Interleukin 16 in Atherosclerosis and Cardiovascular Disease

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Interleukin 16 in Atherosclerosis and Cardiovascular Disease

Caitríona Grönberg

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Abstract

Background and aim - The development of clinical manifictations due to an eroded or ruptured atherosclerotic plaque, or occluded vessel, are one of the major causes of death world wide. It has been known for some time that atherosclerosis develops due to retention and modification of LDL particles in the vessel wall and the subsequent triggering of the immune system. The research presented within this thesis has focused on IL-16, a signaling molecule, in the immune system. IL-16 has shown pleiotropic functions in inflammatory diseases. IL-16 has been described to have the capacity to induce T cell unresponsivness and increase the regulatory T cell population. Regulatory T cells are known to be protective in atherosclerosis by dampening the immune responses. There has been no extensive research on the role of IL-16 in atherosclerosis disease and the clinical manifestations thereof. The aim of the collected work in this thesis was to investigate if IL-16 can induce anti-inflammatory and atherosclerosis dampening effects, if IL-16 holds potential as a biomarker for prediciting future cardiovascular events, and if IL-16 is altered in an retrospective cardiovascular case-control study.

Results – (I) Administration of IL-16, in an experimental model of atherosclerosis consisting of hypercholesterolemic female mice, increased anti-inflammatory factors and decreased the atherosclerotic plaque burden. Male mice, which were defective of IL-16, displayed an increased atherosclerotic burden compared to control mice. (II) Elevated levels of IL-16, in carotid plaques, from individuals with severe carotid atherosclerosis displayed associations to plaque stabilizing components (collagen, elastin and FoxP3). High levels of carotid plaque IL-16 were associated to a decreased risk of suffering from a cardiovascular event. (III) Individuals suffering from severe carotid atherosclerosis had a decreased risk of suffering from a post-operative cardiovascular event, or a cardiovascular event leading to death, if they had high levels of circulating IL-16 compared to individuals with low levels of IL-16. (IV) In a population-based prospective study four single nucleotide polymophisms (SNPs) were found to be associated with IL-16 plasma levels. High plasma levels of IL-16 were associated with an increased risk of myocaridal infarction in women, compared to women with low levels of circulating IL-16. None of the SNPs were associated with an increased risk of cardiovascular events. One of the identified SNPs was associated with a decreased risk of all-cause mortality during the 20-year follow-up period. (V) In a retrospective case-control study, including individuals suffering from diabetes, IL-16 was 50% elevated in individuals whom also suffered from cardiovascular complications compared to the individuals only suffering from diabetes. Plasma levels of IL-16 were associated with surrogate markers of atherosclerosis which was further supported by SNP analysis.

Conclusion - IL-16 in plasma did not display associtions to a decreased risk of cardiovascular events in a prospective population-based study, rather the opposite. We have presented supporting evidence of a protective role of IL-16 in severe and experimental atherosclerosis, reinforced by the associations between high IL-16 levles and increasing amounts of stabilizing factors in the atherosclerotic plaque. We also present supporting evidence, in an experimental setting, for an anti-inflammatory role and plaque burden limiting role of IL-16. The data presented within this thesis warrents futher investigation of the plaque stabilizing properties of IL-16 in severe atherosclerosis and the role of IL-16 in promoting anti-inflammatory mediators.

Key words: Athersoclerosis, cardiovascular disease, interleukin- (IL)16, immunity,T cells, myocardial infarction, stroke.

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Interleukin 16 in Atherosclerosis and Cardiovascular Disease

A fire fighter counteracting an inferno in the vessel wall.

Caitríona Grönberg

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To my family
Hampus, Áine & Éirinn
Mamma, Pappa, Úna & Thomas

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Preface

Atherosclerosis affects us all sooner or later, the question is whether the atherosclerotic plaque will cause clinical symptoms, such as stroke and myocardial infarction. It has been known for some time that atherosclerosis develops due to deficient lipid clearance and modification, triggering the immune system, and due to vessel wall defectiveness. Atherosclerosis is an intriguing and multifaceted disease, demonstrated by the fact that one individual can have several different atherosclerotic plaques in several different locations although only a few of them might be prone to rupture and cause an acute clinical event.

The main focus for many researchers around the world is to find techniques to identify the vulnerable plaques and to promote plaque stabilizing properties allowing prevention of clinical events. Research in the field has generated great insight and help to millions of patients every year. The new treatments have however modified the disease and researchers are now struggling to "catch up" with new therapies to be able to coevolve with the disease.

The research presented in this thesis is focused on a signaling molecule, interleukin 16 (IL-16), in the immune system and whether this cytokine can hold potential to both predict cardiovascular events but also if it may be used as a therapy to limit atherosclerosis progression.

In the following pages you will find my perception of atherosclerosis, the immune system and interleukin 16. This thesis does not present all the answers to the role of interleukin 16 in this fascinating and deadly disease, although it raises some possibilities and a lot of intriguing questions.

Original Papers

I. **Caitríona Grönberg**, Lukas Tomas, Sara Rattik, Úna Eriksson, Ingrid Yao Mattisson, Anahita Abdali, Emelie Larsson, Linda Andersson, Ragnar Alm, Lena Sundius, Ingrid Söderberg, Hardy Kornfeld, Gunilla Nordin Fredrikson, Jan Nilsson, and Harry Björkbacka. *IL-16 Reduces Atherosclerotic Development in Hypercholesterolemic Apolipoprotein E Deficient Mice.* Manuscript.

II. **Caitríona Grönberg**, Eva Bengtsson, Gunilla Nordin Fredrikson, Mihaela Nitulescu, Giuseppe Asciutto, Ana Persson, Linda Andersson, Jan Nilsson, Isabel Gonçalves, and Harry Björkbacka. *Human Carotid Plaques with High Levels of Interleukin 16 are Associated with Reduced Risk for Cardiovascular Events*. Stroke 2015 Oct;46(10):2748-54.

III. **Caitríona Grönberg**; Giuseppe Asciutto, Ana Persson, Gunilla Nordin Fredrikson, Jan Nilsson, Isabel Gonçalves, and Harry Björkbacka. *Endarterectomy Patients with Elevated Levels of Circulating IL-16 Have Fewer Post-operative Cardiovascular Events*. Submitted.

IV. **Caitríona Grönberg**, Gunilla Nordin Fredrikson, Marju Orho-Melander, Gunnar Engström, Olle Melander, Jan Nilsson and Harry Björkbacka. *Interleukin 16 Levels in Plasma and Polymorphisms in Relation to Incident Cardiovascular Events: A Prospective Population-Based Cohort Study*. Manuscript.

V. **Caitríona Grönberg**, Emma Ahlqvist, Gunilla Nordin Fredrikson, Isabel Gonçalves, Andreas Edsfeldt, Helen M Colhoun, Angela C. Shore, Carlo Palombo, Andrea Natali, Maria Wigren, Eva Bengtsson, Gerd Östling, Kunihiko Aizawa, Francesco Casanova, Margaretha Persson, Kim Gooding, Phil Gates, Faisel Khan, Helen C Looker, Fiona Adams, Jill Belch, Silvia Pinnola, Elena Venturi, Michaela Kozakova, Li-Ming Gan, Volker Schnecke, Jan Nilsson, Harry Björkbackaon behalf of the SUMMIT consortium. *Elevated Levels of Circulating IL-16 are Associated with Vascular Complications of Diabetes*. Manuscript.

Published or submitted papers not included in this thesis

1. **Caitríona Grönberg** and Harry Björkbacka. *Atherosclerosis: cell biology and lipoproteins*. Curr Opin Lipidol. 2012 Oct;23(5):505-8.
2. Andreas Edsfeldt, Mihaela Nitulsecu, Helena Grufman, **Caitríona Grönberg**, Ana Persson, Marie Nilsson, Margareta Persson, Harry Björkbacka and Isabel Gonçalves. *Soluble urokinase plasminogen activator receptor is associated with inflammation in the vulnerable human atherosclerotic plaque*. Stroke. 2014 Dec;43(12):3305-12.
3. Sara Rattik, **Caitríona Grönberg**, Maria Gomez, Harry Björkbacka, Gunilla Nordin-Fredrikson, Jan Nilsson and Maria Wigren. *Apolipoprotein B-100 peptide p210 inhibits proliferation of naive T effector cells and promotes induction of tolerogenic antigen presenting cells and regulatory T cells in vitro*. J Clin Cell Immunol 2015;6:3.
4. Giuseppe Asciutto, Maria Wigren, Gunilla Nordin-Fredrikson, Ingrid Yao Mattison, **Caitríona Grönberg**, Ragnar Alm, Harry Björkbacka, Nuno Dias, Andreas Edsfeldt, Isabel Gonçalves and Jan Nilsson. *Apolipoprotein B-100 anitbody interaction with atherosclerotic plaque inflammation and repair processes*. Stroke 2016;Apr;47(4):1140-3.
5. Maria Wigren, Sara Rattik, **Caitríona Grönberg**, Lukas Tomas, Ingrid Yao Mattisson, Ingrid Söderberg, Ragnar Alm, Lena Sundius, Irena Ljungcrantz, Harry Björkbacka, Gunilla Nordin Fredrikson, and Jan Nilsson. *Lack of ability to present antigens on MHC class II molecules aggravates atherosclerosis in ApoE-/- mice*. Submitted.

Abbreviations

AF	amourosis fugaux
APC	anitgen presenting cell
ApoA	apolipoprotein A
ApoB	apolipoprotein B
ApoC	apolipoprotein C
ApoE	apolipoprotein E
Au	arbitrary unit
B cell	B-lymphocytes
C57Bl/6 mice	mice on the genetic background of C57 black 6
CD	cluster of differentiation
CD25	IL-2 receptor alpha chain
CD4	expressed on Th cells, IL-16 ligand
CD8	expressed on Tc cells
CPIP	carotid plaque imaging project
CRS	cardiorenal syndrome
CTLA-4	cytotoxic T lymphocyte antigen 4
CV	cardiovascular
CVD	cardiovascular disease
DAMP	danger associated molecular pattern
DC	dendritic cell
de novo	starting from the beginning
DNA	deoxyribonucleic acid
DR	death receptor
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell
ECM	extracellular matrix
eGFR	estimated glomerular filtration rate
FASL	Fas ligand, CD95
FoxP3	forkhead box protein 3, Treg transcription factor
GATA3	GATA binding protein 3, Th2 transcription factor
HFD	high fat diet
HLA-DR	human leukocyte antigen - antigen D related, MHC class II
IFN-γ	interferon gamma
IL	interleukin
IL-10	interleukin 10, cytokine synthesis inhibitory factor
IL-12	interleukin 12

IL-16	interleukin 16, lymphocyte chemoattractant factor
IL-2	interleukin 2
ILC	innate lymphoid cell
in vitro	outside a living organism
in vivo	inside a living organism
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
MDC	Malmö diet and cancer study
MHC	major histocompatibility complex
MI	myocardial infarction
MMP	metalloproteinase
NK cell	natural killer cell
NOD mice	non-obese diabetic mice
oxLDL	oxidized low-density lipoprotein
PAMP	pathogen-associated moleculer pattern
PBMC	peripheral blood mononulear cell
PD-1	programmed cell death receptor 1, CD279
PDZ domain	structural domain in signaling proteins
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RNA	ribonucleic acid
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SR	scavenger receptor
SUMMIT	surrogate markers for micro- and macrovascular hard end points for innovative diabetes tools
T cell	T-lymphocyte
T1D	type 1 diabetes
T2D	type 2 diabetes
T-bet	T box 21, transcription factor for Th1 cells
Tc	cytotoxic T cells, CD8+
TCR	T cell receptor
TG	triglyceride
TGF-β	transforming growth factor beta 1
Th	T helper cell, CD4+
Th1	T helper cell type 1
Th2	T helper cell type 2
TIA	transient ischemic attack
TLR	toll-like receptor
Treg	regulatory T cell
WBC	white blood cell
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cell
CD9	tetraspannin, alternative IL-16 ligand

Background

The word atherosclerosis comes from the Greek language and in direct translation would be “disgusting/sticky hard tissue, or mass, of the arteries”. Factors that compose life and factors that we expose ourselves to (genes, nutrient intake, immune system, stress and toxins) determine the risk an individual has of developing cardiovascular disease. Cardiovascular disease (CVD) represents the clinical manifestations of atherosclerosis. How can this disgusting hard tissue be a result of the same factors essential for life? All of these factors will be discussed in the chapters below except for one, which is the ability to reproduce. The fact that atherosclerosis causes clinical symptoms, which generally appear in individuals after reproductive age, implies that atherosclerosis will never resolve due to natural selection. With a constant increase in life expectancy and more effective treatment of infectious diseases, there will also be an increasing population with CVD. Today the clinical complications of atherosclerosis are the main cause of death worldwide, therefore more research is needed to understand, find and treat this disease.

Lipoprotein metabolism

Lipids such as cholesterol and triglycerides, which are essential for the cells, are transported in the circulation enclosed by phospholipids and lipoproteins. The lipids are enclosed by these proteins to facilitate the dissolvement in the blood, due to the fact that the lipids are hydrophobic. As the body digests nutrients in the gut, TGs are packed together with lipoprotein (apolipoprotein B48) to form chylomicrons which are then passed on to the circulation.[1] Chylomicrons and very low-density lipoproteins (VLDL) are the main carriers of TGs. The remnants of chylomicrons are taken up by the liver. The master regulator of cholesterol in the body is the liver, where VLDL is formed by fusing triglycerides (TGs), cholesterol and apolipoprotein B100.

As the chylomicrons and VLDL deliver some of its lipids to cells, they become denser and hydrolyzed and turn into low-density-lipoprotein (LDL). The major source of cholesterol, from the liver, is delivered to the periphery by LDL. The

apolipoprotein B-100, on the surface of LDL, binds to receptors on cells and the LDL is taken up by endocytosis. The left-overs from this transportation of lipids are called high-density lipoprotein (HDL). HDL has the capacity to clear excess cholesterol, and chylomicron remnants, from the circulation by binding scavenger receptor class B type 1 in the liver (Figure 1).[1] The excess cholesterol is then depleted from the system together with bile salts.

Most of us have heard about bad cholesterol (LDL) and good cholesterol (HDL), this is however misleading as they are both essential for the body although due to excessive intake of cholesterol and fat (or by familial hypercholesterolemia) the balance is shifted and the LDL can become toxic.[2, 3]

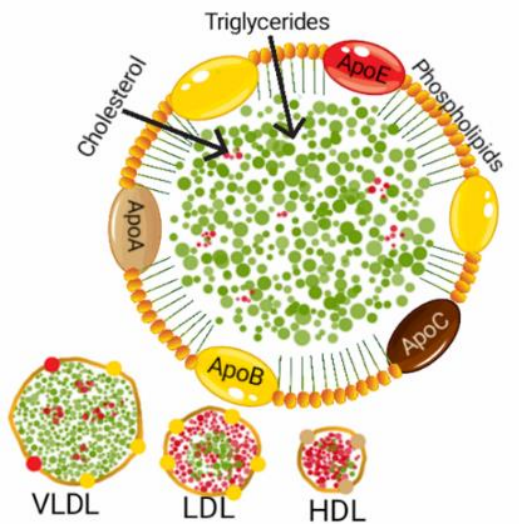

FIgure 1. The chylomicron (largest particle) transports triglycerides and cholesterol encapsuled in phospholipids and apolipoproteins cells. VLDL contains mainly triglycerides, wheras LDL and HDL have increasing fraction of cholesterol compared to the triglyceride level.

Atherosclerosis

The healthy artery

The healthy artery is built up of three layers; the adventitia, tunica media and tunica intima (closest to the lumen). The outermost layer, adventitia, consists of extracellular matrix (ECM) proteins, immune cells, fibroblasts, and vasa vasora which supplies nutrients to the vessel.[4] The intermediate layer, tunica media, consists of smooth muscle cells (SMCs) surrounded by a basement membrane and

ECM. The ECM consists of several different types of collagens, elastin, fibronectin and proteases.[5, 6] The tunica intima is the inner most layer closest to the blood flow and is made up of endothelial cells (ECs)(Figure 2). The three layers work together to supply the elasticity and compression needed to maintain blood flow and pressure.[7]

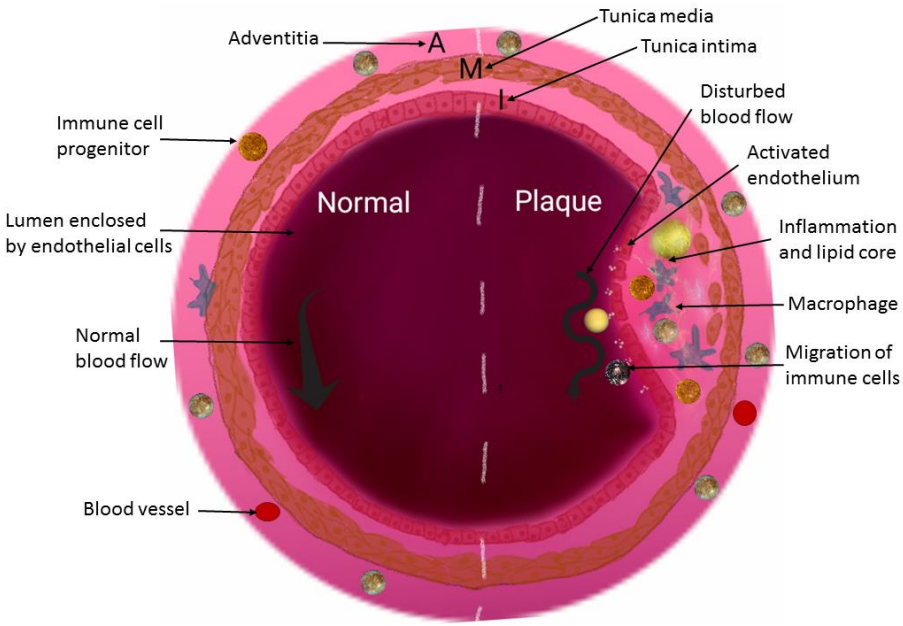

Figure 2. A normal vessel wall (left) with the tunica intima (I) innermost to the lumen surrounded by SMCs in the tunica media (M) and the adventita (A). Formation of an atherosclerotic plaque (right) with a disturbed blood flow, attachement of white blood cells to the endothelial layer and accumulation of foam cells and immune cells in the subendothelial layer.

Atherosclerotic plaque development

The concept of atherosclerosis was observed and described more than a hundred years ago, even in experimental models, as well as the connection between atherosclerosis and the accumulation of lipids in the vessel wall.[8, 9] The atherosclerotic lesion is initiated as fatty streaks in the vessel wall, in the subendothelial cell layer, the occurrence of which can start in adolescence.[10] The fatty streaks can be found in large to medium sized arteries and consists of lipids and phagocytic white blood cells, called macrophages. There are some extra vulnerable places in the arteries where the initiation of atherosclerosis normally takes place (Figure 3).[11] Such places are characterized by regions with disturbed or altered blood flow, which can trigger endothelial permeabilization and invasion of white blood cells.

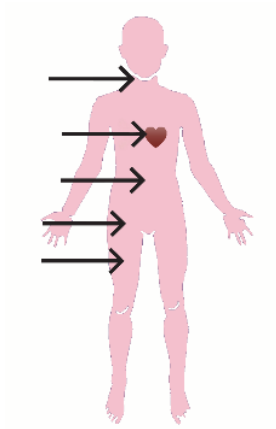

Figure 3. Atherosclerosis prone sites leading to CVD, in the arterial tree are manily the arteries in the; carotids, coronary, aorta, iliaca and femoral.

There are different theories to why atherosclerosis develops, Tabas and Williams presented the response-to-retention model in 1995.[12] They propose that the key factor for the development of atherosclerosis is the retention of apolipoprotein B100 in the subendothelial space. The trapped lipoproteins bind to ECM where they are retained and undergo modifications and oxidation and are engulfed by macrophages.[13] Ross and Glomset have presented a model based on the response-to-injury concept, which focuses on a disturbance of the endothelial layer (injury or by activation) leading to an increased influx of cholesterol and immune cells.[14]

The LDL that enters the vessel wall may be subjected to oxidation and modification. The oxidized LDL (oxLDL) is thought to be the main immunogenic factor in atherosclerosis, leading to increased responsiveness of the macrophages. The oxLDL is recognized as foreign, non-self material, due to the modifications on the surface making leading to binding, and activation of pattern-recognition receptors (PPRs), scavenger receptors (SRs), and toll-like receptors (TLRs).[15-17] The uptake of the oxLDL, which resembles pathogens for the macrophages, will induce a release of pro-inflammatory factors from the macrophages and will further attract immune cells by activation of the ECs.

The SMCs respond to the inflammation by migrating to the intima and by producing ECM proteins to repair the wound. As the macrophages in the fatty streaks take up more lipids, they will swell into lipid-laden foam cells. The foam cells, which are stuck in the vessel wall, are prone to die a messy death, called necrosis.[18] The necrosis results in a release of a dangerous mixture of intracellular proteins and oxLDL into the subendothelial layer, which will further contribute to inflammation and the formation of a toxic necrotic core.[19]

Stable vs. vulnerable plaque

We can live with atherosclerotic plaques for decades without noticing that they are present in our arteries. Some people may even have large atherosclerotic plaques without having any symptoms. These plaques are referred to as stable plaques, and consist of higher levels of SMCs, which produce ECM and a thicker fibrous cap (enclosing the plaque from the lumen) (Figure 4). The only time the individual will notice a stable plaque is due to a massively enlarged plaque and that it thereby restricts the blood flow.

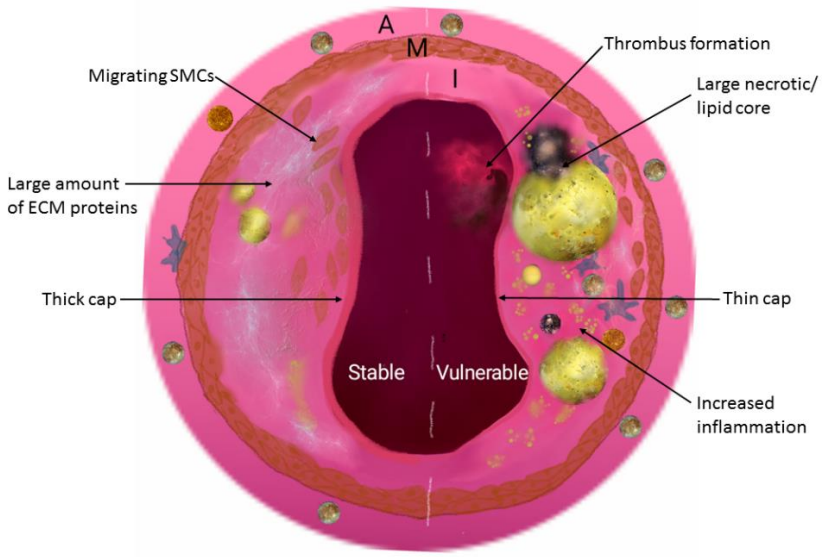

Figure 4. A stable plaque (not prone to rupture) is charecterized by SMCs, ECM proteins, and a thick fibrous cap (left). A vulnerable plaque is characterized by immune cell infiltration, large lipid and necrotic core and a thin fibrous cap (right).

A vulnerable plaque is characterized by a large necrotic/lipid core, more inflammation and a thin fibrous cap. Macrophages have the possibility to produce matrix metalloproteinases (MMPs) which can degrade ECM and the fibrous cap.[20, 21] If the cap ruptures, the plaque content enter the lumen and during contact with the blood flow, cause a thrombosis (blood clot) formation. The thrombus can occlude the lumen and cut off blood flow at the site of the rupture or it can be flushed with the blood flow and occlude smaller vessels further on in the arterial tree. The occlusion causes ischemia, and symptoms from such thrombus formations are known as stroke and myocardial infarction (MI). There is immense research focused on identifying vulnerable plaques and how to promote these plaques to switch into the stable phenotype, where silencing the immune system is thought to be one key factor.[22, 23]

CVD

CV events

As atherosclerosis progresses it is possible that the atherosclerotic plaque erodes, leaks or blocks the arteries and clinical symptoms may arise. Cardiovascular disease (CVD) includes symptoms in the brain such as ischemic stroke, transient ischemic attack (TIA) and amourosis fugax (AF), which all can be caused by atherosclerotic plaques situated in the carotid arteries. MI and unstable angina are caused by arteries being obstructed in the vessels surrounding and supplying the heart with blood. Peripheral artery disease is an atherosclerotic disease affecting the extremities of the body and can lead to intermittent claudication. Interestingly, not all of the atherosclerotic plaques lead to a cardiovascular (CV) symptom.

Risk factors

As mentioned before, the atherosclerotic plaques may be relatively large without affecting the individual. The Framingham Heart Study was initiated in1948 to identify risk factors associated with increased risk of suffering from CVD.[24, 25] The study concluded that the following risk factors are of interest; age, male sex, smoking, total cholesterol, HDL cholesterol, and systolic blood pressure with the addition of diabetes mellitus and body-mass-index (when cholesterol levels are not available).[3, 26] The risk factors established in the Framingham Heart Study seems to be reliable when calculating risk for CVD in a previously healthy individual, however, when the individual has established CVD or is on medication for one or several of these risk factors, they do not seem to predict risk as well.

Treatment

Today the current treatment is to suggest lifestyle changes, remove or stent the atherosclerotic plaques or/and to medicate with aspirin, beta-blocker, ACE inhibitors and statins. Aspirin decreases the platelets ability to form aggregates and thereby decreases the risk for thrombus formation. Beta-blockers block the response to stress hormones in the heart, arteries and kidneys by interfering with the receptors for adrenalin and thereby not allowing the sympathetic system to "over heat". ACE inhibitors decrease the contraction of blood vessels and reduce blood pressure by inhibiting the conversion of angiotensin I to angiotensin II. Statins have been proven to lower lipid levels through inhibition of cholesterol production in the liver, by reducing LDL through an up regulation of LDL

receptors in the liver.[27] Statin treatment has also been associated with increased levels of the athero-protecitve regulatory T cells.[28, 29]

Recently an already approved compound, cyclodextrin, has been proposed as a possible new treatment for cholesterol in atherosclerotic plaques. The authors present evidence, in an experimental setting, for an increase in cholesterol efflux from macrophages and reduction in atherosclerotic progression after treatment with cyclodextrin.[30] As hypercholesterolemia, hypertension and diabetes is dramatically increasing, the usage of medication escalates. The treatment will most probably change the phenotype of the atherosclerotic plaques and the need for new biomarkers and therapies are essential to be able to coevolve with the disease and to resolve CVD.

Immune system

The immune system is an essential part of the body and it is responsible for protecting the body from invading hostile particles. It is a myriad of cells and molecules that patrol and "talk" with each other to ensure that the body is not being infected. The immune system has traditionally been divided into two parts, the innate, which is the first line of defense, and the adaptive which is responsible for remembering previously encountered "wrong doers". Most of the cells that are a part of the immune system are produced in the bone marrow, by hematopoietic cells, and are then relocated to lymphoid organs (thymus, spleen, lymph nodes) and into other tissues.

Innate immunity

First line of defense

The innate immune system is our first line of defense, and therefore needs to be located where the body is most likely to come across pathogens (skin, gut, liver and lungs). Examples of such cells are mast cells, macrophages and neutrophils. Mast cells in the skin and lungs are responsible for the rapid response to allergens involved in symptoms such as hay fever, contact allergy and bee stings. People suffering from these illnesses are of course not so pleased with the immune system but without these cells harmful pathogens would have a free passage and could destroy the body. We also have tissue resident phagocytes, such as macrophages and dendritic cells, which engulf foreign particles to degrade and present them to effective killer cells which can seek out and kill pathogens. The phagocytic cells can recognize these foreign particles by cell surface receptors called DAMPs

(danger-associated molecular pattern molecules), PAMPs (pathogen-associated molecular pattern molecules) and TLRs (toll like receptors).[16]

Common for all types of cells in the immune system is that they communicate with each other by releasing molecules, called chemokines and cytokines, which attracts other immune cells and fine-tunes them to respond in an appropriate way. The phagocytic cells also have the possibility to present small parts of the engulfed particles on their cell surface to enable the adaptive immune cells to respond.

Previously it was thought that the innate immune system did not have any cells which had the ability of creating immunological memory, however during the recent years more evidence has been presented for an emerging field of cells representing innate phenotypes but also displaying capacity to confer memory or to produce the same effector molecules as the adaptive immune system.[31] Among these cells are B1 cells, innate lymphoid cells (ILCs) and natural killer cells (NKs).[32-34] These cells are outside the scope of this thesis, even if they are extremely interesting.

Macrophages and oxLDL

In atherosclerosis the LDL particles are oxidized and modified so that the macrophages no longer recognize the LDL. This entails that the macrophages react to the oxLDL by engulfing and presenting fractions of the LDL to the adaptive immune system. Under a "normal" infection there would most probably be a peak of invading pathogens which would reduce after time as the infection was cleared. This is not the case for the macrophages located in the vessels recognizing the oxLDL.

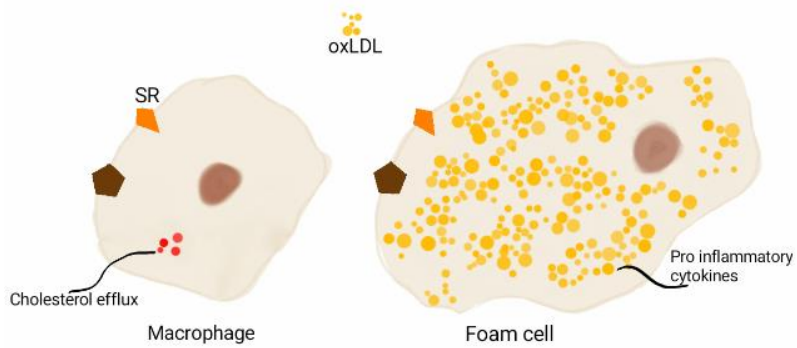

Figure 5. Normal macrophage (left) with receptors recognizing foreign particles and with functioning cholesterol efflux. Foam cell (right) located in the vessel wall, filled with lipids, releasing pro-inflammatory molecules. SR – scavenger receptors, oxLDL – oxidized LDL.

Increased cholesterol intake will most likely increase the levels of oxLDL available for the macrophages in the vessel wall. As a consequence of this the macrophages in the vessel wall will engulf more and more oxLDL and finally swell into lipid laden foam cells.[35] The foam cells will eventually burst and a highly toxic mass of cell debris and lipids will leak out and further activate the immune system in the vessel wall, and a destructive cycle has been initiated (Figure 5).[36, 37]

Adaptive immunity

The adaptive immunity has the capacity of producing memory cells which are derived from cells that have encountered their specific antigen. There are two main populations of cells belonging to the adaptive immunity, B and T cells. These cells have a total repertoire of over ten billion antigen receptors. Several of the different subtypes of B and T cells have been described to affect atherosclerosis.[38]

B cells

B cells are responsible for producing antibodies that bind to specific sequences on cells or particles. The antibody-particle complex is then either cleared away by phagocytic cells or forms an immune complex. The first time a B cell encounters its pathogen it takes approximately a week to produce the specific antibody required to bind to the pathogen of interest.

The second time the body is infected with the same pathogen there are memory B cells, which were produced from the primary infection, that are specialized to recognize this specific pathogen and a rapid antibody production is assembled. It is also possible to introduce a part of the pathogen, which is not as toxic as the entire pathogen, to produce memory B cells. This memory response is the key for how vaccination functions in such an effective way.

There are several known subtypes of B cells where B1 cells have been associated with athero-protection and B2 and regulatory B cells (Breg) have displayed both pro- and anti-atherosclerotic properties.[39-43]

T cells

Stem cells, which produce T cells, are located in the bone marrow and are later transported to the thymus. In the thymus the T cells mature and undergo positive and negative selection for binding to antigen, here apoptosis (programmed cell death) plays a crucial role in clearing defective T cells.

Table 1. Cytokines in atherosclerosis discussed within this thesis.

	Producing cells	Responsive cells	Role in atherosclerosis
IL-2	T helper cells	T cells	Pro/anti[34, 44-46]
IL-4	Th2	Th2, B cells	Pro/anti[47-49]
IL-5	Mast cells, Th2, ILC2	B1 cells, eosinophils	Anti[50, 51]
IL-10	Monocytes, T cells, Bregs	Macrophages, Th	Anti[43, 52-54]
IL-12	APCs	Th1, NK	Pro[55, 56]
IL-16	CD8 T cells, immune cells	Th cells, CD4 expressing cells	Pro/Anti[57, 58]
IL-17	Th17	Th17, macrophages, endothelial	Pro/anti[59-63]
IFN-γ	NK, TH1, Tc	Th1, macrophages	Pro[64-66]
TNF-α	Macrophages, Th, NK	Several cells expressing TNFR1/2	Pro[67, 68]
TGF-β	Macrophages, Th, NK	Th17, Itregs, B cells, macrophages	Anti[69-72]

Positive selection in the thymus of T cells determines whether the cells become cytotoxic or helper T cells (CD8 or CD4 expression respectively).[73] Negative selection deletes T cells carrying T cell receptors (TCRs) recognizing self-antigen with high affinity or induces Forkhead box 3 (FoxP3) expressing T cells, called regulatory T cells (Tregs).[74, 75] The tightly regulated selection of T cells is essential as self-reactivity (cell reacting towards antigen in the individual's own cells) can be potentially extremely destructive for the organism.

Both helper T cells (Th, CD4+ T cells) and cytotoxic T cells (Tc, CD8+ T cells) patrol the body and produce memory T cells after encountering an antigen matching their TCR.[76] Once a T cell has encountered the right match for its specific TCR it takes approximately 3 to 7 days to generate the effector and memory populations from the first naïve T cell recognizing the antigen.[77-79]

One main difference between cytotoxic T cells and helper T cells is the capacity to recognize peptides presented on MHC class I and MHC II respectively. Cytotoxic T cells can recognize peptides presented on any type of cells with MHC I and can kill the cells that display a match to their TCR. Helper T cells needs antigen to be presented on MHC II by antigen-presenting cells (APC), such as macrophages, dendritic cells (DCs) and B cells, to be able to perform their effector functions. Both CD4+ and CD8+ T cells have been found in the atherosclerotic plaque.[80, 81]

Th1

Depending on which factors are available at the encounter with the antigen, the T helper cells can be divided into different subtypes; Th1, Th2, Th17 and regulatory T cells. Th1 cells are produced to fight intracellular pathogens, such as viruses. Phagocytic cells produce among others interleukin (IL)-12 and TNF-α when presenting the antigen to T cells and prime the T cells towards a Th1 response.[82] The Th1 cells are characterized by producing large amounts of interferon gamma (IFN-γ) and have the T-box 21 (T-bet) transcription factor readily transcribed. Macrophages react to the IFN-γ by producing pro-inflammatory cytokines, nitric oxide and matrix metalloproteinases MMPs.[18, 83] Th1 cells have been implied to be harmful in the atherosclerotic disease, which was demonstrated in studies by generating *Tbet* or *Ifnγ* deficient, atherosclerotic prone, mice (Table 1).[64, 84]

Th2

Th2 cells are traditionally evoked as a response to extracellular pathogens and are important in the humoral response of B cells. Th2 cells are characterized by their production of IL-4, IL-5 and IL-13 and Th2 cells have the GATA binding protein 3 (GATA3) as their hallmark transcription factor. The role of Th2 cells in atherosclerosis have yielded both pro and anti-atherosclerotic results. [47-49, 55, 85] IL-5 deficiency in atherosclerosis prone mice did however increase atherosclerosis and there was also a reduction in natural antibodies.[50] Studies on IL-5 and IL-13 are not easily interpreted as we now know that ILC type 2 have the same cytokine profile and have been demonstrated to be athero-protective (Table 1).[33, 34]

Tregs

The first time I heard about regulatory T cells was at a course in advanced immunology in the year of 2007. In this course the teachers presented regulatory T cells as a really hot field in immunology. Now, nearly ten years later, the regulatory T cells (Tregs) are still a hot topic especially in the field of atherosclerosis.[86] The reason for this is that the Tregs have the ability to reduce responsiveness of the T effector cells and APCs, which is of course beneficial in the non-resolving inflammatory setting of atherosclerosis.

Tregs are generally described as expressing the transcription factor FoxP3 and the cell surface markers CD4 and CD25. The Tregs can either be produced in the thymus, natural Tregs, or in the periphery, which are called induced Tregs. There is however an ever emerging field of markers and subtypes of these immunosuppressive Tregs being described.[86, 87]

There are several potential mechanisms where the Tregs may be beneficial in reducing atherosclerosis. The most commonly described is their ability to produce

the anti-inflammatory cytokines IL-10 and transforming growth factor beta 1 (TGF-β), which suppress effector cells. Tregs are known to consume large amounts of IL-2, reducing the availability for other proliferating T cells and thereby decreasing the cells proliferative capacity.[88] Cell to cell contact can also induce immunosuppression.in which Tregs express CTLA-4 and by binding CD80 and CD86 on APCs they can reduce the pro-inflammatory response in the APCs.[86]

There is substantial evidence for a protective role of Tregs in atherosclerosis, both in humans and in experimental models.[86, 88-93] In mice there has even been evidence that Tregs play a role in the clearance of VLDL and chylomicrons from the circulation.[94] In humans, statin treatment has not only been seen to reduce the cholesterol levels but also in increasing the circulating Tregs after treatment.[29, 95]

Apoptosis and anergy

Apoptosis

Apoptosis is essential for a functioning development of the fetus and for clearance of unneeded or exhausted cells in the body. Apoptosis is a highly controlled clearing mechanism, where particles of the cells are packaged and released for other cells to clear. The opposite of apoptosis is necrosis, where the cell dies in an uncontrolled fashion, releasing cell debris and toxins into the surrounding tissue.

Apoptosis can be induced by the extrinsic pathway, or by the intrinsic pathway. The extrinsic pathway is initiated by ligands binding to death receptors (DRs) on the surface of cells, followed by an intracellular activation of caspases (Figure 6).[96] The intrinsic pathway is promoted by mitochondrial dysfunction and the release of cytochrome c, which will in turn activate caspases (Figure 6).[97] Both pathways are known to traditionally result in caspase-3 activation, which will in turn result in certain effector functions of apoptosis. Recent studies, however, suggest the possibility of caspase-3 to have an important role in silencing the immune system by decreasing interferon production.[98, 99]

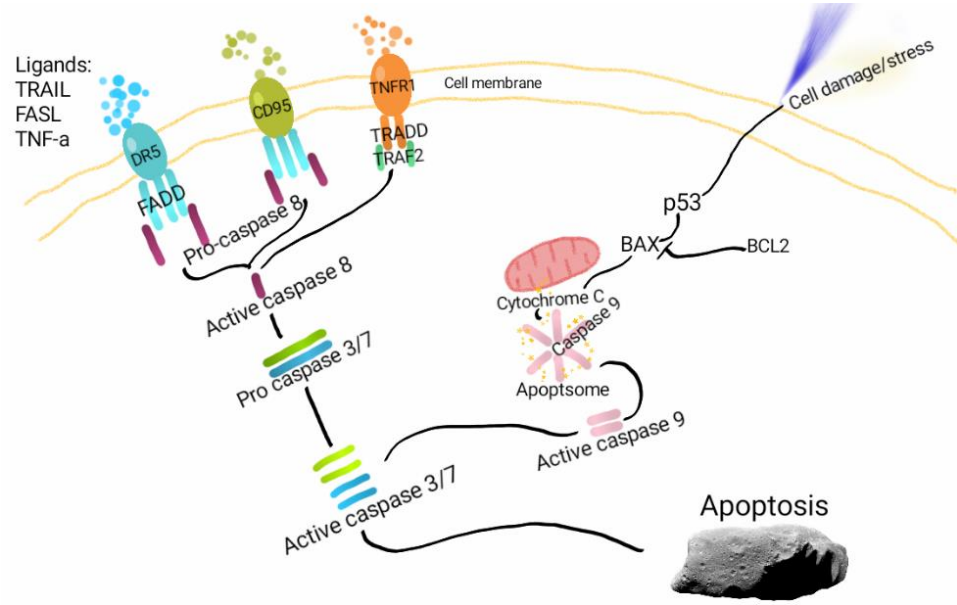

Figure 6. Apoptosis can either be initiated by extrinsic factors binding to death receptors (DRs) or by cellular stress, which induces cytochrome c release from the mitochondria (intrinisic). Both the extrinsic and the intrinsic pathway activate caspase-3 and 7 and activates the ”find me”, ”eat me”, ”tolerate me” signals produced by the apoptotic cell.

Anergy

Anergy is a term used for cells, in this case T cells, that have been silenced and are not able to undertake their effector functions. Anergy is normally initiated after an incomplete activation of a T cell. T cells also undergo deletion if they recognize self-antigen, although not all of the self-antigens are present in the thymus and therefore some of the T cells will encounter self-antigen in the periphery. To prevent harmful auto-reactivity, anergy is essential.

In the spleen and lymph nodes anergic $CD4^+$ T cells are characterized by increased expression of CD44, FR4, CD73, Nrp1, Ki67, CD69, PD-1, CTLA-4, and Nur77 compared to naïve T cells.[100] Caspase-3 activation has also been reported to induce anergic T cells.[101] Recent studies have showed a role of anergic T cells in promoting Tregs.[100] To date I do not know of any evidence of anergic T cells presented as athero-protective. The data supporting the protective role of Tregs in atherosclerosis, however, makes the anergic T cells a really promising target to study in atherosclerosis.

IL-16

Lymphocyte Chemoattractant Factor

Interleukin (IL-)16 was originally described as Lymphocyte Chemoattractant Factor (LCF) in 1982 by David Center and William Cruikshank, although was later given the name IL-16 in 1995.[102, 103] The newly discovered cytokine was described as having chemoattractant properties in naive T4+ lymphocytes (CD4+ T cells).[104] Early studies in human T cells also showed an increase of IL-2 receptor (CD25) and HLA-DR (MHC class II) on T cells treated with IL-16, as well as chemoattractant properties for monocytes expressing CD4.[104]

Further studies demonstrated a specific binding of IL-16 to the CD4 molecule which could be blocked by anti-CD4 antibody, the study also showed that the binding interacted with the D4 domain.[105] There are a number of different cells expressing CD4, which include T helper cells, monocytes, macrophages, dendritic cells, eosinophils, epithelial cells, NK cells, mast cells. The tetraspanin CD9 has also been discussed as a possible receptor for IL-16 on mast cells, as has the involvement of CCR5 on T cells.[106-108]

Pre-form and secreted IL-16

IL-16 is a unique cytokine with no significant sequence homology to other known cytokines. The IL-16 gene is located on chromosome 15 and is produced in a pre-form (631 amino acids). The pre-form is cleaved by caspase-3 to produce the secreted C-terminal (121 amino acids) part and the N-terminal part which has the ability to translocate into the nucleus and to induce cell-cycle arrest (Figure 7).[109-112] The secreted form can be found pre-made in the cytosol of $CD8^+$ T cells and in fibroblasts, while other cell types cleave the pre-form to produce the bioactive IL-16 shortly before secretion.[113, 114]

There is high homology within IL-16 between different species and human IL-16, and the blocking antibody, has been proven to be effective in rodent experiments.[115-117] IL-16 is rare in the cytokine world due to the PDZ-like motif and is the first extracellular protein found to have a PDZ motif.[110, 118] PDZ motifs are important in anchoring receptor proteins in the membrane to cytoskeletal factors and regulate biological processes such as ion channel signaling and transduction. The mechanism by how IL-16 is secreted is not fully understood, however recent work on neutrophils show extracellular IL-16 only after secondary necrosis.[119]

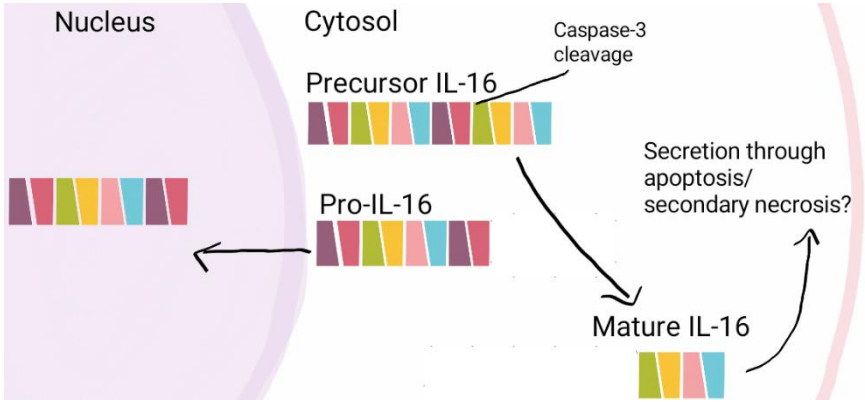

Figure 7. The pre-form of IL-16 is cleaved by caspase-3 resulting in a N-terminal part (which can translocate into the nucleus) and a C-terminal part, which corresponds to the secreted fraction.

IL-16 effects on T cells and disease

The first studies on IL-16 were focused on asthma and allergy, which in the 1990's switched to focus on human immunodeficiency virus (HIV).[120-123] Studies on IL-16 in asthma have associated IL-16 with disease severity as well as the opposite in both human and experimental studies.[124-127] Studies on IL-16, and IL-16 derived peptides, gave promising results demonstrating a reduced proliferative response after pre-treatment with IL-16 before polyclonal activation of T cells, although no therapies have been published in this field after the initial studies.[128-131]

Two very exciting studies were published in the year of 2007. To prove an immunomodulatory role of IL-16 McFadden et al. demonstrated a preferential migration of $CD25^+$ $CTLA\text{-}4^+$ T cells towards IL-16. It was further demonstrated, in the same study, that the cells attracted by IL-16 expressed higher levels of FoxP3 and that IL-16 had the ability to generate a *de novo* production of $FoxP3^+$ T cells.[132] The second study generated a fusion protein combing IL-16 with a neuroantigen to generate a tolerogenic vaccine in experimental autoimmune encephalomyelitis (EAE), which delayed disease onset and progression.[133]

As IL-16 possess the ability to reduce T cell responsiveness and induce immunomodulation the next logical step would be to investigate IL-16 in other autoimmune disease.[130, 132, 133] The role of IL-16 in diabetes and atherosclerosis, where silencing of the immune system could be beneficial, are diseases of the time we live in and are the next logical step in the investigations. There are only a few studies investigating IL-16 in diabetes. Vendrame et al. presented evidence that peripheral blood mononuclear cells (PBMCs), from type 1

diabetic patients, produced lower levels of active caspase-3, which correlated to the decreased levels of IL-16.[134] Non-obese diabetic mice (NOD), treated with a blocking IL-16 antibody, displayed less type 1 diabetes.[115] There is also evidence for increasing IL-16 plasma levels in obese individuals and decreased levels of IL-16 in CD8+ T cells from individuals that smoke with a subsequent increase in bronchoalveolar lavage.[135, 136]

IL-16 in CVD

IL-16 seems to be involved in several of the key risk factors for CVD, however the involvement of IL-16 in atherosclerosis, and metabolic disease, has just recently started to be investigated. At present there is one study investigating the potential role of IL-16 as a prospective biomarker for CVD. In this particular study, IL-16 is presented in an algorithm-based approach combining IL-16 with several other markers to be able to detect individuals with intermediate risk of CVD.[57] Findings of Cross et al. associate increased IL-16 plasma levels with an increased risk of coronary heart disease during a five-year follow-up reporting a twofold increased risk after adjustment for Framingham risk factors.[57]

There have been several studies associating IL-16 gene polymorphism (rs8034928, rs3848180, rs1131445, rs11556218, rs4778889 and rs4072111) with coronary artery disease (CAD) and ischemic stroke.[137-140] None of the genetic polymorphism studies have investigated the SNPs in a prospective cohort.

Recently there was a study investigating the role of IL-16 on vascular smooth muscle cells (VSMCs) which demonstrated an increased migration and invasion of VSMCs after IL-16 treatment, with a specific induction of metalloproteinase (MMP)-9 and activation of transcription factors nuclear factor (NF)-κβ, activator protein (AP)-1, as well as the cell-cycle-inhibitor p21WAF1 in VSMCs.[141] Tamaki et al. showed that IL-16 can possibly impact cardiac fibrosis and myocardial stiffening in patients with preserved ejection fraction, which was further confirmed in experimental animal models.[116]

There is one very interesting unpublished study on *Ldlr* deficient mice investigating the role of IL-16 in atherosclerosis development by abolishing the circulating IL-16 through DNA vaccination. The results seemed to astound the authors where they described an increased lesion formation in the carotid artery and in the aorta, with increased levels of circulating T cells after abrogating IL-16 levels (Wanrooij. Thesis. 2007. http://hdl.handle.net/1887/12357). These results do however fit very well with the data presented in paper I within this thesis.

Methods

I would like to start by emphasizing that in scientific research the most important factor is the study design. With a correct and thoroughly thought through study design you can be sure that the read out will answer your hypothesis. If the study design is not optimal, there can be difficulties in both achieving an answer to the hypothesis or even to prove that the finding is true, due to lack of statistical power. I think that the PhD years have given me this insight and that the study design is no simple matter, but essential.

Experimental atherosclerosis

In this thesis I have used both experimental models, in this case laboratory mice, and human samples. The reason we use mice, to study atherosclerosis, is that they are mammals, and are relatively close to humans evolutionary. The mice are easy to house and can generate offspring rapidly. There are a lot of tools and methods established for working and investigating the role of the immune system and atherosclerosis in mice. These mice have a life expectancy of approximately 2 years, which is advantageous when studying a disease that develops over time.

In **paper I**, we have used a mouse model that spontaneously develops atherosclerotic plaques, due to a deficiency in the *Apoe* gene. The deficiency results in a lack of clearance of cholesterol particles in the circulation, causing excess cholesterol to enter the vessel wall and initiate atherosclerotic lesions. The mice are further pushed into an atherosclerotic phenotype by giving them a high fat diet (HFD, 21% fat and 0.15% cholesterol) resembling a Western diet.

The mice are on C57Bl/6 background which has been proven to be the most susceptible for atherosclerosis, this is important as wild mice are resistant to atherosclerosis, most probably due to their high levels of HDL.[142-145] Furthermore, female mice develop larger lesions than male mice, which is evident in **paper I**.[146] The mice that are referred to as wild-type, in **paper I**, are C57Bl/6 mice.

The mice display plaque formation in the aortic root, aortic arch and finally in the descending aorta. The morphology of the plaques resembles the human

atherosclerotic plaques although with one difference; they do not tend to rupture and produce thrombus formations. This implies that the mice are a tool for investigating plaque initiation, progression and phenotype although not for studying the events associated with the human cardiovascular disease.

Cohorts

At the University of Lund and the Skåne University Hospitals (SUS), in collaboration with other universities, there have been great efforts to produce high quality databases of human material. I have had the opportunity to work with three of these databases (Figure 8). All individuals included, in any of the cohorts, gave written informed consent.

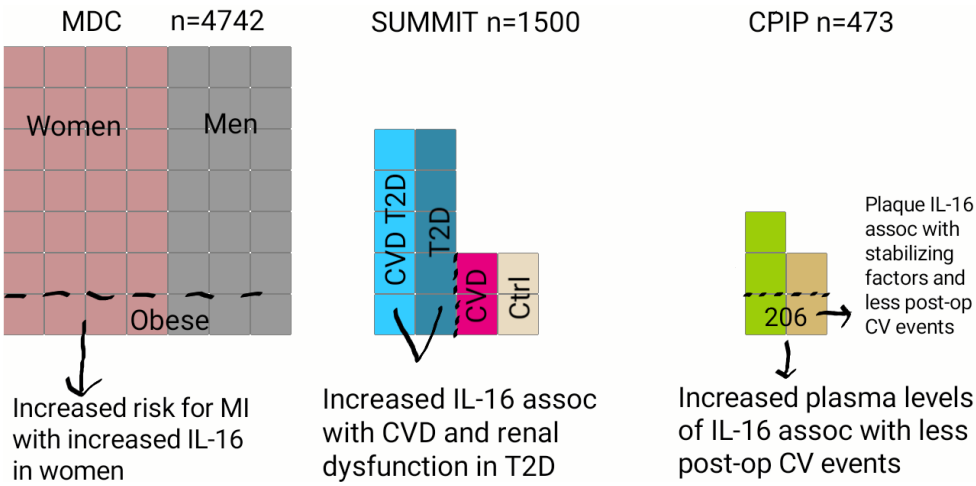

Figure 8. Overview of the clinical cohorts used in this thesis, the number of individuals analyzed in each cohort and the associations to IL-16.

CPIP

In **paper II** and **III** I have worked with the cohort called Carotid Plaque Imaging Project (CPIP). This biobank was, and is currently, collected from patients undergoing carotid endarterectomy (the carotid plaque was surgically removed) at the Vascular Department at SUS, Malmö. It is designed to prospectively investigate secondary prevention in patients with severe carotid atherosclerosis. Inclusion criteria were carotid stenosis degree above 80% (asymptomatic) or carotid stenosis above 70% with stroke, TIA or AF occurring within one month

before surgery (symptomatic). The patients had blood taken the day before surgery and the carotid plaque was snap frozen in liquid nitrogen until further processing.

The most highly stenotic part was saved for sectioning and histological analysis whereas the rest was homogenized and analyzed for other components such as lipids, RNA and protein levels. This database is extremely valuable because it contains both general patient data on risk factors, circulating factors, and an extensive characterization of the plaque phenotype by ultrasound, histology, protein, lipid and RNA profiling. The patients in this cohort are generally old, have considerable medication treatment and have advanced atherosclerosis.

Plasma samples (n=473), and carotid homogenates from 206 patients were assessed on the O-link platform in 2014, where a total of 92 analytes were measured, including IL-16. The individuals in the CPIP cohort, were generally a few years older and more extensively medicated (statins, anti-hypertensive and beta-blockers) than the individuals included in the SUMMIT study (**paper V**). Another difference in the CPIP cohort (**paper II-III**) is that not all of the individuals have had CV events, or diabetes, compared to the individuals in the SUMMIT study (**paper V**).

MDC

The Malmö Diet and Cancer Study (MDC) was initiated in the 1990's to facilitate research on how environmental factors predict diseases like cancer, diabetes and CVD in a population-based prospective study on primary prevention of the Malmö inhabitants. The entire study consists of approximately 30 000 individuals and 6103 of these were randomly selected to be a part of the cardiovascular arm of MDC. Blood samples were taken and plasma and cells were stored until further investigated. In 2014 plasma samples from 4742 individuals were analyzed on the O-link platform to determine the levels of 151 analytes in the circulation. Data was collected in 2013 from the National registries to follow-up on morbidity, mortality causes and events.

SUMMIT

The SUMMIT study (surrogate markers for micro- and macrovascular hard end points for innovative diabetes tools) is the latest and in some ways the trickiest cohort that I have worked with. The cohort is a multicenter study investigating cardiovascular complications of diabetes in 1500 individuals in Dundee, Exeter, Lund and Pisa. The design of the study is a retrospective case-control study including four different groups; relatively healthy individuals, individuals with

CVD, individuals with type 2 diabetes (T2D), and individuals with a combination of CVD and T2D. There was no retrospective limit in time for the occurrence of the CV event or the onset of T2D, which results in a relatively heterogeneous diseased population.[147]

Plasma samples were taken from all of the individuals and circulating cells were analyzed on 211 individuals included in Lund. Plasma samples were sent to O-link to analyze 92 circulating factors where IL-16 was included. The strength of the cohort is that it is a multicenter study, although the multicenter aspect also makes the analysis complicated. It became evident that the different centers had slightly different populations when analyzing risk factors and medication.

In **paper V** we associated the prevalence of CVD and T2D with IL-16 plasma levels. It would be very interesting to reassess the findings, within the same cohort after a few years, on follow-up data describing the incidence of CVD and the progression of surrogate markers of vascular complications.

Statistics

Statistical workflow

To be able to answer the hypothesis that IL-16 could be a biomarker in CVD I needed to acquire some basics in statistics. Before I even could start to test my hypothesis, in the different cohorts, I had to contemplate on a number of different matters.

Confounders

The first matter was which potential confounders should I adjust for? A confounder is something that could impact the factors, directly or indirectly, that you are measuring although they do not necessarily have to impact them (Figure 9). Without adjusting for confounders these factors can cause associations to arise even though there might not be an association. Therefore, it is essential to try to identify possible confounders so not to draw invalid conclusions from the tested hypothesis.

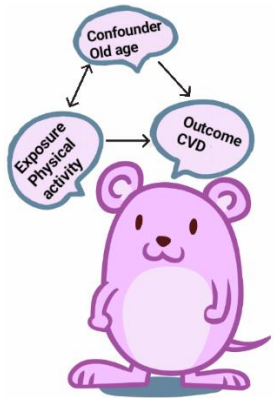

Figure 9. A confounding factor can impact both the measured variable (exposure) and the measured outcome.

There are several different strategies to use to determine which confounders should be used. The simplest is to do a literature search and accept the generally established factors (and/or proven) involved in a disease. The classical example would be the Framingham risk factor score, which was developed from a study investigating a relatively healthy population in the 1950's and factors associated to CVD 5-30 years after the individuals were given a baseline examination. The Framingham risk factors should therefore be suitable to use in the population-based MDC cohort.

There is also the more tedious way of comparing all the risk factors measured with both the associations to disease and with the factor of interest (in this case IL-16). At a biostatistics course I was given the advice to include all associations with a P-value under 0.2. In such an immense material as MDC, with hundreds of variables in the database, this would take some time and most probably generate too many confounders. The general rule of thumb is that it is possible to adjust for 1 confounder or factor per 100 individuals in the analysis. In the MDC this would allow me to adjust for 46 confounders when analyzing all 4742 individuals together.

A middle of the road approach is to analyze the general risk factors that have been described and the factors known to impact the variable of interest (in my case IL-16). This approach will most probably generate less confounders than the previously described method. This is the approach I have used to analyze the CPIP cohort, as this cohort is a severely diseased and medicated population, I do not believe that the Framingham risk factors are sufficient enough. I have also chosen to set the cut off for the confounders at a p-value of 0.05 as is generally expected as the level of statistical significance.

Baseline clinical characterization

The second matter is if the variables are normally distributed or not. If they are not, one can then either: choose to logarithmically transform them, or to use them in statistical tests that are non-parametric (the statistical test takes into account that the variable is not normally distributed). A normal workflow for me would be to describe the cohort by investigating the risk factor distribution and other factors such as medication and to present the findings in a baseline clinical characterization table. I then continue to check if there is any significant difference in the levels of the tested factor (IL-16) in the groups of interest (i.e. CV events vs. controls). Next I associate the levels of the factor of interest with other risk factors or variables to see if there are any associations. Here I also establish which confounders are essential to include.

Regression analysis

Depending on the cohort design, I can either perform a binary logistic regression, where I answer the question if my tested factor (IL-16) is independently associated with prevalent CVD after adjusting for potential confounders. If the cohort is of a prospective character, I use Kaplan Meier curve to test if IL-16 is associated with incidence of CV event over time. If the Kaplan Meier curve is significant and none of the sub fractions (e.g. tertiles) of the IL-16 crosses each other I can conclude that the sub fractions are statistically separated from each other and that the survival distribution is different in the population, I can then progress with analyzing my data in a Cox regression.

The Cox regression resembles the binary logistic regression with the addition of time from measurement until event or censoring. In both the binary logistic regression, and in the Cox regression, I test the continuous factor and the factor divided into groups based on the level of IL-16 (dichotomized, tertiles or quartiles depending on the cohort size), as well as the trend across the n-tiles. If the p-value is significant (the 95% confidence interval does not intercept 1) then the factor is significantly associated with events independently of the confounders that I have determined previously. A value greater than 1 describes an increasing event rate with increasing IL-16 levels, whereas a value below 1 describes a decreasing event rate with increasing IL-16 levels.

Associations

The last matter is that the data generated by producing statistical analysis in **paper II-V** are based on association, which have been based on one single measurement at one time-point. These associations do not have to describe any causality, and always needs to be confirmed in other cohorts, different populations, and in experimental studies before any strong conclusions can be drawn. For the role of IL-16 in atherosclerosis and CVD, I have tried my best to test whether IL-16 has

potential as both a biomarker and as a possible therapy in experimental atherosclerosis, by investigating plaque burden in atherosclerosis prone mice treated with IL-16.

Techniques

To test the hypothesis that IL-16 is associated with a decreased risk of developing CVD and atherosclerosis, we have used several different techniques.

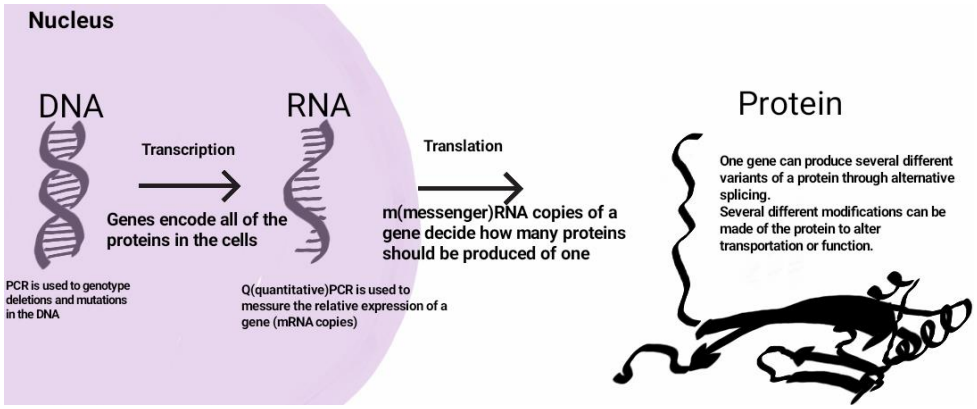

Figure 10. The central dogma explaining how all living organisms cosists of DNA encoding genes which are transcribed into mRNA and translated into protein.

We have investigated DNA, mRNA and protein levels (extracellular and intracellular) in lymphoid organs, in the circulation and in tissues associated with atherosclerosis (human carotid plaques, and aorta, aortic root sections, and carotids from hypercholesterolemic mice) (Figure 10).

By altering the DNA in C57Bl/6 mice we have been able to investigate how deficiency of A*poe* and *Il16* affects atherosclerosis. The altered genotype is consistently checked to assure that the defectiveness remains in the mice by running polymerase chain reaction (PCR) to amplify DNA with specific primers designed to amplify the region of interest (genes coding for *Apoe* and *Il16*).

In both **paper I** and **II** we measured mRNA levels in the carotids. The mRNA measurements are a measurement of how much a gene is transcribed, and is normalized to genes that are expressed at constant rate in all cells (housekeeping genes) to be able to indicate if the gene of interest is up or down regulated under certain conditions. This method is called quantitative real time PCR (qPCR). The technique is based on primer binding to a specific sequence, in the gene of interest,

and of a probe binding in between the primer pair. As the mRNA is amplified the probe will detach and emit a fluorescent signal which is detected for each amplification cycle. The amount of fluorescence is relative to the copy number of the PCR product and then further normalized to the amount of housekeeping gene copies. Measuring mRNA does not resemble the absolute amount of the translated protein, and therefore measuring protein content is also important to be able to explain the biological implications.

We have measured protein levels in several different ways. One of the "easiest" techniques is by measuring proteins by ELISA (enzyme-linked immunosorbent assay), which utilizes specific antibodies binding the protein of interest. These antibodies are then detected by a secondary antibody which has an enzyme conjugated to it allowing a substrate to be degraded and produce a shift in colour which is detectable. The colour intensity is related to a known serial dilution of the protein of interest and thereby the amount in the test samples can be calculated. This technique has also been adapted to facilitate the measurement of several different proteins in the same sample, here we have used both MesoScale and Luminex technology.

The MesoScale technology utilizes electrochemiluminescense, by tagging the protein of interest with Sulfo-Tags, which emit light when they are stimulated with electrochemical stimuli. Each well consists of specific spots, corresponding to the site where the specific protein of interest is detected. The light intensity in each of these spots is then compared to the standard curve and an estimated concentration can be calculated. The draw-back of this technique is that it is not possibly to rerun the plate if any errors occurred during measurement.

The Luminex technology utilizes fluorescent beads, which carry antibodies specific for the protein of interest. The assay requires lasers and sensors to detect the emission wavelengths of the beads carrying the captured protein. There are two really advantageous sides of Luminex. Firstly, the numbers of beads available are up to a hundred, which allows for 100 different proteins to be assessed in the same sample. The other benefit of using Luminex is that the beads can be reanalyzed if any errors occurred during the first measurement.

Measuring proteins in aortic root section or in sections from human carotids by immunohistochemistry utilizes the same technique of a specific antibody designed to measure the protein of interest and detecting the cleavage of the added substrate.

To identify immune cells, the most commonly favorable method is flow cytometry (in daily use called FACS). This method also utilizes the specific binding of antibodies to protein on the surface or inside the cell. The advantage of flow cytometry is that several antibodies for different proteins can be combined with

size and density measurements of cells, and thereby each cell can be thoroughly characterized, which is needed for immune cells, as many of them carry shared proteins on their cell surface.

The most recently invented technique, to be used in this thesis, is the technique developed by O-link (http://olink.com). This method utilizes antibodies for specific binding to the protein of interest. The antibodies have specific DNA probes attached to them and when two antibodies have bound the same protein these probes can hybridize, extend and amplify the specific sequence of the protein of interest allowing a read out similar to the qPCR. The main advantage of this system is that it has the capacity to measure 92 analytes in 1 µl of sample due to multiplexing and high sensitivity which is of great interest while analyzing human material.

Aims and key findings

The aim of the collected work in this thesis was to investigate the potential of IL-16 as a biomarker in the different stages of the atherosclerotic disease in humans, and to validate these findings in an experimental setting. Given the limited knowledge of the relationship between IL-16 and development of CVD we wanted to investigate the role of IL-16 in primary prevention at population level, in established CVD and in cardiovascular complications of diabetes.

Paper I

Aim: To determine the role of IL-16 in the formation and progression of atherosclerotic plaque in hypercholesterolemic *Apoe* deficient mice.

Key finding: IL-16 has the ability to decrease atherosclerotic burden in hypercholesterolemic *Apoe* deficient mice (Figure 11), which is most probably due to modulation of T cell polarization towards an anti-inflammatory state.

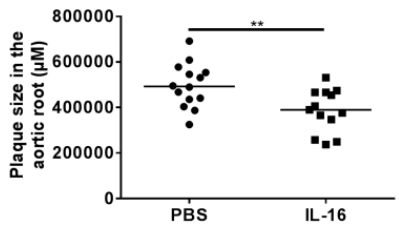

Figure 11. IL-16 treatment decreases plaque burden in the aortic root of hypercholesterolemic ApoE deficient female mice.

Paper II

Aim: To establish if increased mRNA and protein levels of IL-16 in human carotid plaques are associated with a more stable plaque phenotype and a decreased risk of CV events in 206 individuals undergoing endarterectomy.

Key finding: IL-16 was associated with plaque stabilizing factors collagen, elastin and FoxP3 expression. High IL-16 protein levels in human carotid plaques were independently associated with a decreased risk of suffering a CV event during a two-year follow-up after surgery (Figure 12).

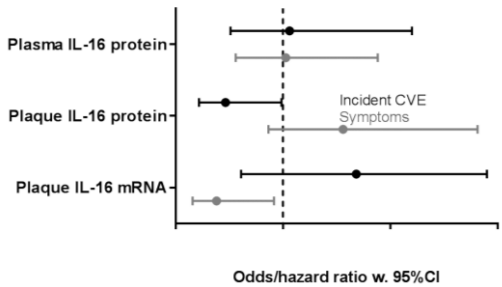

Figure 12. IL-16 protein in homogenates from human carotid plaques (n=206) is independetly associated with decreased incidence of cardiovascular events. IL-16 mRNA in homogenates from human carotid plaques (n=206) is independtly associated with asymptomatic patients. Black lines display the hazard ratios (with 95% CI) for incidence of cariovascular events and grey lines depicts the odds ratios (with 95% CI) for associating with symptomatic carotid plaques.

Paper III

Aim: To investigate if plasma levels of IL-16 can predict CV outcome in 473 patients who have undergone surgery to remove the carotid plaque.

Key Finding: High levels of IL-16 in plasma were independently associated with a decreased risk of suffering from CV events and death due to CV events, during a follow-up period of three years (Figure 13).

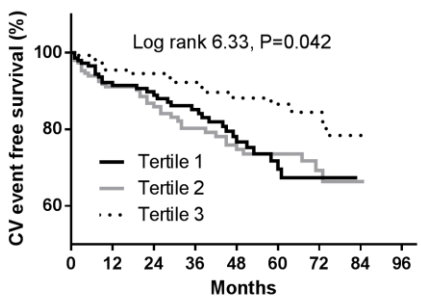

Figure 13. Kaplan-Meier curve depicting cardiovascular event free survival during follow-up of IL-16 plasma tertiles in individuals who underwent endarerectomy (n=473).

Paper IV

Aim: To study if plasma levels of IL-16 can be used as a prospective biomarker, of CVD, in the population-based MDC study.

Key finding: Increased levels of IL-16 in plasma does not have the capacity to predict CV risk in a population-based prospective setting when investigating 4742 individuals with a follow-up time of approximately 20 years. High levels of IL-16 in plasma were increased with CV risk factors and displayed independent associations with an increased risk of MI in women (Figure 14). We also identified four SNPs, which were associated with IL-16 plasma levels, however they did not associate with an increased risk of CV events.

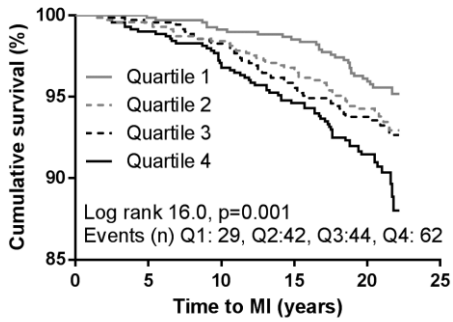

Figure 14. Kaplan-Meier curve depicting myocaridal infarction event free survival of women divided in quartiles depedning on IL-16 plasma levels (n=2874).

Paper V

Aim: To assess if plasma IL-16 is a possible marker of vascular complications in diabetes in a multi-center study.

Key finding: Individuals with combined CVD and type 2 diabetes had increased levels of IL-16 in plasma compared to individuals suffering from only type 2 diabetes (Figure 15). This was further supported by the SNP rs11556218, which was associated with reduced plasma levels of IL-16, and with decreased levels of surrogate markers of atherosclerosis. Individuals with high levels of IL-16 in plasma had a dramatically increased risk for renal damage, which was independent of risk factors.

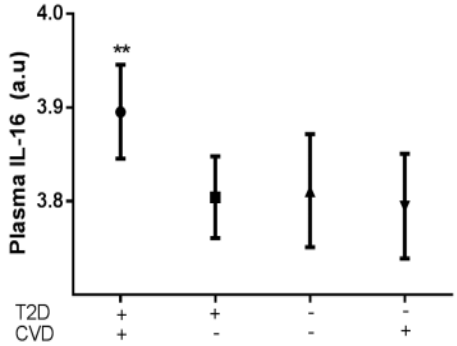

Figure 15. Plasma levels of IL-16 (a.u.) are increased in individuals with cardiovascular complications of type 2 diabetes.

Results and discussion

In the following sections I will present, and discuss, the data describing the role of IL-16 in atherosclerosis and cardiovascular disease. From studies in an experimental model of atherosclerosis, I will present our findings of the role of IL-16 in dampening plaque progression, and the connection to apoptosis and T cells. I will then continue to present the associations found with IL-16 in secondary prevention in individuals with severe carotid atherosclerosis. I will present the inherited predisposition of IL-16 in the form of genotyped single nucleotide polymorphisms (SNPs) and their associations to IL-16 plasma levels. Finally, I will present whether or not IL-16 plasma levels can be used in primary prevention of cardiovascular risk and in cardiovascular complications in diabetic subjects.

IL-16 in experimental atherosclerosis

In **paper I** we tested whether IL-16, in a controlled environment, impacted atherosclerosis formation and plaque phenotype in an experimental model of atherosclerosis. The benefit of studying atherosclerotic development in mice is that there is limited "background noise" due to the identical genetic and environmental background. The drawback is that the mice never generate atherosclerotic plaques leading to a CV event. Administration of IL-16 to hypercholesterolemic *Apoe* deficient female mice reduced the atherosclerosis burden compared to control treated mice. This was further supported by the fact that *Il16* and *Apoe* deficient hypercholesterolemic male mice had increased atherosclerotic burden compared to control mice.

We could not identify any differences in the compositions of the plaques, situated in the aortic root, when investigating inflammation (MOMA and CD4 staining) or collagen (Van Gieson staining). In the brachiocephalic artery (which we use as the equivalent to the carotid in humans) there was increased expression levels of genes associated with apoptosis (caspase-3 and BAX) in mice treated with IL-16 compared to control. There was also a significant decrease in caspase-3 gene activity in mice deficient in both *Il16* and *Apoe* compared to *Apoe* deficient mice. The results indicate a connection between apoptosis and IL-16 in experimental

atherosclerosis (**paper I**) and the connection is also present in human carotid plaques (**paper II**).

Apoptosis

Is apoptosis beneficial in atherosclerosis? Promoting controlled apoptosis in the atherosclerotic plaque could be advantageous as this will clear cell components in an anti-inflammatory fashion. The opposite to apoptosis, necrosis, is thought to be harmful in the atherosclerotic disease and believed to promote vulnerable plaques. Immune cells are readily produced from stem cells in the bone marrow, and will be replenished in the circulation, this is however not the case of all of the other cell types in the body. For example, apoptosis in cardiomyocytes is not beneficial due to that less than 50% of the cardiomyocytes are renewed after birth.[148] Death of the cardiomyocytes will therefore, most probably, result in a weakened heart increasing the risk of morbidity and mortality. This is of course not a desirable consequence of IL-16 treatment.

Table 2. Spearman correlations between IL-16 and death receptors.

	MDC		**SUMMIT**		**CPIP plasma**		**CPIP plaque**	
n	4742		1435		558		202	
	r	P-value	r	P-value	r	P-value	r	P-value
TRAIL	0.537	0.0E0	0.232	6.3E-19	0.159	0.00017	0.505	1.9E-14
TRAIL-R2	0.533	0.0E0	0.397	1.9E-54	0.268	1.1E-10	0.454	1.1E-11
TNF-R1	0.625	0.0E0	0.576	2.2E-127	0.328	1.8E-15	-0.008	ns
TNF-R2	0.622	0.0E0	0.572	3.3E-125	0.363	8.4E-19	0.272	0.00014
FAS	0.586	0.0E0	0.490	2.0E-87	0.394	3.7E-22	0.399	4.2E-9

Evidence put forward in this thesis supports the connection between IL-16 and increased apoptosis, both in mice (**paper I**) and in humans (**paper II**), as well as in Table 2 and Figure 16. There is, however, a risk of undesirable side effects. IL-16 seems to be a consequence of activated caspase-3, as has been described previously. We have seen that IL-16 has a role in inducing apoptosis by detecting an enhanced gene activity in genes involved in the apoptotic pathway from studies on IL-16 treated mice (**paper I**).

Further characterization of the atherosclerotic plaques from IL-16 treated, or deficient mice, is needed to conclude that IL-16 induces apoptosis. This can for example be done by TUNEL or active caspase-3 measurements in the atherosclerotic plaques. To be able to consider IL-16 as a potential therapy further

studies on different cell types are essential. The experimental studies should also focus on if IL-16 has the capacity to increase risk factors known to promote cardiovascular disease and vascular complications, so that the net effect eventually will be an undesired overall risk for cardiovascular disease.

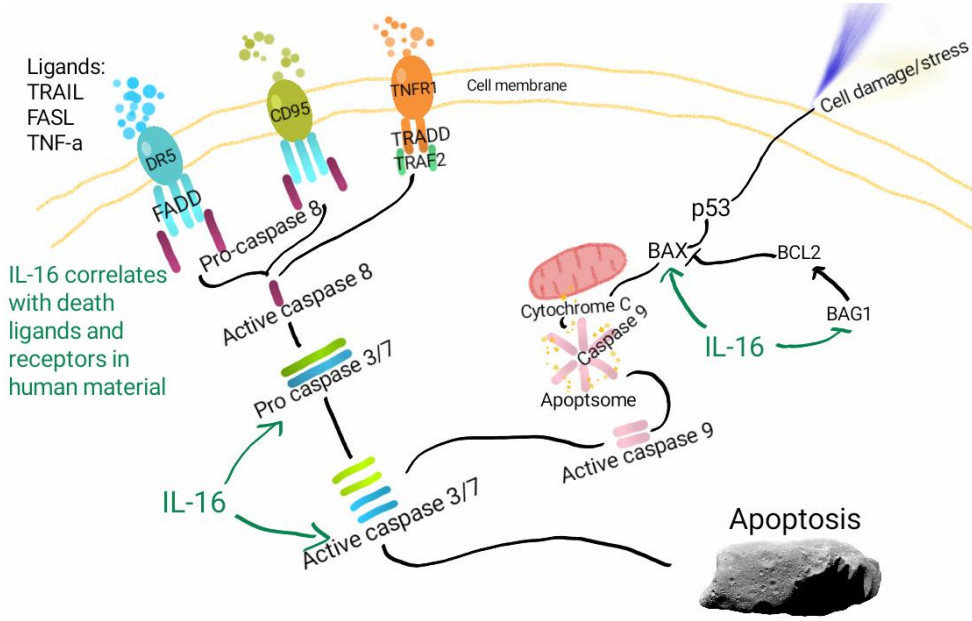

Figure 16. Evidence presented in this thesis (green) of the connection between IL-16 and apoptosis.

IL-16 and T cells

IL-16 increases the CD44 expression on T cells *in vivo*, or switches cells phenotype from naïve to effector/memory cells (**paper I**). This happens within 24 hours and is therefore not a traditional response to activation, as this is traditionally thought to take at least a couple of days or up to a week to reach a maximum peak in the response.[149, 150] IL-16 has been reported to increase Tregs and to induce unresponsiveness in polyclonal activated T cells.[130, 132] IL-16 has also been reported to play an anti-inflammatory role in EAE, although these data are to some extent contradictory to other studies showing preferential migration of Th1 cells to IL-16.[106, 107, 133, 151, 152] IL-16 has also been reported to be involved in pathogenesis in diseases like diabetes and RA.[115, 134, 153-157]

An increase in the effector/memory population of the T cells has been proposed to promote atherosclerosis, and the associations between IL-16 and increased levels of CD44 expressing T cells and atherosclerotic burden in **paper I** is puzzling.[158] A similar association between IL-16 and effector/memory T cells was seen in a sub-cohort of the SUMMIT study (Table 3).

Table 3. Spearman correlations between IL-16 plasma levels and number of circulating T cell populations in 211 individulas of the SUMMIT cohort.

	Correlation	P-value
CD4+CD45RA+	-0.164	0.017
CD4+CD45RO+	0.142	0.039
CD4+CD62L+CD45RO-	-0.160	0.020
CD4+CD62L+CD45RO+	0.220	0.001
CD4+CD197+CD45RO-	-0.126	0.067
CD4+CD197+CD45RO+	0.130	0.060
CD8+CD62L-CD45RO+	-0.138	0.046
CD8+CD197+CD45RO-	-0.133	0.054

The mice which were treated with IL-16, in **paper I**, displayed increased percentages of $CD44^+$ T helper cells. To further analyze these cells, we investigated makers known to be associated with cells identified of anti-inflammatory capacity. The $CD44^+$ T cells, from IL-16 treated mice, had a higher fraction of regulatory T cells and T cells expressing the immune regulator CTLA-4 compared to control treated mice. The increase of $CD44^+$ Tregs and CTLA-4^+ T cells, in **paper I,** could explain the decreased levels of atherosclerosis in IL-16 treated mice. The anti-inflammatory properties of IL-16 were further supported by the decrease in circulating TNF-α and the decreased release of IFN-γ from stimulated splenocytes (**paper I)**.

In a sub-cohort of the MDC study (n=540), PBMCs were analyzed by flow cytometry and correlated to IL-16 levels in plasma. There are clear sex-specific differences where women with increased IL-16 levels in plasma have more cytotoxic T cells as well as Tregs (Table 4). CD8+ T cells have been described to have pre-made bioactive IL-16. There are also evident differences in sex-specific correlations between HDL and T cells, increasing HDL levels associates with decreasing T cell levels in women but no associations were found in men (Table 3).

Table 4. Sex-specific Spearman correlations for IL-16 with T cell populations as well as sex-specific Spearman correlations with HDL. A total of 222 men and 318 women were studied for T cell numbers and IL-16 levels in the circulation.

	IL-16		**HDL**	
	Men	Women	Men	Women
CD3+ T cells	0.028	0.138*	-0.079	-0.247***
CD3+CD4+ T cells	-0.026	0.045	-0.048	-0.153**
CD3+CD8+ T cells	0.044	0.161***	-0.071	-0.257***
CD3+CD4+IFN-γ+ T cells	-0.115	-0.016	-0.092	-0.105*
CD3+CD4+IL4+ T cells	-0.141*	0.007	-0.060	-0.077
CD3+CD4+CD25+ T cells	-0.072	0.005	-0.018	-0.033
CD3+CD4+CD25+FoxP3+ T cells	-0.010	0.118*	0.025	-0.076

In a linear regression model IL-16 was positively associated (B=0.055, P=0.026) and HDL was inversely associated (B=-0.156, P=0.000002) with the number of cytotoxic T cells when adjusting for Framingham risk factors in women. No significant associations were found in men except for smoking (B=0.125, P=0.0004). Regulatory T cells were independently associated with IL-16 (B=0.003, P=0.033) and age (B=0.001, P=0.027) in women, whereas no associations were found between IL-16 and Tregs in men.

The evidence for a connection between IL-16 and caspase-3, and the knowledge that IL-16 is involved in increasing the Treg population, as well as inducing unresponsiveness in T cells, is very interesting.[130, 132] There is a proven role of caspase-3 in anergy and evidence of anergic T cells generating regulatory T cells.[100, 101] Further investigation is needed to elucidate the potential role of IL-16 in inducing anergic/regulatory T cells. It is also interesting to investigate the potential anti-atherosclerotic properties of anergic T cells.

It is fascinating that the sub-types of T cells correlate differently with IL-16 once analyzing men and women separately. Further studies investigating the association between lipid metabolism, sex-hormones, immunology and IL-16 could potentially hold valuable information.

IL-16 in severe carotid atherosclerosis

To be able to study how IL-16 impacts plaque phenotype and CV risk we had the opportunity to measure IL-16 in carotid plaques surgically removed from 206 patients, presented in **paper II**, in the CPIP cohort. The carotid plaques have been

extensively studied and characterized allowing us to associate IL-16 levels to markers known to stabilize, or to promote, plaque rupture.

In **paper II** we measured both IL-16 mRNA and protein levels in the carotid plaques. Increasing IL-16 mRNA levels were associated with asymptomatic patients, and increased expression of CD4 and FoxP3. Increased protein levels of IL-16, in the carotid plaques, were associated with elevated levels of collagen, elastin and caspase-3 activity. Collagen and elastin are ECM proteins generally accepted to promote stabilization of the atherosclerotic plaque, which should lead to less rupture prone plaques and less clinical manifestations of CVD.

The aim was to investigate if the increased IL-16 protein levels also associated with less CV events by recording CV events for approximately 2 years after removing the carotid plaque (**paper II**). During follow-up the individuals with levels of IL-16 above median, in the carotid plaque, displayed association with a decreased risk of suffering a CV event after adjusting for potential confounders (HR 0.47, 95% CI 0.22-0.99, P=0.047). No significant associations were detected between IL-16 mRNA expression levels in the carotid plaque, or with plasma IL-16 levels, and CV events during follow-up.

In **paper III** we had the possibility to analyze plasma samples from 473 individuals undergoing carotid endarterectomy and to follow these individuals for approximately 3 years. During this time CV events and CV events leading to death were recorded. The associations between high levels of IL-16 and a decreased risk of CV events was significant in a Cox regression when comparing to the lowest tertile and adjusting for potential confounders (HR 0.535, 95% CI 0.27-0.81, P=0.007). An even stronger reduction in risk was found between the highest tertile of plasma IL-16 and risk of dying due to CV complications (HR 0.252, 95% CI 0.09-0.70, P=0.008) compared to individuals within the lowest tertile, after adjusting for potential confounders.

The individuals included in these studies (**paper II-III**) are all affected by severe atherosclerosis. The CPIP study is a large and well characterized database, although the design of the study has not included any healthy individuals as controls, allowing us only to draw conclusions based on this highly selected, and diseased, population. Plasma levels of IL-16 from healthy individuals that are age and sex matched would have been valuable, however carotid vessels from healthy individuals do not have plaques, which make a comparison difficult and complex.

It is also puzzling that IL-16 plasma levels do not display any association to the primary CV event, demonstrated by the lack of association with the asymptomatic group. There is a possibility that IL-16 is elevated as a repair mechanism in both the symptomatic and asymptomatic group. Further experimental and mechanistic

studies are needed to evaluate the potential of IL-16 in severe atherosclerosis and the clinical complications thereof.

In conclusion, IL-16 displays associations with increased stabilizing factors and a decreased risk of post-operative CV events. Our data support the hypothesis that high levels of IL-16 confers a protective role in severe carotid atherosclerosis, in an elderly population.

IL-16 gene polymorphism

The aim was to identify SNPs associated with IL-16 plasma levels to further strengthen the conclusions drawn from the associations regarding IL-16 plasma levels. The SNPs also hold potential as a screening method if they are significantly impacting the risk of suffering from CVD. In **paper IV** and **V**, we measured IL-16 in plasma and SNPs located in, or close to, the IL-16 gene in the MDC and SUMMIT studies. SNP data can indicate if certain alleles are associated with increased, or decreased, levels of IL-16 in plasma and can therefore support a predisposition from birth to have altered risk of CVD due to IL-16 plasma levels.

By researching literature, we identified four SNPs associated with IL-16 plasma levels (rs4072111, rs8034928, rs3848180, and rs1131445) in the prospective population-based MDC study (**paper IV**). All but one were associated with increased levels of IL-16 in plasma, whereas rs3848180 was associated with a reduction in plasma levels after adjusting for Framingham risk factors. None of the SNPs were associated with an increased risk of MI, stroke or all-cause mortality after adjusting for Framingham risk factors. Rs1131445 in men, which associated with increased levels of IL-16, was significantly associated with a 40% reduction in risk of suffering a MI. The same SNP (rs1131445) was associated with a 23% risk reduction for all-cause mortality in the entire population, and a 35% risk reduction in women.

In the SUMMIT study (**paper V**), rs11556218 (which was not available on the chip used to identify SNPs in the MDC study) was associated with reduced levels of IL-16 in plasma and was inversely associated with markers of atherosclerosis, supporting a harmful role of IL-16 in vascular complications of type 2 diabetes. Interestingly, rs11556218 has previously been described to be associated with increased risk of ischemic stroke, which makes it a potential candidate for further analysis in the MDC and CPIP cohorts.[140]

In **paper IV** and **V**, we identified in total five SNPs that were associated with IL-16 plasma levels. The data presented are based on associations and do not prove causality. There are several different steps between the DNA sequence, where we

have measured the SNPs, and the functional IL-16 protein which can impact the levels of the circulating IL-16 (Figure 8). There is also a possibility that it is not the identified SNP that controls the associations with the plasma levels of IL-16, but instead a sequence or SNP that is in linkage disequilibrium with the measured SNP. Finally, one needs to be aware of the fact that we are testing 4 or 5 SNPs with several different outcomes (variables) which increases the number of statistical tests that have been performed. As the p-value is set as being significant if under 0.05 there is a chance of 1 in 20 of getting a false positive significance.

There have been several reports on IL-16 associated SNPs in case-controls studies of CVD but only one of them have investigated the associations to plasma levels of IL-16.[137-140] To my knowledge the two studies presented in **paper IV** and **V** are the two largest cohorts that have been analyzed for SNPs associated with IL-16 plasma levels and further associated with CVD. **Paper IV** is also the first to perform an extensive prospective study of the role of IL-16 in CVD, and **paper V** is the first to investigate the role of IL-16 in vascular complications of T2D.

The presented results imply that the inherited genetic material determines the IL-16 plasma levels later in life, however these SNPs do not seem to be strong predictors of CV risk in a large population-based prospective study (**paper IV**). In a case-control study (**paper V**) the SNP rs11556218 was associated with reduced levels of plasma IL-16 and less atherosclerotic manifestations, supporting a harmful role of IL-16 in vascular complications of type 2 diabetes.

IL-16 and cardiovascular risk factors

There are several known risk factors which are used to determine the future risk of suffering from a CV event, of which the most commonly used is the Framingham risk score which includes; age, sex, smoking, cholesterol, HDL and systolic blood pressure. To understand the role of IL-16 in CVD we therefore first explored the associations between IL-16 plasma levels and risk factors in MDC, which is a prospective population-based cohort of relatively healthy individuals. Interestingly, in **paper IV**, IL-16 levels were significantly lower in females, and as a consequence the analysis was split on sex so not to mask any sex-specific differences.

All of the Framingham risk factors were associated with increasing IL-16 levels, except for smoking, in the MDC study. After expanding the analysis to include diabetes mellitus, BMI, LDL, diastolic blood pressure, white blood cell count and lymphocyte count, all of the risk factors associated positively with increasing IL-16 levels in plasma in both men and women. In women the risk factors most

closely associated with IL-16 levels were BMI, cholesterol, LDL, HDL (inversely) and triglycerides. Men displayed a stronger association between IL-16 with systolic and diastolic blood pressure, which was then followed by BMI, HDL (inversely) and triglycerides.

In **paper V**, where we investigated the vascular complications of T2D, it became evident that renal dysfunction was highly associated with increasing IL-16 levels in individuals with T2D, due to the increasing levels of creatinine and decrease in eGFR with increasing IL-16 plasma levels. In the SUMMIT study (**paper V**) IL-16 displayed associations to HDL (inversely), LDL, cholesterol and systolic blood pressure although the majority of individuals were medicated (i.e. with statins and metformin).

In a prospective population-based study (**paper IV**), consisting of relatively healthy individuals, IL-16 was associated with most of the measured risk factors known to impact CVD. In an elderly population already suffering from disease which was more medicated, creatinine was associated with IL-16 plasma levels in individuals suffering from T2D (**paper V**) or from severe atherosclerosis (**paper III**). It is left unclear if the high levels of IL-16 are a compensatory response to the increased levels of risk factors, or if it is the actual increase in risk factor levels that drives the augmented IL-16 levels.

IL-16 as a biomarker in primary prevention

To determine if IL-16 could be a potential biomarker in primary prevention we performed Cox regressions adjusted for potential confounders by Framingham risk factors and diabetes mellitus. There were no significant associations between increased risk of suffering from a CV event with high IL-16 levels in men. There was a 64% increased risk for suffering from a MI for women within the highest quartile of IL-16, compared to the women in the lowest quartile, of the individuals included in the MDC study (**paper IV**).

The increased risk for MI remained significant after adjusting for all of the risk factors that were associated with IL-16. There were no associations to stroke, CV death or all-cause mortality with high IL-16 levels. So far we could conclude that IL-16, measured in plasma, was not a universal biomarker for CVD in primary prevention, but perhaps a sex-specific marker for MI in women.

IL-16 and obesity

We have described that IL-16 levels in plasma correlates to hypercholesterolemia, and it has previously been shown that IL-16 is increased in individuals that are overweight.[135] Due to the increasing evidence supporting a connection between IL-16 and lipid metabolism, I wondered if IL-16 could predict CV risk in obese subjects in the MDC cohort and therefore I selected individuals with body-mass-index (BMI) over 30 (n=597). Obese individuals displayed a tendency of increased risk of MI with increasing IL-16 levels in tertiles (Kaplan Meier Log rank test 5.132, P=0.077). Next I performed a sex-specific analysis, which displayed no association with increasing IL-16 levels with risk for MI for men (n=215), whereas there was a striking association in obese women (n=380) with increasing IL-16 levels for risk of MI (Log rank test 9.916, P=0.007) (data not included in paper IV).

In a Cox regression, women within the highest tertile of IL-16, had a 3-fold increased risk for MI compared to the lowest (HR 2.972, 95% CI 1.239-7.126, P=0.015) after adjusting for potential confounders. Men displayed no significant association between IL-16 levels and MI (HR 0.974, 95% CI 0.466-2.038, P=0.945). In **paper IV**, we reported an association between IL-16 and MI in all obese and non-obese women collectively (HR 1.640, 95% CI 1.04-2.57, P=0.032), however after only selecting women with a BMI below 30 there was no significant association between increasing IL-16 tertiles and increased risk of MI (HR 1.277, 95% CI 0.842-1.917, P=0.255), suggesting that there is a link between obesity, increased IL-16 levels and risk of MI (data not included in paper IV). Though, it is possible that the exclusion of the high risk obese groups impairs the statistical power by reducing the event rate.

The data presented in **paper IV** supports the perception that CV risk factors may impact IL-16 plasma levels, or vice versa, in the MDC population-based prospective study.

As has been mentioned before, the results presented in **paper II-V** are based on associations and do not allow us to prove causality. With this said the number of individuals included in the MDC study (**paper IV**) is, to my knowledge, the largest of its kind to investigate the potential of IL-16 as a biomarker for primary prevention. There is one study investigating the potential of IL-16 in a relatively healthy population to identify individuals with intermediate risk of suffering from CV event within a 5-year follow-up period. IL-16 was, in this study, combined with several other biomarkers in an algorithm based approach and was associated with increased risk of suffering from CVD.[57]

There is a general consensus that the risk prediction of CVD in women is understudied. It is also known that women during fertile age, prior to menopause,

have a decreased risk of CVD. This is most probably due to increased HDL levels as a result of estrogen levels and this protection decreases after menopause. More research is needed to gain insight into the development and risk prediction of CVD in general, and also more specifically into the characteristics of the disease in women.

In conclusion, the value of measuring IL-16 in plasma for determining primary prevention of CVD is however most likely limited, although IL-16 might be valuable to measure in obese women to predict future risk of MI.

IL-16 is associated with vascular complications of CVD

Since there were evident connections between high IL-16 levels with several of the risk factors known to impact CVD, we went on to study the role of IL-16 in established atherosclerosis. In **paper V**, we investigated if IL-16 could be a marker of vascular complications in subjects suffering from type 2 diabetes in the SUMMIT study, including 1500 individuals.

In **paper V** we report a significant increase of circulating IL-16 in subjects with CVD and diabetes, compared to subjects with only CVD, diabetes or controls. In a binary logistic regression, the subjects with diabetes within the highest quartile of IL-16 levels had a 50% increased risk for CVD compared to the lowest quartile, after adjusting for Framingham risk factors. We could also confirm that plasma levels of IL-16 are associated with increased amount of risk factors in subjects with diabetes. Creatinine, eGFR and systolic blood pressure were the risk factors displaying the strongest associations with IL-16. Furthermore, we report associations between increasing IL-16 levels with surrogate markers of atherosclerosis, such as ABPI (inversely) and with increased plaque area of the common carotid artery.

In the CPIP study (**paper III**), plasma levels of IL-16 in 473 individuals displayed significant associations with increasing serum creatinine, supporting the strong associations found in the SUMMIT study (**paper V**). The increased, respectively decreased, levels of creatinine and eGFR with increasing IL-16 levels purported us to reflect upon whether kidney damage was associated with IL-16.

Since diabetes is known to affect vasculature in the whole body we had the privilege of having data on renal dysfunction, in the SUMMIT study (**paper V**), reported as microalbuminuria, proteinuria or renal failure. To our surprise the quartile including subjects with the highest levels of IL-16 in plasma had more than a 6-fold increased risk of suffering from renal dysfunction compared to the individuals with the lowest quartile, after adjusting for risk factors.

It is known that heart and kidney function is closely linked. The term cardio-renal syndrome (CRS) is used to describe the condition of the two-way relationship between CVD and the worsening of kidney function (Figure 17). Several different factors are thought to play a role in CRS including the immune system, cytokines and apoptosis.[159, 160]

It is clear that individuals with both diabetes and CVD have an increased risk of suffering from renal dysfunction. The association between IL-16 and increased risk of CVD could indicate a stronger association with renal dysfunction. In a linear regression model IL-16 was as significant a marker for renal dysfunction as was CVD. IL-16 was only exceeded by creatinine in predicting risk of renal dysfunction, whereas other risk factors did not predict risk of renal damage to the same level.

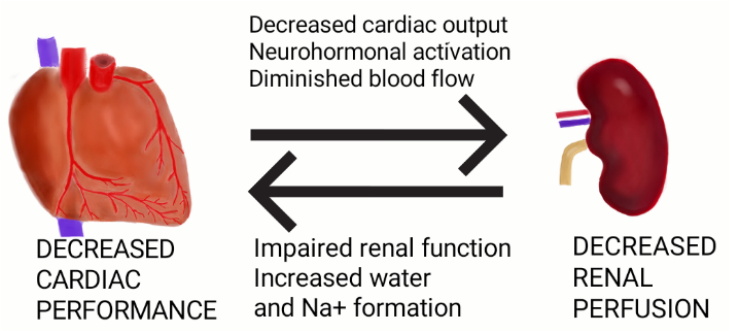

Figure 17. Cardiorenal syndrome - the interplay between decreased heart function and decreased renal function.

Whether the increased IL-16 levels are a result of the increased risk factor levels or a consequence thereof we cannot answer. Furthermore, there is a possibility that confounding factors, which might have not been taken into consideration, are responsible for the presented associations between IL-16 and risk factors. There are a few other studies describing an association between IL-16 and obesity, as well as with diabetes.[115, 134, 135]

It was evident that IL-16 was strongly associated with renal damage. If IL-16 is responsible for driving renal damage, CVD, or both, we cannot answer in this study. There is even a possibility that the increased IL-16 levels are a compensatory factor trying to reduce the vascular damage. Zhao et al. have proposed a compensatory role of IL-16 in the outgrowth of endothelial progenitor cells in individuals with kidney disease.[161] Further experimental studies are called for to elucidate the role of IL-16 in vascular complications of diabetes, as well as in the connection to CV risk factors.

Our data support the hypothesis that IL-16 is associated with an increased rate of prevalent CVD in individuals with T2D in a multicenter study. The presented data are based on statistical tests, calculating the probability of co-variance and do not predict if IL-16 has a driving or inhibiting effect on the disease. There has, however, been evidence presented for a pathological role of IL-16 in an experimental study in non-obese diabetic (NOD) mice where the authors used the human IL-16 blocking antibody 14.1.[115] The same antibody, 14.1, was used in **paper I** included within this thesis, where we failed to measure any reduction of circulating levels of IL-16 after 14.1 treatment. To my knowledge there have been no larger studies investigating the associations between IL-16 and cardiovascular complications in diabetic subjects.

The multicenter aspect of the SUMMIT study (**paper V**) was one of its strengths although also one of the complicating matters of this study. It became evident that the four different study centers (Dundee, Exeter, Lund and Pisa) differed slightly in the enrolled populations when accounting for age, gender, medication and so forth. The difference in the study center populations made the confounding factors difficult to determine. Further research on the role of IL-16 in vascular complications of IL-16 is needed, preferably both by re-evaluating our findings in other cohorts as well as validation in experimental studies.

In conclusion, the levels of IL-16 in plasma were higher in individuals with cardiovascular complications of T2D, as well as associated with surrogate markers of atherosclerosis. To understand how IL-16 impacts vascular complications from diabetes, more research is called for to identify the underlying mechanisms, and the potency of IL-16 as a biomarker.

Pro and cons of IL-16 in the atherosclerotic disease

The collected work of this thesis has aimed to establish a role of IL-16 in atherosclerosis and CVD. The hypothesis from the start was that IL-16 carry anti-inflammatory functions, by inhibition of T cell responses. In **paper I**, the net effect of IL-16 in experimental atherosclerosis was anti-inflammatory and perhaps thereby resulted in a decrease in atherosclerotic burden. The results from **paper I** were also reflected in the association between IL-16 and stabilizing plaque components presented in **paper II**. High levels of IL-16 in carotid plaques were associated with a decreased risk of suffering a post-operative CV event during a two-year follow-up period (**paper II**).

As carotid plaque homogenates require surgical removal, and thereafter thorough processing, plasma samples are preferable in a clinical setting, when discussing

biomarkers. Therefore, we tested whether plasma IL-16 levels, from 473 individuals with severe carotid atherosclerosis, could predict future CV events (**paper III**). High IL-16 levels in plasma associated with a decreased risk of suffering from CV events or CV events so severe leading to death as final outcome (**paper III**). **Paper I-III** supports a protective role of IL-16 in atherosclerosis and CVD.

The results presented in **paper I-III**, are contradicted by the results from IL-16 in subjects with established T2D and with cardiovascular complications (**paper V**). The data presented in **paper V**, indicates that IL-16 is elevated in individuals with T2D who also suffer from CVD, compared to individuals only suffering from T2D. Furthermore, IL-16 was connected to renal dysfunction and associated with surrogate markers of atherosclerosis. One SNP, rs11556218 which associated with decreased levels of IL-16 in plasma, was inversely associated with surrogate markers of atherosclerosis, further supporting a disease driving role of IL-16 in T2D (**paper V**).

Paper IV is the largest study, to my knowledge, performed on circulating IL-16 as a biomarker for primary prevention. There was no association between SNPs, associated with IL-16 plasma levels, with an increased risk for future CVD. There was even one SNP (rs1131445) which associated with decreased risk for all-cause mortality during the 20-year follow-up period. Furthermore, it was evident that women had decreased levels of IL-16 compared to men, and that IL-16 correlated to several of the known cardiovascular risk factors (**paper IV**). Women with high levels of IL-16 had a 64% increased risk of suffering from a MI, compared to the individuals with low levels. Obese women had an even further increased risk of suffering from CVD if they had elevated levels of IL-16 (**paper IV**). The conclusion from **paper IV** is that IL-16 may mainly predict risk in obese women but IL-16 is not useful in a "normal", heterogeneous population to predict future CV risk.

I believe that IL-16 possesses anti-atherogenic properties, evident in **paper I-III**. The reason why IL-16 is elevated in subjects with T2D with cardiovascular complications, and in obese women, is because IL-16 is a firefighter trying to put out an already raging fire.

Conclusions and Future Perspectives

In this thesis, I have shown that;

I. IL-16 decreases the atherosclerotic burden in hypercholesterolemic mice.

II. IL-16 levels depend on which gene polymorphisms (SNPs) are inherited from parents to offspring and are closely associated with risk factors driving CVD.

III. IL-16 is associated with stabilizing factors in human carotid plaques and is associated with less post-operative CV events and CV event leading to death.

IV. IL-16 is not a potential prognostic biomarker in a relatively healthy population, but perhaps it should be further evaluated as a biomarker for CVD in obese women.

V. In subjects with type 2 diabetes, IL-16 is associated with several vascular complications and should be further validated as a biomarker for risk prediction in subjects with diabetes.

Furthermore, there are strong associations between increasing IL-16 levels and many of the risk markers for atherosclerosis, especially cholesterol, HDL (inversely) and LDL levels. In the future I believe that the connection between lipid metabolism, risk factors and the association to increasing IL-16 levels will remain extremely interesting and should be further investigated. As previously mentioned, the study design of paper II–V present associations and do not prove causality and need to be further tested.

The relatively rapid effect of IL-16 on T cells is, to my mind amazing, and should be further evaluated as a future therapy. The manuscripts presented in this thesis only highlight the effect of IL-16 on T cells. There are still, however, many potential effects of IL-16 on other cells affecting atherosclerosis development, which have not been included here as they are outside the scope of this thesis.

Finally, there is a protective role of IL-16 in experimental and advanced atherosclerosis, which is most likely connected to T cells and apoptosis. A few more years and I will have solved the remaining question marks ☺.

Populärvetenskaplig sammanfattning

Interleukin 16 - brandmannen som släcker farliga eldsvådor i kärlväggen.

Immunförsvaret påverkar risken för hjärt-kärlsjukdom. En signalsubstans i immunförsvaret, IL-16, har visat sig öka antalet anti-inflammatoriska immunceller. Vi har bevis för att IL-16 minskar åderförfettningen i kärlväggen samt minskar risken för att drabbas av hjärt-kärlsjukdom.

Vår kropp består av många olika typer av celler, där vissa av cellerna har som uppgift att skydda oss mot farliga inkräktare som bakterier och virus. Dessa celler ingår i vårt immunförsvar. Under vissa förhållande så blir cellerna farliga för kroppen då de tolkar kroppsegna byggstenar (i detta fall det onda kolesterolet) som inkräktare. När detta sker i kärlväggen kan en lokal inflammation uppstå och kan resultera i startskottet för åderförfettning (arteroskleros), vilket senare kan ge upphov till hjärtinfarkt och stroke. En av orsakerna som gör sjukdomen fascinerande är att celler har förmåga att kommunicera med varandra genom att utsöndra protein som programmerar omgivningens gensvar. Kan dessa kommunikationsfaktorer vara vägen till att hitta, förstå och lindra sjukdomsförloppet?

Över tid så kan inflammationen i kärlväggen leda till att ett plack uppstår. Immunförsvaret kommunicerar med proteiner och ett sådant protein är interleukin-16 (IL-16). Om placket brister kan det innebära allvarliga konsekvenser för individen i form av hjärtinfarkt eller stroke. Placket kan ge upphov till komplikationer på olika sätt, antingen kan det täppa till kärlet, eller kan en blodpropp bildas som en följd av placket, som sedan transporteras med blodet och fastnar i ett trängre kärl. När blodet inte kan transporteras till hjärnan eller hjärtat uppstår syre och näringsbrist vilket leder till att vävnaden dör.

Komplikationer av åderförfettning är den största dödsorsaken i världen och mycket forskning fokuserar på att hitta, karakterisera och behandla åderförfettning. Denna avhandling har haft som fokus att utvärdera om IL-16 kan vara en lämplig markör för att identifiera risken för att drabbas av åderförfettning samt om IL-16 kan påverka placket till att bli mindre bristningsbenäget.

Det finns flera faktorer som bidrar till om en individ har en högre risk att drabbas av åderförfettning och dessa inkluderar manligt kön, rökning, hög ålder, höga nivåer av det ”onda” kolesterolet (LDL), högt blodtryck, lider av diabetes, fetma eller har defekta nedärvda gener som påverkar kolesterolnivåerna i blodet. En av orsakerna till varför hjärt-kärlsjukdom är så vanligt är att sjukdomen ger sig oftast till uttryck när personen har passerat fertil ålder. Detta innebär att sjukdomen inte kommer försvinna ur populationen på grund av naturlig selektion. I den tid vi lever, med mycket fysisk inaktivitet och tillgången till mat i överflöd, bidrar detta till en växande grupp individer med stor risk för att drabbas av hjärt-kärlsjukdom.

För att förstå sjukdomsförloppet så behöver man bakgrundskunskapen att i kroppen finns immunceller som ansvarar för bort städningen. Dessa städarceller kallas makrofager och de har som uppgift att äta upp allt de träffar på som kan vara farligt för kroppen, så som bakterier eller delar av andra döda celler. När kroppen transporter fett till celler så packas det i speciella ”fartyg” (låg-densitets lipoprotein, LDL). Dessa fartyg kan ändra utseende p.g.a. att det är väldigt stormigt (oxideras) eller för att de stöter på hinder (modifieras). Fartygen kommer då att ändra skepnad så att makrofagerna inte känner igen dem som ett inhemskt (kroppseget) material och kommer att hindra det genom att förtära och bryta ner hotet, och därmed minska risken för allvarliga konsekvenser.

Om detta sker i kärlväggen så uppstår det en fettansamling inuti makrofagerna och de blir skumceller. Skumcellerna är klumpiga, inte mobila, och kommer tillslut att spricka av alla fartyg som de har inuti sig. Ut från skumcellerna läcker då en blandning av nedbrutna fartyg och cellrester som uppfattas som inkräktare av andra immunceller. Nu har en negativ spiral startat i kärlväggen och ytterligare makrofager kommer anlända till platsen för att städa upp, men dessa kommer gå samma öde till mötes. Det bildas helt enkelt en kyrkogård av vrakrester som kommer kalla på ytterligare hjälp från immunförsvaret.

Hjälpsignalerna som immuncellerna skickar ut kallas för kemokiner och cytokiner. Kemokiner hjälper cellerna att hitta till platsen genom att gå mot en ökad gradient av kemokinerna. Cytokinerna berättar vilken typ av inkräktare immuncellerna har stött på och hur det inkommande försvaret bör förbereda sig. En sådan signalsubstans, eller cytokin, är IL-16. De celler som leder angreppet kallas för T celler. Det finns flera olika typer av T celler och beroende på vilken T cell som attraheras till platsen så kommer immunförsvaret styras till att svara på hotet på olika sätt. Vissa T celler är för aktiv krigsföring och driver på det aterosklerotiska sjukdomsförloppet (dessa benämns som T hjälpar celler typ 1) och andra har förmåga att dämpa den inflammatoriska responsen mot hotet (regulatoriska T celler).

Det finns bevis för att IL-16 binder till T celler som har en specifik faktor på ytan som kallas CD4. CD4 finns på alla T hjälpar cellerna samt en del andra celler som

ingår i immunsystemet. IL-16 har förmågan att hämma T hjälpar cellernas förökning (delning) samt att prioritera ett regulatoriskt T cells svar. Det finns forskning som visar att ett av de proteinerna som är involverat vid celldöd (caspase-3) också är involverat vid aktiveringen av IL-16. Det finns fortfarande frågetecken på hur IL-16 tar sig ut från cellerna där det har producerats och hur exakt IL-16 påverkar andra celler. Eftersom man vet att IL-16 kan öka antalet regulatoriska T celler, och att dessa celler kan verka skyddande mot sjukdomsförloppet, har arbetena som är presenterade i denna avhandling fokuserat på IL-16s roll i ateroskleros och komplikationerna där av.

I papper IV var frågeställningen huruvida höga IL-16 nivåer i blodet kunde förutsäga risken för att drabbas av hjärtkärlsjukdom upp till tjugo år efter att proverna togs. Till vår förvåning så visade sig höga nivåer av IL-16 vara kopplat till ökad risk för kvinnor att drabbas av hjärtinfarkt, men inga samband fanns för män. Vidare så kunde vi se starka kopplingar mellan ökade IL-16 nivåer med flera riskfaktorer kopplade till hjärt-kärlsjukdom. Designen på studien gör det svårt för oss att säga om de höga nivåerna var en orsak av hjärtkärlsjukdomen, eller om det var en konsekvens av den, men slutsatsen är att IL-16 kan inte användas som en universal markör för att identifiera personer med risk för hjärt-kärlsjukdom. Vi hade även tillgång till analyser av arvsmassan, från dessa individer, där vi kunde identifiera mutationer i arvsmassan som var kopplade till mängden IL-16 i blodet. En av dessa mutationer, som var kopplad till en ökad mängd IL-16, visade sig vara korrelerad till en minskad risk för dödlig utgång för både män och kvinnor.

Vidare ställde vi oss frågan om prover tagna från personer, som redan har drabbats av hjärt-kärlsjukdom kunde förutsäga risken för att få ett nytt kliniskt symptom orsakat av åderförfettningen i kärlväggen. Här hade vi förmånen av att ha tillgång till plack i halspulsådern. Placken var tagna från patienter med kliniska symptom av placken, eller från patienter med en plackstorlek som täppte till minst 80 % av kärlet. Det visade sig att höga nivåer av IL-16 i placket, samt i blodet, minskade risken för att få en komplikation av åderförfettningen samt att det minskade risken av att dö av komplikationer av åderförfettningen.

Eftersom placket börjar bildas redan vid en ung ålder, långt innan man utvecklar symptom, så pågår mycket forskning på vad som bidrar till att placket är stabilt och inte riskerar att brista. Vi kunde koppla samman de höga nivåerna av IL-16, i placket, med ökade mängder av bindvävsprotein (plack stabiliserande) samt med regulatoriska T celler. Slutsatsen av papper II och III är att höga nivåer av IL-16, i en population som lider av kraftig åderförfettning, är kopplat till minskad risk för nya kliniska symptom av åderförfettningen.

Vi vet att individer som lider av diabetes har ökad risk att få en defekt kärlvägg. I papper V, undersökte vi om det fanns ett samband mellan hjärt-kärlsjukdom och IL-16 hos personer med diabetes. IL-16 var även i denna population starkt kopplat

till riskfaktorer som påverkar hjärt-kärlsjukdom. Det visade sig att individer med höga nivåer av IL-16 hade en 50 % ökad risk för att lida av både diabetes och hjärt-kärlsjukdom jämfört med individer som enbart hade diabetes. Det som verkligen var överraskande var att de personer som hade höga IL-16 nivåer i blodet hade 6 gånger högre risk för att lida av nedsatt njurfunktion. Slutsatsen från denna studie är att det finns en koppling mellan höga IL-16 nivåer i blodet med nedsatt kärlfunktion som antagligen påverkar både njurfunktion och hjärt-kärlsjukdom.

Papper II-V i avhandlingen är baserade på associationer som beräknas med statistiska metoder och indikerar sannolikheter för ett samband. Studier som är baserade på associationer, i mänskliga populationer, är svåra att dra några definitiva slutsatser ifrån. Vi vet med andra ord inte med säkerhet att IL-16 är skyddande mot hjärt-kärlsjukdom i individer som redan lider av allvarlig åderförfettning.

För att svara på frågan om IL-16 kan hämma åderförfettningsprocessen behövde vi utföra ett mer kontrollerat försök. I papper I har vi använt oss av möss som är åderförfettningsbenägna och är genetiskt identiska. Vi har injicerat IL-16 i början av sjukdomsprocessen och sedan kvantifierat mängden fett och plack i kärlväggen hos dessa möss. Slutsatsen var att behandling med IL-16 minskade mängden, och storleken, på placken i kärlväggen. För att bekräfta dessa fynd genererade vi en mus som saknade funktionellt IL-16. Det visade sig att hanar som saknade IL-16 hade mer plack än de som hade IL-16, medan honorna inte visade någon skillnad i plackstorlek.

Vad drar jag för slutsats av de studier jag har varit involverade i angående IL-16 påverkan på åderförfettning? Jag har inte alla svaren och jag anser att mer forskning behövs för att förstå IL-16 roll i hjärt-kärlsjukdom. Åderförfettning är en komplex och fascinerade sjukdom som drabbar många människor årligen. Det verkar inte som att IL-16 kan användas som en indikator för att beräkna risken för hjärt-kärlsjukdom, hos relativt friska individer, men mer forskning behövs för att titta närmre på varför IL-16 är kopplat till en ökad risk för hjärtinfarkt hos kvinnor. Jag förordar också att mer forskning bör bedrivas på kopplingen mellan njursjukdom och IL-16, samt varför IL-16 är starkt kopplat till kolesterolnivåer.

Vi har presenterat bevis på att IL-16 är kopplat till en minskad risk för nya kliniska symptom hos personer som lider av allvarlig åderförfettning och att detta styrks av de data vi har genererat under experimentella förhållanden. I denna avhandling har jag sökt svaret på om IL-16 är en skyddande faktor i hjärt-kärlsjukdom. Jag har inte svaret på den frågan men jag tror att IL-16 kan vara en stabiliserande faktor i placken och mer forskning behövs för att förstå IL-16 i sig självt och hur IL-16 påverkar faktorer som är involverade i ateroskleros.

Science for everyone

Interleukin 16 – a fire fighter counteracting an inferno in the vessel wall.

The immune system impacts the risk of suffering from cardiovascular disease. A signal substance in the immune system, IL-16, has been reported to increase anti-inflammatory immune cells. We have proof of a protective role of IL-16 in cardiovascular disease, both by stabilizing atherosclerotic plaques, and by associating with a decreased risk of suffering from cardiovascular event.

The body consists of numerous cell types, of which certain types of cells are designed to protect us from dangerous intruders such as bacteria and viruses. These cells are a part of our immune system. Under certain conditions the same cells that are designed to protect us, turn against one's own body and interpret essential building-stones as foreign. If this event occurs within the vessel wall, there is a possibility that an inflammation arises and initiates an atherosclerotic plaque, which later can result in myocardial infarction or stroke. The cells in the immune system have an amazing capacity to communicate with each other through releasing proteins which can program the surroundings' response. Is it possible that one of these proteins, interleukin-16 (IL-16), has the propensity to identify, alter and limit the disease severity?

Inflammation in the vessel wall is thought to initiate atherosclerosis and over time an atherosclerotic plaque can develop. If the plaque ruptures the individual is at risk of suffering from a myocardial infarction or stroke, which can massively impact the quality of life or even cause death. The atherosclerotic plaque can either occlude the vessel where it is situated, or it can rupture, causing a blood clot. The blood clot will travel with the blood flow until it blocks a smaller vessel. If this obstruction occurs in the brain the individual can suffer a stroke, or if it happens in the vessels supplying the heart with blood, it can lead to a myocardial infarction. The obstruction causes a lack of oxygen and cells in the tissue die. Complications of atherosclerosis, cardiovascular disease (CVD), are the main cause of death in the world and great efforts are made to identify and to treat this disease. This thesis has focused on evaluating if IL-16 can alter the atherosclerotic

plaque to be less prone to rupture and thereby reduce the risk of suffering from CVD. Furthermore, we have assessed to possibility of utilizing IL-16 as a biomarker for predicting CVD.

There are several different factors that contribute to the risk of developing CVD such as being of the male gender, smoking, high age, high levels of the "bad" cholesterol (LDL), high blood pressure, diabetes and obesity and mutations in genes which control cholesterol levels. One of the reasons behind the large number of individuals suffering of CVD is that the disease normally affects people that have passed child bearing years. This implies that the disease will not disappear due to natural selection. The increasing sedentary lifestyle and problems with obesity will undoubtedly influence the number of individuals suffering from CVD. Every grain of new insight into the disease is therefore extremely valuable.

To understand the disease one has to understand the role of a certain type of immune cells called macrophages. The macrophages eat, or engulf, particles that are seen as a threat to the body. To facilitate the transportation of cholesterol to the cells that need it, the lipids are packed into "cargo ships" called low-density lipoprotein (LDL). The cargo ships can change their appearance due to stormy weather (oxidation), or by crashing into obstacles (modification). The cargo ships will then change their appearance and the macrophages will recognize the cargo ships as foreign (non-self) and will evaporate the threat by engulfing them. If this occurs repeatedly in the vessel wall, the macrophages will eventually swell into lipid laden foam cells. The foam cells are immobilized and finally burst, or die, from all the engulfed cargo ships packed with lipids. The foam cells will release a toxic mess of cargo ships recognized as foreign and cell debris which will further attract immune cells to clear the threat. A negative cycle has now been initiated and more macrophages will enter the site, get stuck and die. This graveyard of ship scraps, lipids and cell debris can eventually form an atherosclerotic plaque.

Macrophages that encounter foreign particles will release signaling molecules which are called chemokines and cytokines. Chemokines help incoming immune cells to locate the site of inflammation. Cytokines are released to prepare the incoming immune cells for the type of pathogen the macrophages have encountered and enabling them to respond in an appropriate way. One of these signaling molecule, or cytokine, is IL-16.

The cells that are responsible for coordinating the attack on the pathogens are called T cells. There are several different types of T cells. Depending on which type of T cell that is attracted to the site, the immune system will respond to the invasion with different tactics. Certain T cells are for active warfare and have been found to be driving the atherosclerotic disease (these cells are called T helper cells type 1, Th1) and other have the ability of dampening the immune response (regulatory T cells).

There is evidence that IL-16 can bind to T cells that express a specific factor on their cells surface which is called CD4. CD4 is expressed on all types of helper T cells and also some other immune cells. IL-16 has the capacity to reduce the T helper cells response to activation and to induce a regulatory T cell response. Research has shown that one of the proteins, caspase-3, involved in controlled cell death (apoptosis) is responsible for producing the active form of IL-16. It is neither fully understood how IL-16 is released from the cells, nor the exact mechanism behind IL-16 functions. Since IL-16 has the capacity to increase the number of regulatory T cells, and these regulatory T cells have been proven to be involved in decreasing the risk of CVD, the aim of this thesis was to investigate the role of IL-16 in the atherosclerotic plaque progression and the complications thereof.

In paper IV the aim was to evaluate if high levels of IL-16 in blood could predict the risk of suffering from CVD up to 20 years after the blood sample was taken. To our surprise, high levels of IL-16 were related to an increased risk of suffering of a myocardial infarction for women although not for men. Furthermore, we could identify strong associations between increased IL-16 levels with several risk factors known to be involved in CVD. Analysis of the mutations associated with the levels of IL-16 resulted in identification of one mutation which was associated with a decreased risk of all-cause mortality in both women and men. The design of the study does not allow us to draw a conclusion if the high levels of IL-16 are causing the CVD or if it is a consequence of the disease, however IL-16 is not a unisex marker for predicting CVD.

Next we asked the question if IL-16 levels in individuals, who have already suffered from CVD, could predict the risk of suffering an additional cardiovascular (CV) event. To answer this question, we had the benefit of measuring IL-16 in carotid plaques surgically removed from patients, who had either suffered a CV event to the brain (stroke, TIA or amourosis fugax) or had at least 80% blockage of the carotid vessel by the atherosclerotic plaque. The results indicated that high levels of IL-16 in the carotid plaques, as well as in the blood, decreased the risk of suffering an additional CV event or death by a CV event. Since the atherosclerotic plaque is initiated at a young age, decades before the clinical symptom arise, a lot of research is focused on identifying factors involved in stabilizing the already existing atherosclerotic plaque, making it less rupture prone.

To understand why high levels of IL-16 were connected to a decreased risk of suffering from a new CV event, we correlated the levels of IL-16 with factors known to stabilize the plaques such as collagen, elastin and regulatory T cells. The results showed that increased levels of IL-16 went hand in hand with an increase in stabilizing factors. The conclusion of the data presented in paper II-III was that

high levels of IL-16, in an elderly population with severe atherosclerosis was associated to a decreased risk of suffering a new CV event.

To further evaluate the possibility of utilizing IL-16 as a risk predictor for CVD we assessed the levels of IL-16 in blood from individuals with diabetes, which is presented in paper V. It is known that individuals suffering from diabetes have an increased risk of developing a defective vessel wall and therefore to suffer from CVD. We found that individuals who had been unfortunate enough to suffer from both a CV event and diabetes had a 50% increase in IL-16 in their blood compared to diabetic individuals without CVD. An interesting finding in this study was that individuals with diabetes and high levels of IL-16 had a six-fold increased risk of suffering from renal dysfunction. The conclusion from the presented results in paper V was that there is an association between IL-16 and a decreased vascular function in diabetic individuals, which probably affects both CVD and kidney function.

The papers II-V presented within this thesis are based on associations, which are calculated with statistic methods and indicate the probability for a connection. Studies which are based on associations in human cohorts are hard to draw any definitive conclusions from. We cannot say for sure if IL-16 is protective against CVD. To be able to answer the question if IL-16 holds potential to reduce the atherosclerotic progression we had to perform an experimental study.

In paper I we used mice which were predispose to atherosclerosis and were genetically identical, which limits the background noise. We injected IL-16 at the initiation of the disease and quantified the amount of lipid and plaque content in the vessel wall. The results displayed evidence for reduced amount, and size, of atherosclerotic plaques in the IL-16 treated mice. We could also see an increase in the amount of regulatory T cells after injecting IL-16. To verify our results, we generated a mouse model deficient in IL-16. Male mice lacking IL-16 had larger atherosclerotic plaques compared to mice with functional IL-16, whereas the female mice did not differ.

What conclusions can I draw from the studies conducted to evaluate the role of IL-16 in atherosclerosis and the clinical events thereof? IL-16 is not a universal marker for predicting CV risk in a relatively healthy population; however, more research is needed to understand why IL-16 is connected to an increased risk for women, and more specifically obese women, to suffer from myocardial infarction. I strongly feel that more research is needed to elucidate the connection between IL-16, cholesterol levels and the risk of developing kidney disease.

In this thesis we have presented evidence that IL-16 is associated with less CV events in a population suffering from severe atherosclerosis and that the protective properties of IL-16 in atherosclerosis. We have also strengthened the results of the

protective properties of IL-16 with experimental data. In this thesis I have tried to answer the question if IL-16 has the ability to reduce progression of atherosclerosis and the clinical complications thereof. I cannot say that this is proven beyond doubt, however I strongly believe that IL-16 can act as a stabilizing factor in the atherosclerotic plaques. Trying to elucidate the role of IL-16 in the immune system, atherosclerosis and CVD is not over, and I hope that this thesis was merely a start to understanding this complex cytokine in this deadly and fascinating disease.

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My sister recently asked if I could write a short description of her for her thesis. The challenge was not the description per se, but rather to keep it short, for my sister has many facets to her character. She studied biotechnology at Swedish University of Agricultural Sciences and shortly thereafter went to an interview for the PhD position she later acquired. Rumours say that the interview was something special. You see, Caitríona herself would never claim success quite as brazenly, which is why I am doing it now. She'll be cringing when she reads this and debating with herself if she should omit some of the description. Don't. She is an older sister, a mother, a scientist, a wife, a daughter, a fantastic cheese cake maker, a gardener, an animal lover. But what always surprises me is not her long list of skills, but her will of steel and stamina. Every morning, early mind you, she sets out to reach goals and do a full day's worth of work, after which she comes home to look after her family. Every morning, relentless. She's a steam engine which simply cannot be stopped, an oak tree which cannot be uprooted, a powerful working horse ploughing the field for seeds to be planted in. Her thesis is one of those seeds.

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