatory process followed by further infiltration of additional inflammatory cells\textsuperscript{25} (Figure 1A).

The internalized LDL can be modified by progressive oxidation and internalized by macrophages\textsuperscript{17}. The oxidized low-density lipoprotein (Ox-LDL) moieties are pro-inflammatory mediators and further augments the recruitment of monocyte-derived macrophages through induction of factors like adhesion molecules, growth factors and pro-inflammatory cytokines. The intimal accumulation of LDL and monocytes leads to the formation of fatty streaks \textsuperscript{24}. Fatty streak formation in the arterial wall begins early in life and is found in half of infants in the first six months of life\textsuperscript{26}, but gradually declines in the subsequent years and might reflect the susceptibility of mother to the risk of CVD\textsuperscript{26}. Recent studies in mice have suggested the possibility of cholesterol crystal formation in the sub endothelial space at early stage of atherosclerosis\textsuperscript{27}. The accumulation of cholesterol crystals may trigger the activation of additional inflammatory signaling cascades and establish an inflammatory milieu together with the infiltrating immune cells\textsuperscript{27}. In atherosclerotic prone ApoE-knock out (KO) mice fed with high cholesterol diet, the crystals were found to accumulate in the sub endothelial immune cells as early as 2 weeks of age\textsuperscript{27}. The deposition of cholesterol crystals was evident both inside and outside of the cells localized in the necrotic core and sub endothelial areas\textsuperscript{27}. The crystals were also abundant in the immune cell rich areas of human atherosclerotic lesions\textsuperscript{23}. Collectively, this indicates that cholesterol emerge early during the atherogenesis and might contribute to the sub-endothelial inflammatory response.
Figure 1B: Schematic representation of progression of atherosclerosis. The formation of a necrotic core during the progression of atherosclerotic plaque.

In the arterial intima, macrophages undergo morphological changes and up-regulate scavenger receptors to internalize modified lipoproteins, thereby forming foam cells, which in combination with increased SMC proliferation results in thickening of the intima. This early stage of the lesion development is often clinically silent. These atherosclerotic lesions are usually stable but asymptomatic lesions may potentially be precursors for the more advanced, unstable and symptomatic atherosclerotic lesions.

The macrophages secrete pro-inflammatory cytokines, which amplifies the local inflammation and generate a self-perpetuating inflammatory response by activating endothelial cells, SMCs, macrophages and recruiting lymphocytes. T-cells in the atherosclerotic lesion are also triggered to elaborate inflammation by the production of interferon-γ and TNF-β that can further stimulate the macrophages, SMC and endothelial cells. Some of the macrophages within the atheroma undergo apoptosis or necrosis and can thereby form a necrotic core. On the luminal side, the necrotic core is...
covered by a fibrous cap consisting of SMCs and extracellular matrix proteins (Figure 1B).

The activated macrophages also produce proteolytic proteins that leads to degradation of extracellular matrix proteins and weakens the protective fibrous cap which increases the susceptibility for plaque rupture. In advanced atherosclerotic lesions, cholesterol crystallization may lead to volume expansion and induce plaque rupture by perforating the outer layer of atherosclerotic plaque\textsuperscript{32}. The rupture of the plaque triggers platelet activation and coagulation, thereby leading to thrombus formation and occlusion of the blood vessel (Figure 1C) \textsuperscript{25}. The formation of necrotic and calcified core, fibrous cap, hemorrhage and micro-thrombi are the characteristics of advanced, symptomatic lesions\textsuperscript{29}.

Figure 1C: Schematic representation of atherosclerotic plaque rupture. Platelet activation and thrombus formation leads to occlusion of the vessel.
**The Innate Immune Response and Atherosclerosis**

As described above, inflammation and immune response are key components in the pathophysiology of atherosclerosis. Accumulation of immune cells such as neutrophils, mast cells, dendritic cells, lymphocytes, monocyte and macrophages is involved in atherosclerosis\(^3^3\). In addition, the non-professional immune cells of the vasculature, such as endothelial cells and vascular SMCs also participate in the activation of immune response by pathogens or endogenous metabolite recognition and immune cell recruitment\(^3^4\).

**Endothelial Cells**

In the normal vessel, the endothelium produce nitric oxide (NO) that plays an important role for the vascular tone by the inhibition of inflammation, thrombosis and cell proliferation\(^3^5\). However, the activation of endothelium/endothelial dysfunction leads to the dysregulation of NO production and activation of reactive oxygen species\(^3^6\). In the adverse condition, including risk factors, such as hypertension and hypercholesterolemia, the severe dysregulation of NO and ROS production may contribute to atherogenesis\(^3^5\).

Several atherogenic factors, including Ox-LDL, may cause endothelial dysfunction and induce expression of leukocyte adhesion molecules by the activation of pattern recognition receptors (PRRs) on the endothelium\(^1^7\). The activation of these receptors may trigger the signaling cascade that ultimately leads to the activation of NF-κB, thereby leading to the production of pro-inflammatory cytokines, adhesion molecules, such as ICAM, VCAM, selectins and chemoattractants\(^3^7-3^9\). MCP-1, the best characterized chemotactic cytokines is produced from endothelial cells, SMCs, and macrophages and is upregulated in all the stages of atherosclerosis, thereby indicating the important role of MCP-1 in the leukocyte infiltration to the intima of vessel wall\(^4^0\). In addition to MCP-1, the release of complement protein C5a from the endothelial cells is important for the recruitment of monocytes to the intima\(^4^1\).

**Smooth Muscle Cells (SMCs)**

Proliferation and migration of SMCs from media to intima is a characteristic pathophysiological feature during atherosclerotic plaque progression\(^4^2\). Following vascular injury, SMCs undergo a phenotypic shift from the normal
contractile phenotype to synthetic phenotype with increased production of extracellular matrix (ECM)\(^{43}\). SMCs also express several receptors that facilitate the lipid uptake and foam cell formation\(^{44}\). These cells are responsible for the production of the ECM in the intima and are a major contributor of the fibrous cap formation\(^{42}\). The inflammatory milieu generated in the advanced atherosclerotic lesions as a result of inflammatory response, may induce apoptosis of SMCs, thereby leading to thinning of the SMC-rich fibrous cap and rupture of the atherosclerotic plaque\(^{17,45,46}\).

**Monocytes/Macrophages**

Infiltration of monocytes to the arterial intima is an important phenomenon in atherogenesis\(^{17}\). The differentiation of monocytes to macrophages in the arterial intima by M-CSF induces expression of scavenger receptors such as CD36, CD68, MARCO, SR-PSOX and facilitate the uptake of modified lipids, thereby forming foam cells\(^{47}\). Macrophages do also play a key role in inflammation and innate immune response by expressing receptors such as Toll like receptors (TLR) and nucleotide oligomerization domain (NOD) like receptors (NLRs) that recognizes a broad range of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). The recognition and uptake of processed ligands through scavenger receptors (SR-A1) can lead to its presentation to T cells, thereby forming a link between the innate and adaptive immune response\(^{48}\).

**Immune Response**

The human body is a functionally coordinated, complex system that contains enriched resources of energy for microbes. Innate and adaptive immune response play an inevitable functional role in balancing the commensals and defending the invading pathogenic microbes to maintain immune homeostasis by establishing an interacting network of pro- and anti-inflammatory mediators. The prime role of the innate immune system is to recognize conserved PAMPs of microbes and host endogenous DAMPs by PRRs, which are localized in a number of immune and non-immune cells\(^{49}\). Among the different types of PRR known to date, TLRs and nucleotide NLRs are the most well studied PRRs\(^{50,51}\).
Toll-like receptors were the first group of PRRs to be characterized and were named after the Toll receptor of fruit flies, which was discovered for its role in the development of the fly and later recognized for its contribution in innate immunity\textsuperscript{52}. This discovery was a breakthrough for researchers to explore the innate immune response in modulating the adaptive immune response. TLRs are membrane bound sensors that recognize PAMPs from the extracellular components and endosomes\textsuperscript{53}. Among the 13 so far known TLRs, only 10 are functional members in humans\textsuperscript{54}. TLR 1/2/4/5/6 are expressed on the cell surface whereas TLR3/7/8/9 are expressed on intracellular membranes of endosomes, lysosomes, and endoplasmic reticulum (ER)\textsuperscript{54}. In addition to TLRs, the cytoplasmic protein family of the Nod-like receptor (NLR) has been identified as intracellular surveillance proteins\textsuperscript{55}. Like TLRs, NLRs functions as intracellular PRRs to recognize PAMPs and DAMPs for initiating innate and adaptive immune response\textsuperscript{49}.

**NLRs**

NLRs are involved in the detection of a variety of ligands. Structurally, NLRs are multi-domain proteins which contains a NACHT domain, an N-terminal effector domain and a C-terminal leucine rich repeat (LRR) region\textsuperscript{56}. During the activation of NLR, the NACHT domain is responsible for the oligomerization and the effector domain for the activation of downstream signalling partners\textsuperscript{57}. The LRR region recognizes NLR stimulating molecules, like PAMPs and DAMPs\textsuperscript{57}. (Figure 2).
**Figure 2:** Schematic representation of the domains in human NLR gene family. The N terminal effector domain varies in between the NLR subfamilies and can consist of either pyrin (PYR), caspase activation and recruitment domain (CARD), acidic domain (AD) or baculovirus inhibitor apoptosis repeat (BIR) domain. The central nucleotide binding domain (NBD/NACHT) and the C terminal leucine rich repeats are common in between the subfamilies of NLR protein. The characteristic amino acid terminal pyrin domain, NACHT and LRR are found in almost all the NLRPs with the exception of NLRP10 lacking the LRR domain and NLRP1 (located on chromosome 17p13.3) which contains an additional domain FIIND (function to find) and CARD.

Based on the current paradigm, the ligand recognition by LRR leads to the release of auto repression of NLR oligomerization due to the internal interaction of NACHT and LRR domain. This results in the exposure of the NACHT domain to NLR homotypic oligomerization and recruitment of downstream adaptor proteins, resulting in the formation and activation of protein complexes called inflammasomes. Among the NLR proteins, NLRP constitutes the largest family with 14 NLRPs. Except for NLRP1 (17p13) and NLRP3 (1q44), most of the NLRP genes are located in two
clusters on chromosome 11p15 (NLRP 6, 10 and 14) and 19q13.4 (NLRP 2, 5, 7, 8, 9, 11, 12, 13 and 14)\textsuperscript{61}. NLRP1, NLRP3, AIM2, and NLRC4/IPAF are some of the most widely examined NLRPs that form a multimeric protein complexes by self-oligomerization with scaffold proteins to form inflammasomes\textsuperscript{59}.

**NLR Signaling**

NLRs are the scaffolding protein, the assembly of which leads to the activation of inflammatory caspases via the NF-κB and MAPK signaling pathways, thereby driving transcription of genes involved both in innate and adaptive immune response\textsuperscript{55}. Upon activation, the NLRs, including Nod1 and Nod2, recruit RICK (also known as RIPK2/RIP2) via CARD-CARD interaction and mediate the activation of NF-κB and MAPK signaling pathways via transforming growth factor β-activated kinase (TAK1)\textsuperscript{62-64}.

Among the NLRs, the caspase-associated recruitment domains (CARD) has emerged as a key regulator of several signaling pathways, including NF-κB signaling and apoptosis\textsuperscript{65,66}. The CARD containing proteins are broadly classified in two groups, based on the functional interaction with caspases and NF-κB signals\textsuperscript{67}. The CARD domain in human caspase-1, is one of the most extensively studied caspase in the programmed cell death and cytokine regulation. The activation of caspase-1 is mediated via the recruitment of CARDs of either ICEBERG or Ipaf/CARD12\textsuperscript{67}. Also, the CARD containing protein mediate NF-κB activation via two different signaling routes. The CARD proteins such as CARD4/NOD1, NOD2 and CARD6 mediate NF-κB activation\textsuperscript{62,68,69} via RIPK2 whereas CARD9, CARD10, CARD11 and CARD14 mediate NF-κB activation through the recruitment of the BCL10\textsuperscript{70-72}.

Considering the role of NLRs in inflammation-driven immune response against the harmful stimuli, emerging evidence implicate the role of NLR family members including NLRP1, 3 and 4 as important component of innate immune response to form inflammasome complexes \textsuperscript{59}. Inflammasome complexes are activated upon sensing relevant stimuli and undergo oligomerization with NLR protein to form a caspase-1 activating scaffold\textsuperscript{73}. The activation of caspase-1 subsequently leads to the procession of precursor pro-IL-1β and pro-IL18 to their biologically active IL-1β and IL18 forms.
respectively\textsuperscript{73}. Among the known inflammasomes to date, the NLRP3 inflammasome is functionally the most well characterized inflammasome that has been studied in a variety of inflammatory diseases. A comprehensive review on the role of inflammasome in different inflammatory disease, the reader is referred to elsewhere\textsuperscript{74,75}.

**Polymorphisms in the NLRP3 and CARD8 Genes**

To date, several SNPs in the NLRP3 region have been genotyped to study the association to several diseases with inflammatory background.

![Schematic representation of polymorphisms in the NLRP3 gene investigated in relation to inflammatory diseases. Exons of NLRP3 gene are displayed as white boxes (not to scale). Upregulation of NLRP3 is indicated as (↑) beside the SNP rs number, polymorphisms with unknown biological function are not labeled. The lower panel represents the different domains of NLRP3, PYD, Pyrin domain; NAD, NACHT associated domain; and LRR, Leucine-rich repeat\textsuperscript{74}.](image)

The functionally well-known Q705K polymorphism (rs35829419) in the NLRP3 gene was found to confer protective effect in several different diseases with inflammatory background, like Alzheimer’s disease and celiac disease, but not in type 1 diabetes or rheumatoid arthritis\textsuperscript{74}. Also, in another study, the SNP rs35829419 revealed a significant association to increased IL-1β levels and showed a trend to the lower levels of CRP in plasma \textsuperscript{76}. Several other SNPs are found to exert deleterious effect to the susceptibility...
of diseases. The rs4353135, rs4266924, rs55646866, rs6672995, rs107635144 and rs10733113 SNPs are located in a regulatory region downstream the NLRP3 gene (Figure 4) were found significantly associated with Crohn's disease (CD) in five European cohorts. Although these SNPs were strongly associated to the risk of CD, the association to NLRP3 expression and IL-1β production was conferred only by rs4353135 and rs6672995 respectively. The risk alleles of rs4353135 and rs6672995 were found to be associated with lower NLRP3 and IL-1β expression respectively in the peripheral blood of healthy donors. On contrary, no significant association was found between the variants and CD in a different sample set of CD patients from UK. The conflicting results due to the lack of replication should therefore be interpreted cautiously.

![Figure 4: Schematic representation of a polymorphism in the CARD8 gene and mRNA isoforms (modified from Bagnall et al 2008 (not to scale)].

Exons of the CARD8 gene are displayed as white boxes. The arrow (→) represents the site of open reading frame (ORF) for the given isoforms.

In addition to the Q705K polymorphism, several studies have shown the association of C10X polymorphism in the CARD8 gene (rs2043211; Figure 4) with different inflammatory diseases including inflammatory bowel dis-
ease, rheumatoid arthritis, and Alzheimer’s disease\textsuperscript{74}. However the pathophysiological role of the polymorphism in association with the diseases remains unknown. The C10X polymorphism is a non-sense mutation in exon 5 of the CARD8 gene that results in a truncated CARD8 protein\textsuperscript{78}. The functional consequence of the truncated CARD8 protein remains to be investigated. The C10X polymorphism leads to the A to T transversion and accounts for mainly two known isoforms of CARD8, T48 and T54\textsuperscript{78}. The T48 and T54 isoforms are the transcription consequence of the polymorphism that leads Cys>Stop at codon 10 and Phe>Ile amino acid substitution at codon 52 respectively. In addition to T48 and T54, three additional isoforms of CARD8 includes T47, T51 and T60 with varying transcriptional consequence of C10X variants\textsuperscript{78}. Also, the transcription of T47 begins downstream of the C10X variant and remain unaffected from the transcriptional consequence of the variant leading to almost functional CARD8 protein\textsuperscript{78}. This might also explain the expression of CARD8 in the individuals that are homozygous for the rare C10X variant. However, a detailed functional analysis of the different isoform of CARD8 in relation to CVD remains to be performed.

**NLRP3 Inflammasome**

Cryopyrin associated periodic syndrome (CAPS) is a group of autosomal dominantly inherited diseases comprising of familial cold autoinflammatory disease (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurological cutaneous articular syndrome (CINCA), earlier known as neonatal onset multiple inflammatory syndrome (NOMID). The three diseases share overlapping characteristics and clinical symptoms of recurrent fever, increased white blood cell count and inflammation. The underlying common cause of CAPS is gain of function mutations in the NLRP3 gene, which leads to increased release of IL-1β\textsuperscript{79,80}. The gene is composed of 9 exons, where exon 1 corresponds to the PYD domain, exon 2-3 encodes the NACHT domain and exon 4-9 encodes the LRR domain\textsuperscript{81} (Figure 3). The majority of the CAPS related mutations in NLRP3 region are found in exon 3, that corresponds to the NACHT domain (http://fmf.igh.cnrs.fr/IS-SAID/infevers/), indicating the importance for this region for the function of the NLRP3 protein.
The NLRP3 scaffold protein (118kDa) is mainly expressed in the cytosol of monocytes, granulocytes, dendritic cells, T and B cells, osteoblasts and epithelial cells\textsuperscript{82,83}. The NLRP3 scaffold protein is widely known to be a part of a trimeric protein complex, the NLRP3 inflammasome, which consists of ASC (PYCARD) adaptor protein and caspase-1 protein\textsuperscript{73}. Several different factors such as PAMPs, like bacterial lipopolysaccharide and different microorganisms (\textit{C.albicans, S.cerevisiae, L.monocytogenes, S.aureus, P.gingivalis}) and viruses (adenovirus, Sendai virus, influenza virus) are implicated to activate the NLRP3 inflammasome\textsuperscript{84-93}. Furthermore, DAMPs, like, monosodium urate, uric acid, elevated glucose levels, extracellular ATP, cholesterol crystals, calcium pyrophosphate dehydrate (CPPD), and different pollutants (silica, UV radiation, asbestos, skin irritants) have also been known as NLRP3 inflammasome activators\textsuperscript{94}. The assembly of NLRP3 multiprotein complex, leads to auto cleavage of procaspase-1 to active caspase-1 followed by processing of the premature proinflammatory cytokines IL-1\(\beta\), IL-18 and IL-33 to their active forms\textsuperscript{95-97}.The processing and release of IL-1\(\beta\) by NLRP3 inflammasome signalling requires two signals. The priming signal 1 (e.g., toll-like receptor [TLR]\textsubscript{4} agonists and certain inflammatory cytokines like tumor necrosis factor [TNF]-\(\alpha\)) primes the inflammasome by inducing the expression of pro IL-1\(\beta\) and pro IL-18. The NLRP3 inflammasome activation signal 2 (NLRP3 activators, i.e. DAMPs and PAMPs) promote the assembly of the NLRP3 inflammasome and caspase-1 activation to process the pro IL-1\(\beta\) to its mature form\textsuperscript{98}. Several mechanisms including, lysosomal destabilization, due to phagocytosed particles and crystals\textsuperscript{99,100}, mitochondrial damage, due to intracellular K\(^+\) efflux and Ca\(^{2+}\) mobilization\textsuperscript{101-105}, and ROS induction by mitochondria and NLRP3 activators\textsuperscript{106,107} can contribute to the assembly of the NLRP3 inflammasome protein complex for the downstream processing of IL-1\(\beta\) (Figure 5).

Among the negative regulators of the NLRP3 inflammasome, studies have found certain proteins of microbial origin\textsuperscript{108,109}, and endogenous origin like the TRIM family proteins, nitric oxide, microRNA, IFNs, CD40 ligands and autophagy, that serve as checkpoints to prevent the accidental over expression and hyperactivity of the inflammasome\textsuperscript{110-117}. 
Figure 5: Pathway for the activation of NLRP3 inflammasome complex: The activation of the NLRP3 inflammasome can be triggered by crystalline structures, ATP, reactive oxygen species (ROS), muramyldipeptide (MDP) and pathogen associated molecular patterns (PAMPs). The NLRP3 inflammasome activates IL-1β via caspase-1, which results in caspase-1 dependent cell death (pyroptosis) and the cleavage of glycolysis enzymes that result in macrophage activation.

CARD8 (Cardinal/TUCAN)

The caspase recruitment domain (CARD) was first identified as a protein-protein interaction motif in caspases, as key proteins in the regulation of apoptosis. The CARD motif is also known for its function as a scaffolding molecule in signaling pathways to induce inflammatory responses by activating NF-κB. In the past decade, several CARD containing proteins, such as Nod1, Nod2, CARD10, Bcl10, CARD11, CARD14 have been identified and known to functionally responsible for the activation of NF-κB. The CARD proteins together with a linker protein (Bcl10) and effector protein (MALT1) form a signaling complex, known as the signalosome that activates the NF-κB via the activation of IKK complex. The activation of NF-κB subsequently leads to either an apoptotic or a proinflammatory response.
response to combat a certain pathophysiological condition. However, disruption of the signalosome may contribute to important pathophysiological consequences. A study from Delekta and coworkers showed that disruption of such signalosome blocks the thrombin dependent adhesion of monocyte to endothelial cells by preventing the thrombin mediated induction of adhesion molecules such as ICAM-1 and VCAM-1\textsuperscript{123}.

The CARD8 (also known as TUCAN/CARDINAL) protein has in some studies also been associated to the NLRP3 complex, although its role for inflammasome activation is not completely clear\textsuperscript{95}. Initially, CARD8 was shown to be a regulator of NF-κB, caspase-1 activation and NOD2 signaling\textsuperscript{118,124-126}. Though, recent studies have shown that CARD8 negatively regulates NLRP3, studies have also shown that CARD8 has no role on the IL-1β release\textsuperscript{127,128}. The CARD8 gene is located on chromosome 9q13 and consists of 13 exons. The two common CARD8 mRNA isoforms are the T48 isoform that encodes a 432 amino acids long protein starting from exon 5 and the T57 isoform, which encodes a 487 amino acids long protein, starting from exon 4 of CARD8 (Figure 4). Functionally, the CARD8 protein is also involved in the suppression of NF-κB pathway signaling, thereby regulating the inflammatory genes\textsuperscript{124}. In addition, CARD8 is also found to regulate apoptosis by directly interacting with the caspase proteins\textsuperscript{129}, but its role in the regulation of inflammation and CVD is still not known.

**NLRP3 Inflammasome in Atherosclerosis**

Considering the importance of inflammation in atherosclerosis, studies have focused on the role of the NLRP3 inflammasome in the pathophysiology of atherosclerosis\textsuperscript{37,130}. Some initial mouse studies showed conflicting data regarding the role of the NLRP3 inflammasome in CVD. Duewell and coworkers showed in 2010 that hypercholesterolemic Ldlr\textsuperscript{-/-} mice reconstituted with bone marrow from mice deficient in Nlrp3, Asc or IL-1a/b developed less atherosclerosis than those reconstituted with wild type bone marrow\textsuperscript{27}. Similarly, cholesterol-crystal induced inflammasome dependent IL-1β secretion was abolished in macrophages with defective NLRP3 gene\textsuperscript{130}. This indicate that NLRP3 plays an important role in the development of atherosclerosis. However, on the other hand, Menu and coworkers showed that ApoE\textsuperscript{-/-} mice interbred with Nlrp3\textsuperscript{-/-}, Asc\textsuperscript{-/-} or Caspase-1\textsuperscript{-/-} mice did not show any difference in atherosclerosis progression or macrophage infiltration compared to ApoE\textsuperscript{-/-} mice\textsuperscript{131}.
When it comes to human CVD, elevated expression of NLRP3 protein was observed in the aorta tissue of patients undergoing coronary artery bypass graft (CABG) surgery and positively correlated with severity of coronary artery diseases\textsuperscript{132}. Furthermore, increased expression of NLRP3 was found in patients with diabetes, hypertension and smoking habits\textsuperscript{132}. The elevated expression of NLRP3 was shown to positively correlate with the total cholesterol, lipoprotein and negatively correlated to the HDL-c levels in the serum, indicating that cardiovascular risk factors may promote the expression of NLRP3 by regulating the CVD associated risk factors\textsuperscript{132}. The expression of NLRP3 and its downstream released cytokines were found to be significantly increased in the human peripheral blood monocytes from the coronary artery disease (CAD) patients\textsuperscript{133}. Accumulation and crystallization of cholesterol during atherogenesis is a hallmark in the classification of pathological and advanced atherosclerotic lesions\textsuperscript{29}. Also, these crystals were able to induce NLRP3 mediated caspase-1 dependent IL-1$\beta$ release in the primed human PBMC and macrophages\textsuperscript{27,130}, thereby indicating the possibility of cholesterol crystals to act as an endogenous danger signal in the atherosclerotic lesions. The crystals may induce the translocation of the lysosomal proteolytic content into the cytosol, which is sensed by the NLRP3 inflammasome via an unknown mechanism\textsuperscript{27}. Studies have also shown that oxidized LDL, can prime cells for NLRP3 inflammasome activation\textsuperscript{27,134}. Also, the CD36 that is implicated in the pathophysiology of atherosclerosis can recognize Ox-LDL and initiate both signal 1 and 2 for the complete activation of NLRP3 inflammasome in the mice models\textsuperscript{135,136}. In addition to CD36, the Ox-LDL can upregulate P2X7R and promote the production of NLRP3 mediated IL-1$\beta$ release by inducing PKR phosphorlylation in THP-1 macrophages\textsuperscript{137}. In a recent study, IL-1$\beta$ production through NLRP3 inflammasome activation was shown to promote myocardial inflammation and systolic dysfunction \textsuperscript{138}. Taken together, previous findings suggest a potential role of the NLRP3 inflammasome in the atherosclerotic process.

**NLRP3 Downstream Signaling in Atherosclerosis**

The significance of investigating the role of NLRP3 in the pathophysiology of CVD can be partly attributed to importance of NLRP3 mediated IL-1$\beta$ signaling in inflammatory diseases. A large number of studies have indicated the biological and pathological significance of IL-1$\beta$ in vascular inflammation and atherosclerosis. Studies have suggested that lack of IL-1$\beta$ reduces the severity of atherosclerosis to 30\% in mice by regulating the expression
of adhesion and chemotactic molecule in the aorta\textsuperscript{139}. Also, the selective deficiency of IL-1\(\alpha\) or IL-1\(\beta\) in bone marrow derived cells inhibited atherosclerosis in mice, possibly by the inhibition of the release of cytokines from macrophages\textsuperscript{140}. Studies have also shown that the overexpression of the IL-1Ra, the natural blocker of both IL-1\(\alpha\) and IL-1\(\beta\), significantly reduces the aortic inflammation and atherosclerotic lesion area in the ApoE-KO mice respectively\textsuperscript{140-142}. Moreover, the complete absence of IL-1R1 significantly reduced the progression of atherosclerosis in mice deficient in IL-1Ra, when subjected to high fat diet and \textit{P.gingivalis} infection\textsuperscript{143}.

In addition to IL-1\(\beta\), IL-18, which is another member of the IL-1 family, is known to be processed as a result of NLRP3 activation\textsuperscript{144,145}. IL-18 is highly expressed in human carotid atherosclerotic plaque and is associated with plaque stability\textsuperscript{146}. The endogenous inhibitor of IL-18, IL-18 binding protein (IL-18BP) have shown to inhibit plaque development, progression and induces plaque stability in the ApoE-KO mice\textsuperscript{147}, thereby suggesting the antiatherogenic property oh IL-18BP\textsuperscript{148}. However, a recent publication by Wang \textit{et al} gave a new angle to the Il-18 signal in atherosclerosis\textsuperscript{149} suggesting the participation of IL-18 in atherogenesis by binding to the IL-18 receptor and Na-Cl co-transporter in ApoE-KO mice\textsuperscript{149}. Moreover, IL-18 has been shown to induce the expression of IFN-\(\gamma\) and CXCL16 in macrophages, NK cells and SMCs, suggesting the pro-atherogenic role of IL-18 in the upregulation of scavenger receptors in the vascular and immune cells\textsuperscript{150-152}.

Unlike IL-1\(\beta\) and IL-18, another member of the IL-1 family, IL-33, has been shown to exhibit a protective role in the development of atherosclerosis by inducing IL-5 and ox-LDL antibodies\textsuperscript{153}. This study also showed that the role of IL-33/ST2 signaling in the production of protective autoantibodies in atherosclerosis by regulating the Th1/Th2 balance\textsuperscript{153}. The full length precursor IL-33 is also a nuclear factor that binds to heterochromatin and has transcriptional repressor properties\textsuperscript{154}. The protein is highly expressed in healthy endothelial cells of the blood vessel and is downregulated when exposed to the pro-inflammatory stimulants\textsuperscript{155}. In context to NLRP3 activation, the processing of IL-33 by caspase-1 leads to the inactivation rather activation of IL-33, thereby explaining the functional differences between the IL-33 from the other NLRP3 mediated caspase-1 processed cytokines\textsuperscript{156}.
Though, the precursor IL-33 is potent inducer of pro-inflammatory cytokines in mast cells and other immune cells\textsuperscript{157-159}, the pro- or anti-inflammatory effects of IL-33 depends on the disease and the disease model\textsuperscript{160}. 

\textit{Innate immunity in human atherosclerosis and myocardial infarction: Role of CARD8 and NLRP3.}
AIM OF THIS THESIS

The overall aim of the thesis was to elucidate the influence of NLRP3 inflammasome and CARD8 protein in the pathophysiology of atherosclerosis and to the risk of myocardial infarction.

The specific focus of each study were as follows:

- To study the NLRP3 inflammasome in the pathophysiology of human atherosclerosis and to assess the association of variants located in the downstream regulatory region of NLRP3 to the risk of MI. (Paper I)

- To investigate the effect of Q705K polymorphism in the NLRP3 gene to the risk of developing MI. (Paper II)

- To investigate CARD8 in human atherosclerosis and the association of CARD8 variant, rs2043211 to the susceptibility to MI. (Paper III)

- To elucidate the role of CARD8 in the regulation of inflammatory cytokines and chemokines in vascular cells. (Paper IV)
MATERIALS AND METHODS

Biobanks and Ethics

In the present study, we have used three different cohorts with human material, which will be described below. The sampling and the baseline characteristics of the BiKE, FIA and SCARF cohorts have previously been described \(^{161-163}\). All studies had been ethically approved and were conducted in accordance with the declaration of Helsinki.

Human carotid plaque tissue and control tissues were from the Biobank of Karolinska Endarterectomies (BiKE) cohort at Karolinska University Hospital, Stockholm, Sweden. The carotid plaque tissues (n=106) were obtained from the patients with >70 % carotid artery stenosis undergoing carotid endarterectomy surgery and the control vessel tissues (n=10) were iliac arteries devoid of macroscopic atherosclerosis from organ donors. PBMC (n=98) were collected from patients at the same time. The tissues were collected and handled in a standardized manner to limit the inconsistency in the quality of data. The clinical parameters of the patients were recorded in the database\(^{164,165}\) and linked to the global gene expression patterns. The clinical presentation of the symptomatic plaque including transient ischemic attack (TIA), stroke, and amaurosis fugax (AF) were evaluated for stroke preventive carotid intervention as per to American and European guidelines\(^{166,167}\). The asymptomatic carotid plaque is usually identified from the cervical bruits during the clinical investigation of nonspecific symptoms and preoperative evaluation for cardiac or other surgery. The use of perioperative carotid lesion over post-mortem tissues limits increased risk of tissue degradation. (Paper I and Paper III)

The Stockholm Coronary Atherosclerosis Risk Factor (SCARF) cohort is a case-control study with 387 MI patients and 387 healthy controls. Patients admitted for acute MI in the coronary care units were identified from the three hospitals (Danderyd Hospital, Karolinska Hospital and Norrtälje Hospital) of northern Stockholm. Age and sex matched healthy control individuals were recruited from the population of the same county. The study was designed to investigate the role of genetic, biochemical and environmental risk factors to the susceptibility of MI. Exclusion criteria for the pa-
tients were type I diabetes mellitus, renal insufficiency, chronic inflammatory disease, drug addiction, psychiatric disease, and concomitant disease. (Paper I and Paper III)

The First-ever myocardial Infarction study in AC-county (FIA) cohort, a prospective nested case-control study, comprised of DNA from 555 MI patients and 1016 controls from the Northern part of Sweden. For the FIA cohort, cases of first definite MI (fatal and non-fatal) and suspected fatal MI that occurred prior to January 1, 2000 were included in the study. Cases were identified through screening of hospital discharge records, general practitioners’ reports, and death certificates, in accordance with WHO and MONICA criteria\textsuperscript{168}. For each case, two healthy control individuals were selected matched for sex, age and geographic area\textsuperscript{169}. Exclusion criteria for cases were stroke, acute myocardial infarction (AMI), or cancer within the five years prior to or one year after AMI. (Paper I, Paper II and Paper III)

**Global Gene Expression Analysis and Polymorphism Imputation**

Several methods can be used for quantifying the mRNA content in a sample, such as Northern blots, expression arrays, real-time PCR and high-throughput RNA sequencing. However, the microarray gene expression platform was selected based on the cost effectiveness, accuracy and precision. Purification and quantification of RNA was performed using Qiagen RNeasy kit and Nanodrop respectively. The mRNA expression of the inflammasome components, including CARD8 in the samples from BiKE biobank, was assessed using Affymetrix HG-U133 plus 2.0 gene chip arrays. The Affymetrix HG-U133 plus 2.0 array includes probe set focusing the 3’ region of the gene and the intensity of their hybridization is the value for the quantity of entire gene. The expression data were normalized by using the RMA algorithms with log2 transformation (Paper I and Paper III). Imputation was performed for the C10X polymorphism rs2043211 in the CARD8 gene based on the information from proximal SNP (rs6509365) and linkage disequilibrium ($r^2=0.925$) obtained from 1000 genome project using MACH 1.0 algorithm. However, due to the lack of good proximal SNP for Q705K polymorphism, the technique was not used in the Paper II. The quality of imputation was an Rsq value of 0.70 (Paper III).
Genotyping

To date, numerous genome wide association studies (GWAS) have been performed with respect to various diseases and have uncovered several genetic susceptibility loci associated with increased disease risk. However, the SNPs identified with GWAS technique probably account for a fraction of the hereditary factors, since the remaining heritability lie in genetic alterations that do not achieve genome wide significance\textsuperscript{170}. Therefore, we performed association studies using a candidate gene approach. Candidate gene studies are suited for detecting genetic variants underlying common and complex diseases where the risk of association with the gene of interest might be relatively small.\textsuperscript{171}

The genotyping of the polymorphism were performed using TaqMan\textsuperscript{®}SNP Genotyping Assay with 7900HT real time PCR system (Applied Biosystems). The assay contained two primers for the polymorphism of interest and two probes to distinguish between the alleles. The probe mixes comprised of reporter dyes (VIC and FAM dyes for allele 1 and 2 respectively), a minor groove binder to increase the melting temperature of the probe length and a non-fluorescent quencher to inhibit the fluorescent signal generated by the reporter dye that is in proximity. The fluorescent signals are generated by hybridization after the hybridized probes are cleaved from the complementary sequence, followed by release of VIC or FAM fluorescence (homozygous genotype) or both (heterozygous genotype). (Paper I, Paper II and Paper III)

Cell Culture

For several years, endothelial cell culturing has been used as an \textit{in vitro} model to study the pathophysiology of atherosclerosis. Human Umbilical Vein Endothelial cells (HUVEC) are the adherent primary cells that can be readily cultured in large amounts. The culturing and growth of HUVEC depends largely upon the culturing medium. HUVEC can be cultured in serum free growth medium for 12 hours, however the absence of growth factors for longer hours can trigger apoptosis in the cells. In our experiments, the HUVEC were cultured in a low serum medium containing several growth supplements such as VEGF, IGH, EGF, heparin sulfate, FGF, hydrocortisone hemisuccinate and ascorbic acid (Lifeline Cell Technologies,
Walkersville, MD, USA) (Paper IV). The growth factors are essential to simulate the physiological environment of the HUVECs. Studies have shown that the deprivation of growth factors can differentiate the HUVEC in to smooth muscle-like cells\textsuperscript{172,173}.

**Human Atherosclerotic Carotid Plaque Model**

Human atherosclerotic lesions comprises of a variety of cell types that contribute to a complex inflammatory milieu. To assess the relevance of NLRP3 inflammasome activation, we used an *ex-vivo* model of human atherosclerotic carotid plaque obtained from endarterectomies. The major advantage of this model is the possibility of assessing the influence of various moieties on aggravating inflammation due to the inflammatory cascade and pathways due to cellular interplay. Though the *ex-vivo* model is efficient in investigating the local inflammatory cascades, there are certain limitations associated with the model, like lack of systemic influence, long-term analysis and hemodynamics (Paper III).

**Gene Silencing using siRNA**

Gene silencing using small interfering RNA (siRNA) has emerged as a powerful tool to study the gene function in cultured cells. The method was developed by Andrew Z Fire and Craig C Mello, for which they were awarded the Nobel Prize in Physiology or Medicine in 2006\textsuperscript{174}. Silencing of genes using synthetic small interfering RNA (siRNA) mediate RNA interference (RNAi) in mammalian cells by hybridizing and degrading the target complementary mRNA and thereby suppressing the expression of a target gene. The silencing of the gene expression using siRNA depends on a number of factors such as transfection efficiency, transcription rate of selected genes, cytotoxicity and rate of cell division\textsuperscript{175}. In brief, the short double stranded oligo siRNA are complex bound with liposomes in the transfection reagent to traverse the cell membrane\textsuperscript{176}. The positive charged liposomes encapsulate the negatively charged siRNA and forms a siRNA-lipid complex, promoting efficient endocytosis of siRNA by the cells. Once internalized, the siRNA integrates with RNA induced silencing complex (RISC) and undergoes strand separation of the siRNA molecule. The antisense strand hybridizes to the complementary target mRNA in the cell and degrade the targeted mRNA, thereby knocking down the expression of the target gene\textsuperscript{174}. The negative control siRNA used had no homology with any vertebrate gene.
Addressing the issue of off-target effects of siRNA\textsuperscript{177}, the use of several pooled siRNA targeting the same gene at different locations helps to limit the off target effects of siRNA and confirm the phenotype specificity. The use of several individual siRNA targeting different exons of the same gene can minimize the risk of missing valid targets by interfering with all the possible isoforms of the gene. (Paper IV)

**Immunostaining**

Immunostaining has emerged as a powerful tool to investigate a cellular marker and co-localization of target proteins in the tissue and has provided diagnostic, prognostic and predictive information of several diseases, including atherosclerosis\textsuperscript{178}. The basic principle used in the technique is that the primary antibody binds to the tissue constituent (protein/epitope). The secondary antibody from the same species as that of the primary antibody recognizes the tissue bound primary antibody. The label (enzyme, fluorescence and avidin-biotin) conjugated to the secondary antibody enables to visualize the presence of target proteins.

**Immunohistochemistry**

Immunohistochemical staining was performed using commercially available antibodies. Paraffin embedded sections were deparaffinized, rehydrated and subjected for antigen retrieval in a boiling antigen retrieval buffer. The pretreatment with antigen retrieval agents ensures the uncovering of the antigen sites of the protein formed during the formalin fixation of the tissues. Nonspecific background staining was reduced by using blocking reagent to reduce the amount of endogenous peroxidase activity, which may result in false-positive results. After blocking, the slides were incubated with primary antibody for 1 hour at room temperature followed by incubation with secondary horseradish peroxidase (HRP) polymer to visualize with 3,3’-diaminobenzidine (DAB) substrate. HRP together with DAB method utilizes a HRP conjugated secondary antibody against the primary antibody and DAB as substrate to visualize the staining of the target protein. The sections were counterstained with hematoxylin to stain the nucleus and mounted. The DAB staining produces a dark brown reaction for the target protein and the nucleus appears deep blue-purple color. (Paper IV)
Immunofluorescence

Indirect immunofluorescence (IF) was performed to detect proteins in cultured cells. The technique requires fluorescent dye conjugated antibodies that can be used directly or indirectly to bind the target antigen. The technique can be used both on live and fixed cultured cells depending upon the type of fluorescence techniques. Usually direct IF technique is employed for staining of live cells. Though the staining is quick and simple, the signals produced are usually low when compared to the indirect IF. Mammalian cells contain flavin coenzymes and reduced pyridine nucleotides that is responsible for biological autofluorescence. Fixing the cells and tissues with aldehydes may also produce high level of autofluorescence, which can be minimized by washing the fixed cells with 0.1% sodium borohydride in phosphate-buffered saline prior to antibody incubation. A significant problem with most fluorescence-based techniques is photobleaching. The generation of reactive oxygen species in the specimen results in photochemical destruction of the fluorophore which thereby losses it fluorescent capacity. Photobleaching can be minimized by using antifading reagents and reducing the excitation light intensity and duration. (Paper IV)

Western Blot

Western blot, also known as protein blot or immune blot is a common technique used to detect and quantify proteins. In brief, the proteins are denatured by heat and using an ionic detergent SDS (sodium dodecyl sulphate) and a reducing agent DTT (dithiothreitol) before loaded on to a gel containing SDS. The SDS bind to the denatured uncoiled protein molecule and DTT cleaves the disulfide bond to eliminate the tertiary and quaternary protein structures. The denatured protein samples are electrophoresed to separate the proteins based on the size. The proteins are then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane using electrophoretic transfer. When compared to other membranes, such as nitrocellulose membrane, PVDF has higher binding capacity and durability. During the electrophoretic transfer, the gel containing the proteins is placed in direct contact with the membrane and sandwiched between the electrodes submerged in the conducting buffer of the electrophoretic chamber. After the transfer, the membrane containing the protein is subjected to a blocking buffer to block the unreacted areas of the membrane and to improve the sensitivity of the assay by reducing the background interference. The two most frequently used blocking agents are non-fat milk and bovine serum
albumin (BSA). When detecting phosphorylated protein, BSA is preferred over milk to limit the interference of phosphoprotein casein in milk. After blocking, the membrane is incubated with primary antibodies. The selection of primary antibodies depends on the protein epitope and the number of proteins to be detected. For multiplex detection on membrane, the choice of antibody depends on the host species that was used to raise the antibodies and the labels conjugated to the secondary antibody. The primary antibodies raised in two different species can be used to probe the membrane together or separately and the corresponding species specific secondary antibodies are used for detection. Among the different labels conjugated with secondary antibody, such as biotin, enzymes and fluorescent probes, the HRP conjugated secondary antibodies are widely used due to its high sensitivity and the transient reaction product formation when subjected to chemiluminescent substrates. Recently, the development of the IRdye fluorophores labeled IgG secondary antibody have facilitated the multiplex detection with high sensitivity and low background. (Paper IV)

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Enzyme-Linked Immunosorbent Assay (ELISA) is a microplate based assay technique used for quantifying and detecting proteins, antibodies and peptides. Among the different types of ELISAs\(^\text{179}\), the sandwiched ELISAs are most commonly used due to high sensitivity and the direct use of sample without purification. The technique utilizes a protein specific capture antibody immobilized in the microplate well to capture the target protein from heterogeneous mixture of sample. The biotin conjugated detection antibody binds to target protein epitope and produce color in the presence of substrate. The technique is more quantitative than western blotting and confers several advantages. The calibrated standards enables quantification of the protein and the use of two antibodies specific for the target protein increases the sensitivity of technique. The high sensitivity also facilitates the detection of low abundant proteins using ELISA (Paper I, III, IV).

**Statistics**

The choice of statistical test depends on the scientific question, the data structure and the study design. Student’s T-test is a parametric test for the normally distributed data and is widely used to compare means of two independent groups. The non-parametric test for a similar data would be
Mann-Whitney U test. Two tailed Student’s T-test was used for analyzing the difference in mRNA expression of inflammasome proteins in the carotid lesion compared to healthy vessels and also between the control and CARD8 knock down cells. The significance level was P<0.05. The association between the gene mRNA expression and genotype in plaque and PBMC was analyzed using a nonparametric regression model known as additive linear regression model. For the *ex-vivo* carotid plaque experiments, the analysis was performed using Mann Whitney U-test. The statistical analysis of cytokine profile includes the STATISTICA 7.1 software (StatSoft). The association between cytokines and genotyped SNP was performed using linear regression with an additive genetic model and adjusted for age, sex and status using PLINK. A web-based calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to test the deviation of the SNP from the Hardy-Weinberg equilibrium. Association between SNPs and MI were analysed using $\chi^2$ test with SPSS software package (SPSS Inc) and EpiInfo software 2008 (Centre for Disease Control and Prevention). For the statistical comparison of more than two groups, One-way ANOVA with Bonferroni post-test was performed using Graph-Pad Prism (version 5.01).
RESULTS AND DISCUSSION

The NLR scaffolding proteins, CARD and NLRP are known to impart inflammation driven immune responses by forming a protein complex\textsuperscript{182}. NLRP3 inflammasome has been widely studied for its role in the pathophysiology of inflammatory diseases, but its role in CVD is still not fully understood. In some studies the CARD8 protein has also been shown to be functionally associated with NLRP3 inflammasome\textsuperscript{95,127}, although its role in the NLRP3 inflammasome activation remains controversial\textsuperscript{128}. The main aim of this thesis was to investigate NLRP3 and CARD8 in the pathophysiology of atherosclerosis and to the risk of MI.

**NLRP3 Inflammasome Components and CARD8 are Expressed in Human Atherosclerosis**

In the present thesis, elevated mRNA expression of several NLRP3 inflammasome-related genes in human atherosclerotic plaque tissue was revealed. The expression of *NLRP3, CARD8, ASC (PYCARD), CASP1, IL-1β*, and *IL18* mRNA were found to be markedly increased in atherosclerotic lesions compared to control vessels (Paper I and Paper III). The expression of NLRP3 and ASC was also verified on the protein level in carotid plaque, where the expression was found in the intima with some infiltrating CD68 positive macrophages (Paper I; Figure 6). Expression of the *NLRP3* inflammasome-related components in atherosclerotic lesions therefore suggest a possible role of NLRP3 inflammasome activation in human atherosclerosis, though the possibility of activation of other inflammasomes (like NLRP1 and AIM2 inflammasomes) cannot be excluded. In atherosclerotic lesions, the expression of NLRP3 and ASC was shown in the extracellular space of the necrotic area (data not shown). The expression of these proteins in the extracellular space might be due to the secretion from the cell, or a result of dead cells in the necrotic core. Studies have shown that the activation of NLRP3 inflammasomes may however lead to extracellular accumulation of NLRP3 and large ASC complexes, called ASC specks, which may act as possible danger signals to amplify the inflammatory response\textsuperscript{183,184}. Also, the expression of NLRP3 was found to be co-localized with the expression of CD68 in the atherosclerotic plaque (Paper I). Taken together with the association between the levels of *NLRP3* mRNA expression and CD68 mRNA
in the atherosclerotic lesion, indicates the recruitment of macrophages medi-
ate a self-perpetuating inflammatory response, thereby increasing the
plaque burden (Paper I).

Figure 6. Expression of NLRP3 (Red), ASC(Red) and CD68(Green) in hu-
man carotid plaque. Arrows indicate the expression of NLRP3 (black ar-
row, upper panel), ASC (black arrow, lower panel) and CD68 (blue/white
arrow in upper and lower panel). The scale bar for the 1.54x and 80X ob-
jectives are 1mm and 20µm respectively.

The accumulation of macrophages is a hallmark in the atherogenic process
and contribute to the initiation, progression and plaque rupture of ather-
sclerotic lesions. Also, the role of macrophages in plaque progression and
plaque destabilization might be partially due to the consequence of inflam-
matory milieu generated as a result of NLRP3 inflammasome activation.
Our results are consistent with several other studies on the expression of
NLRP3 by CD68 positive macrophages. The expression of smooth
muscle actin (SMA) was evident in the intimal region with sparse expression
of NLRP3 and ASC (Figure 7). The expression of NLRP3 and ASC in the
SMC in the intima region may in addition indicate that SMC can produce
NLRP3 inflammasome dependent IL-1β secretion in the atherosclerotic lesion (Paper I). Furthermore, the significantly increased expression of NLRP3 was observed in symptomatic patients when compared to asymptomatic patients, suggesting the possibility of aggravating inflammation in the advanced lesion\textsuperscript{188} (Paper I).

In the present thesis, we also studied CARD8 in atherosclerosis and myocardial infarction. CARD8 was in some early studies found related to the NLRP3 inflammasome complex, but more recent contradictory results exist\textsuperscript{128}. Significantly elevated expression of CARD8 mRNA was evident in atherosclerotic lesions compared to the expression in transplant donor vessels (Paper III). Our results were also verified on the protein level, where the expression of CARD8 was found in the endothelial cells in the atherosclerotic lesion (Paper IV).

\textbf{Figure. 7.} Expression of NLRP3 (Red), ASC (Red) and SMA(Green) in the human carotid plaque. Arrows indicate the expression of NLRP3 (black arrow, upper panel), ASC (black arrow, lower panel) and SMA (blue/white arrow in upper and lower panel). The scale bar for the 1.54x and 80X objectives are 1mm and 20\textmu m respectively.
In addition to endothelial cells, some other cells expressed CARD8 in the lesion, but the specific cell type remains to be investigated. In the normal vessel, the CARD8 expression was predominantly found in the endothelial layer but also in the smooth muscle cells (Figure 8). In the cultured endothelial cells, the expression of CARD8 was mostly present in the cytoplasm and sparsely in the nucleus, which is in line with studies showing both cytoplasmic and nuclear localization of CARD8 in the cells\textsuperscript{124,126}. The expression of CARD8 in the endothelium could be further confirmed in mRNA microarray data generated from human tissue samples and deposited in the Oncomine databank\textsuperscript{189}.

**Functional Role of NLRP3 inflammasome and CARD8 in Human Atherosclerosis**

As NLRP3 is involved in IL-1β processing, we analyzed the IL-1β release in an *ex-vivo* atherosclerotic plaque model after treatment with LPS, ATP and cholesterol crystals (Paper I). LPS, ATP, and particularly the combination of these two stimuli markedly enhanced the release of IL-1β. Notably, a similar pattern was also seen when cholesterol crystals were used in combination with LPS, which significantly increased the release of IL-1β. These
findings suggest that the NLRP3 inflammasome is activated in human atherosclerotic plaque when exposed to NLRP3 activating ligands. Importantly, even without addition of LPS, there was a significant release of IL-1β upon stimulation with ATP or cholesterol crystals, suggesting that the lesions were already primed for inflammasome activation (Paper I).

In the present thesis we showed altered expression of inflammatory markers in atherosclerotic lesions in relation to CARD8 gene (Papers III and IV). In Paper III we suggested that CARD8 plays a role on the immune response in the atherosclerotic lesion, why we investigated this by CARD8 knock down in HUVECs (Paper IV). The HUVEC cells were selected as a model due to a pronounced expression of CARD8 in the endothelial layer. The knockdown of CARD8 in the HUVEC reduced the basal release of several chemokines and cytokines that are functionally involved in the infiltration of the leukocytes in to the intima. Knock down of CARD8 in HUVEC significantly downregulated the expression of IL-6, Rantes, IP10, MCP-1 and ICAM on the both mRNA and protein levels. Also, preliminary data shows that proteins, including I-TAC, CXCL11, C5/C5a were also possibly regulated by CARD8. Loss of CARD8 in HUVEC thereby seem to abrupt the production and secretion of proteins actively involved in inflammatory response and leukocyte infiltration, thereby indicating the importance of CARD8 protein in regulating inflammatory proteins and leukocyte adhesion proteins in the endothelial cells.

In the last decades, studies has demonstrated the role of CARD proteins including, CARD4,-9,-10,-11,-14,-15 in the activation of NF-κB signaling. We therefore examined the signaling pathway involved in the regulation of cytokines by CARD8 in vascular cells. The expression of IκBα and phosphorylated-p65 was not influenced of the knock down of CARD8 when compared to the control cells, thereby suggesting the limited the role of canonical NF-κB signaling in the CARD8 dependent regulation of inflammatory and chemotactic proteins in HUVECs. However, the possible role of non-canonical NF-κB signaling mediated by p52-RelB complexes remains to be investigated (Paper IV).

Taken together, the NLRP3 inflammasome activation and the increased expression of CARD8 in the atherosclerotic plaque might be a possible contributor to the enhanced inflammatory response and leukocyte infiltration in the plaque progression and instability.
Effect of Genetic Variants of the \textit{CARD8} and \textit{NLRP3} to their mRNA Levels in Atherosclerotic Plaque and PBMC

The association of genetic variants in the \textit{CARD8} and \textit{NLRP3} genes have previously been shown to be related to the risk of diseases with inflammatory background\textsuperscript{74}.

In the present thesis, we therefore investigated the cis-regulatory effect of these genetic variants (rs4353135, rs4266924, rs6672995, and rs10733113) on the \textit{NLRP3} expression in atherosclerotic plaques and PBMC (Paper I, Figure 4). The homozygous rare genotypes of rs6672995, and rs10733113 were significantly associated with increased level of \textit{NLRP3} mRNA expression in PBMCs but not in atherosclerotic lesions. Our results are partially in agreement with Villani and coworkers in the association of the variants with the expression of \textit{NLRP3}, confirming the association of these variants in the downstream regulatory region of \textit{NLRP3} with the expression of the \textit{NLRP3} gene\textsuperscript{77}. However, the mechanism by which the variants increase the expression of \textit{NLRP3} remains to be elucidated.

In addition, the polymorphism rs34829419 encoding Q705K shift in exon 3 region of \textit{NLRP3} gene leads to constitutively activated NLRP3 inflammasome that subsequently leads to excessive production of IL-1\textbeta\textsuperscript{191}. However, the unavailability of the Q705K probe and the lack of good proxy SNPs in the human660W-Quad beadChip limited the analysis of this variant in relation to atherosclerosis, and this analysis could unfortunately not be performed (Paper II).

The association of the \textit{CARD8} polymorphism rs2043211 (C10X) to the expression of \textit{CARD8} was also investigated in atherosclerotic plaque from the BiKE cohort (Paper III). The homozygous rare variant of the \textit{CARD8} polymorphism rs2043211 was associated with lower \textit{CARD8} mRNA expression in the atherosclerotic plaque. The polymorphism generates a premature stop codon that possibly may affect the expression and function of \textit{CARD8}. The lower expression of \textit{CARD8} in homozygous carriers of the minor allele might however be due to the partial rescue of \textit{CARD8} expression by alternative splicing, as suggested by Bagnall and coworkers, who showed that the transcription of one of the isoforms (the T47 isoform) of
the CARD8 gene begins downstream of rs2043211 polymorphism\textsuperscript{78}. However, which isoforms expressed in the atherosclerotic lesions remains to be investigated.

Recent studies have also shown that CARD8 acts as a negative regulator of NLRP3, suggesting that lack of CARD8 may aggravate inflammation by rescuing the tightly regulated inflammatory components\textsuperscript{127}. Also, the role CARD8 in the suppression of caspase-1 and NF-κB\textsuperscript{118,192,193}, led to the assumption that the lower expression of CARD8 in the atherosclerotic lesion might increase the risk of inflammation in the homozygous carriers of the rare genotype. However, our functional data on the knock down of CARD8 in the endothelial cells suggest that the lack of CARD8 can restrict the inflammatory response in the lesion by down regulation of inflammatory and chemotactic proteins (Paper IV).

In addition to this, the variant rs2043211 of CARD8 gene was also associated to the increased mRNA expression of TMEM143 located in the vicinity of the CARD8 gene, conferring the cis-regulatory effect of the SNP to the expression of close proximity genes (Paper III). However, the role of TMEM143 in the pathogenesis of atherosclerosis is still unclear.

**Role of CARD8 and NLRP3 Genetic Variants in the Circulating levels of Inflammatory markers in Plasma of MI Patients and Controls**

In paper I, we examined the functional consequences of the genetic variants rs4353135, rs4266924, rs6672995, and rs10733113 in the NLRP3 downstream regulatory region in the IL-1β release. The minor alleles of the variants rs4266924 and rs10733113 were significantly associated with increased IL-1β levels in plasma of controls but not in MI patients. No association was found between these variants and IL-18, TNF-α, MCP-1 or CRP levels in patients or controls (data not shown). The association of the minor allele of rs4266924 and rs10733113 with increased plasma IL-1β might be explained based on the influence of variants on the increased NLRP3 gene expression, since the variants are associated with increased expression of NLRP3 that is involved in the processing and release of IL-1β (Paper I). Also, the Q705K polymorphism of NLRP3 gene showed gender specific association to increased CRP levels in the unaffected males (Paper II). Considering the role of IL-1β in the production of CRP, the association suggest
the role of Q705K in the IL-1β mediated CRP production. However, the lack of association in plasma of MI patients might be due to several additional so far unknown factors involved in the disease pathophysiology that may over dominate the effect of the genetic variants investigated. Taken together, the study suggest a functional effect of the variants in the regulation of NLRP3 expression and IL-1β production. This is in partial agreement with study from Villani and coworkers showing a significant association of the variants to NLRP3 expression and IL-1β production. Also, the Q705K polymorphism of NLRP3 gene showed a gender specific association with CRP levels (Paper II). The variant Q705K was associated with increased CRP levels in the males and not in females. In order to understand the gender specific association of Q705K polymorphism to CRP levels, more mechanistic studies remains to be performed.

The CARD8 rs2043211 polymorphism was investigated in the SCARF and FIA cohorts (Paper III). The minor allele of rs2043211 in the CARD8 gene was associated with lower levels of CRP in both cohorts and to lower levels of MCP-1 in the SCARF cohort (Paper III). At the time for publication of Paper III, the mechanistic influence of CARD8 on the inflammatory response was still unknown. However, in Paper IV, we found a possible explanation to the fact that the minor allele of CARD8 was associated with lower levels of CRP, since CARD8 was found to in vitro downregulate IL-6, which is an important regulator of CRP production. This therefore suggest that the lower expression of CARD8 imparted by the minor allele of rs2043211 polymorphism possibly may reduce the inflammatory milieu by limiting the of IL-6 dependent CRP production. Also, the role of CARD8 in the regulation of chemotactic proteins such as MCP-1, RANTES, and IP10 is in line with the association of rs2043211 with the lower level of MCP-1 in the serum (Paper III and Paper IV). Studies have shown that healthy individuals carrying the rs2043211 polymorphism were found to be at higher risk of developing bacteremia, possibly due to the compromised immune response of host. Moreover, the rs2043211 polymorphism was found to produce significantly lower levels of IL-1β in the Neisseria meningitidis infected blood when compared to wild type carriers, which is in line with our results where the minor variant of CARD8 rs2043211 is associated with lower levels of MCP-1. However, cautious interpretation of the results must be undertaken since it is still unknown which isoform of CARD8 is expressed. It was previously shown that the T47 isoform may rescue the actual effect of the polymorphism rs2043211. Studies have also
shown that the T47 isoform is functionally similar to the T48 isoform of CARD8 in negatively regulating the NLRP3 during the resting state\textsuperscript{127}, thereby, suggesting an ambiguous role of the CARD8 polymorphism rs2043211 in the inflammatory response (Paper III and Paper IV).

**Association of the Genetic Variants in the CARD8 and NLRP3 Genes to the Risk of Myocardial infarction**

We further examined the association of the variants of CARD8 and NLRP3 gene with the risk of MI in two different MI cohorts (SCARF and FIA; Papers I, II and III). The rs2043211 SNP in the CARD8 gene, and the variants in the downstream regulatory region of the NLRP3 gene showed no significant overall association to the risk of MI in either of the two cohorts investigated (Papers I and Paper III). However, the Q705K polymorphism in the NLRP3 gene conferred protection against MI in females (Paper II), but the mechanistic role of this polymorphism in females remains to be elucidated. Although no significant association was found between the NLRP3 variants and MI, the heterozygous genotype of rs4353135 showed a trend towards higher risk of MI (Paper I). Our results are in general in line with a previous study by Lewis \textit{et al.} on the absence of genetic association of the SNPs to Crohn’s disease and ankylosing spondylitis\textsuperscript{200,201}. Moreover, the gender-specific association of the Q705K polymorphism conferring protection against MI in females can be possibly attributed to factors influencing the gender differences (Paper II). The fact that the present study contains a relatively low number of individuals is a limitation of the study. In particular, the absence of association between the NLRP3 variants and MI in northern Swedish population should be interpreted cautiously due to the possible interaction of these genes with environmental exposures. Furthermore, the FIA cohort origins from a region where genetic drift has been proposed, which may explain a possible divergence from other cohorts\textsuperscript{202}.

Our results showing no significant association between CARD8 polymorphism rs2043211 and MI in SCARF or FIA cohorts, is supported by a previous study showing no association between the rs2043211 polymorphism and cardiovascular events in rheumatoid arthritis patients\textsuperscript{203}. The small trend towards a higher MI risk for carriers of the minor allele in the FIA cohort, but not SCARF, may reflect genetic divergence between the two Swedish regions from which the two cohorts were recruited\textsuperscript{202}. However, conflicting evidence has also been published previously regarding the
rs2043211 polymorphism and several different diseases with inflammatory background, like inflammatory bowel disease, increased severity of rheumatoid arthritis, increased risk of Alzheimer’s disease in women and other inflammatory diseases, suggesting that further studies are required in order to confirm the association of these variants in these diseases\textsuperscript{192,204-209} (Paper III).
Conclusions

- The thesis shows that NLRP3 inflammasome-related genes are highly expressed in human atherosclerotic plaques and are sensitive to activation of NLRP3 when exposed to NLRP3 activating ligands. Our findings support a role for NLRP3 inflammasome in human atherosclerosis, linking cholesterol and inflammation within the atherosclerotic lesion.

- Genetic alterations in the downstream region of NLRP3 gene seem to have a cis-regulatory effect on the NLRP3 gene by increasing the NLRP3 mRNA expression in PBMC and elevating IL-1β levels in the plasma, thereby, suggesting a possible role of the NLRP3 variants in systemic inflammation.

- CARD8 is highly expressed in the atherosclerotic plaque and the rs2043211 polymorphism affects the CARD8 expression and is associated with lower levels of MCP-1 and CRP. However, genetic alterations in the CARD8 gene seem to be of limited importance for the development of MI.

- CARD8 is required for basal expression of cytokines and chemokine’s in endothelial cells in vitro and plays may play a role in the regulation of inflammatory response.
Future Perspectives

The focus of the thesis was to investigate NLRP3 inflammasome and the CARD8 in the pathophysiology of atherosclerosis and to the risk of MI. Despite of the several recent updates in addition to our contribution in the field of inflammasome, the mechanism of NLRP3 activation together with the functional consequence of CARD8 protein remains to be elucidated in the pathophysiology of atherosclerosis. Considering the functional role of CARD8 in the regulation of immune response, more elaborative research might represent CARD8 as a potential therapeutic target in the chronic inflammatory diseases including atherosclerosis.

Several factors contribute in the progression of atherosclerotic plaque. The factors responsible for the increased expression of CARD8 in the atherosclerotic lesion remains to be elucidated. Also, to identify the upstream regulators of CARD8. ANRIL, the gene associated with the atherosclerosis is also shown to regulate the expression of CARD8, however the molecular mechanism by which ANRIL regulates CARD8 remains unknown.

To further elucidate the role of CARD8 mediated immune response in the vascular inflammation, it is important to study the expression of CARD8 isoforms and their functional consequence in vascular and immune cells. Knock outs of CARD8 in both cell line and animal model can broaden the understanding on the effect of CARD8 protein in the regulation of immune response.

To obtain a wide spectrum of the genes regulated by the CARD8 protein, it is essential to perform a microarray and protein array from the CARD8 knock down endothelial cells. This will be helpful in identifying downstream target genes that are regulated by CARD8.
Acknowledgements

The completion of this thesis is the end of my journey in obtaining my PhD, with encouragement, support and inspiration from numerous people including my supervisors, colleagues, friends and families. At the end of this beautiful journey, it is a pleasant task to express my gratitude to all those who contributed to the success of the whole study and made it a wonderful experience for me.

I would like to thank my supervisor Karin Fransén, for the guidance, patience and advice from the time I joined Allan’s lab. Under her guidance, I have successfully accomplished not only this thesis but also gained knowledge on writing grant applications and reviews. I am grateful to her for teaching me to critically review the research papers that led me later to achieve ‘Certificate of excellence in reviewing’ for my very first reviewing of an article from the Spandidos publications. I am continually amazed with her hard work, and skills in handling work pressure especially when it’s close to the deadlines. She has not only helped me in improving my writing skills but also motivated me to accomplish a review article. Her unflinching dedication and being always available to discuss research (even during midnights☺️) always inspires me. I have been extremely fortunate to have a supervisor who cared so much both for my work and also my well-being.

I am extremely thankful to my co-supervisor Allan Sirsjö, for tolerating me for almost 7 years in his lab. I am indebted to him for his constant faith on me and being very supporting throughout the amazing experience of PhD. I have to mention that his enthusiasm and passion for science is very contagious for anyone working with him, and I am enjoying it to the fullest (hoping for nothing less than a Nobel Prize☺️). I greatly appreciate him for being very open to new ideas and suggestion to explore science. The wonderful experience not only includes the academic support but also the numerous fabulous opportunities from teaching to international travels (Melbourne, Cairns, Berlin, Brussels, Greece, Visby, Bangkok, Krakow, Copenhagen, Marstrand) and many fun activities that will remain fond memories forever (The beautiful deep sea dive in the Great barrier Reef is one among them). I could not have hoped for a better supervisor than him.

Thanks to my co-supervisor, Liza Ljungberg for being friendly and very supportive. Working with her was so much fun not only in terms of learning
new scientific concepts but also during travel adventures filled with joy of springs (Sea dive and the fun-filled Bangkok trip).

Special thanks to our collaborators and co-authors for the successful completion of the projects.

I would also like to thank Torbjörn Bengtsson, my former co-supervisor for introducing me to the field of Periodontitis. Working with him was an amazing experience and his constant appreciation for every single work has been a huge motivation to explore science in the field of Periodontitis. I enjoyed very much working with *Porphyromonas gingivalis* infection in an *ex-vivo* arterial vessel to explore the ability of the different mutants infecting the vessel wall. The beautiful images of *P. gingivalis* infection in vessel wall will remain as a lovely memory forever. Thanks to Hazem, Eleonor, Kristin, Boxi and Kaddy for the wonderful time we shared while working on collaborative projects.

I would also like to thank the Cardiovascular Research Centre (CVRC) for the tremendous opportunities and support in terms of activities, such as seminars, presentations and discussions to develop collaborative projects and scientific insight is very much appreciated. Thanks to Magnus Grenegård for his enthusiastic initiatives, Anita Hurtig-Wennlöf for the collaborations, Knut Fälker for helping me with all the western blots, Ashok Kumawat for being easy-going and friendly to approach with any research queries (and for communicating in Hindi), Ulrika and Maria for always being kind to help, Madde for tolerating me in the office and for fun time in Greece together, and Caroline for being so outspoken, lively and helpful. I thank Mulugeta and Stefania for helping me on various occasions in order finish my thesis successfully.

My sincerest thanks to Marie Alkman, for being always helpful with ordering lab reagents despite of bothering her sometimes with too much work. My work wouldn’t have successfully accomplished on time without her help. Thanks to Ulla Eriksson for being generous and helpful. Thanks to Ayako and Ruzan for being very helpful with statistics.

Thanks to Anna Göthlin-Eremo, for helping me with staining and immunosectioning. Her generosity, curiosity and support has helped me to fulfil my fourth manuscript successfully.
Thanks to Dr. Senthil Kumaran for the tremendous support and faith that led me to practice molecular biology techniques, and histiology in his Lab. Thanks Sudha mam for kindness and hospitality. I would also like to thank my teacher Sowjanya Singh, for her guidance, support, prayers and encouragement that helped to have positive attitude towards life.

Life in Sweden would not have been so much fun without my best friend Sravanthi. If it was not her, probably I wouldn’t have thought of coming to Sweden to pursue my education in Sweden. To have such a kind, generous, caring friend in life is a blessing. Thanks to Tanzina for her friendship and guidance to work with cryosectioning and immunostaining. Thanks to my dost Sravya, for tolerating me especially when I was on her nerves from time to time. It was very easy to get connected to her in a short time because of her very friendly and kind nature. Thanks for the fun we had together in Lofoten, the crazy giggles together almost everytime, and bearing with my short time memory loss nature. Thanks to Isak, for being a very generous, curious and helpful person. He has helped me to get acclimatized and to work with new infrastructure at Campus USÖ and also for introducing me to the “Gold mine”. It was fun discussing research with him and his passion for research can be very contagious. Thanks for being a motivating researcher and a good friend. Thanks to Ali for introducing me to Allan’s lab and helping me throughout his stay as a PhD student.

Thank to my family in Sweden, the International group. On several occasions, I could get through my difficult times just because of the prayers, love and care from the members of International group. Thanks to Viendo and Joakim Börjesson for choir singing and hiking adventures, Helen and David Lindner for potluck and Carcassonne, Breezy and Daniel Lindqvist for being there for me all the time, Kaddy and Isak for fun during festivals, food and movies, Maria and David Holford for all your help, Bisrat for support and friendship, Karin and Walter for love and care, Renata and Marcello for friendship and fun times, David and Peter for all the fun we had together in Örebro and Uppsala. I am indebted to David and Peter for making me laugh to the fullest and to encourage me at difficult times. Among others, thanks to Joubert, Pradeep, George Bethel, Solange, Jones, Chris, Esther, Hanah, Hamish, Annika, Josef, Raphael, the newcomers and others for being a part and parcel for this big family.
Research would not have been fun without good colleagues and friend being around and supportive. Thanks to former and present colleagues and friends including Berhane for the nice talks, Sezin for being kind and caring, RongRong for fun and late night work in lab together, Vladimir for being helpful on several occasions, George, Aleem, Nixon, Patience and Ajay for friendship.

Thanks to colleagues at campus USÖ, Naveed, Wessam, John Peter, Sukithar, Alex and KFL for a wonderful work environment and support.

I would also like to thank Rapha church and Brickebergskyrkan for all the prayers that has helped me to accomplish this task successfully.

My school and graduate friends, Keerthi, Asma, Santosh, Monica, Rhadika, Hema, Vakula, Aznee, Divya, Bala, for their support and trust on me.

Thanks to my friend Lydia for her care and concern, Alen and Rathesh family, Devi and Selvan family, Priscilla, Kuma, Vimala aunty and Dhanya for making me feel like home in Sweden. I am very much grateful to Ravi Vumma and family for helping and inspiring me to pursue an academic career in Sweden.

Thanks to my parents for the tremendous sacrifice, prayers and guidance to fulfil every good and beautiful dream of mine. My brother Jeethu (the cool guy), for the care, love and support. He has been an inspiration for many including me for always being calm, humble and meek. My cousins for all the fun and support. Thanks to Andreas for the beautiful Lofoten drive and being supportive throughout my PhD journey. It is true that ‘Behind every successful woman stands a strong man’. I am very grateful to him for the crazy but adventurous and thrilling long drives, and looking forward for more expeditions that remain to be explored. Thanks to God for his wonderful gift, Nathaniel to colour my life. I have enjoyed every journey we took together since he was 1 month old. Thanks to him for being the source of my strength, joy and happiness.

Thanks to Elisabeth Frölander and Sofia Lykkekle for helping me with the administration works, Lennart for helping with the delivery of reagents, and Erik Norgren for helping me with the printing of thesis.
Our study was supported by knowledge Foundation HÖG15, Swedish Research Council, the Center of Excellence for Research on Inflammation and Cardiovascular Disease (CERIC) Linnaeus Center, the Swedish Heart-Lung Foundation, the Foundation for Strategic Research, Uppdrag Besegra Stroke, the Strategic Cardiovascular Programs of Karolinska Institute, Stockholm County Council, Örebro University, Sigurd and Elsa Goljes Foundation, Foundation for old Servants and Magnus Bergvall Foundation.
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