Antibody-mediated Immunity in Cardiovascular Disease

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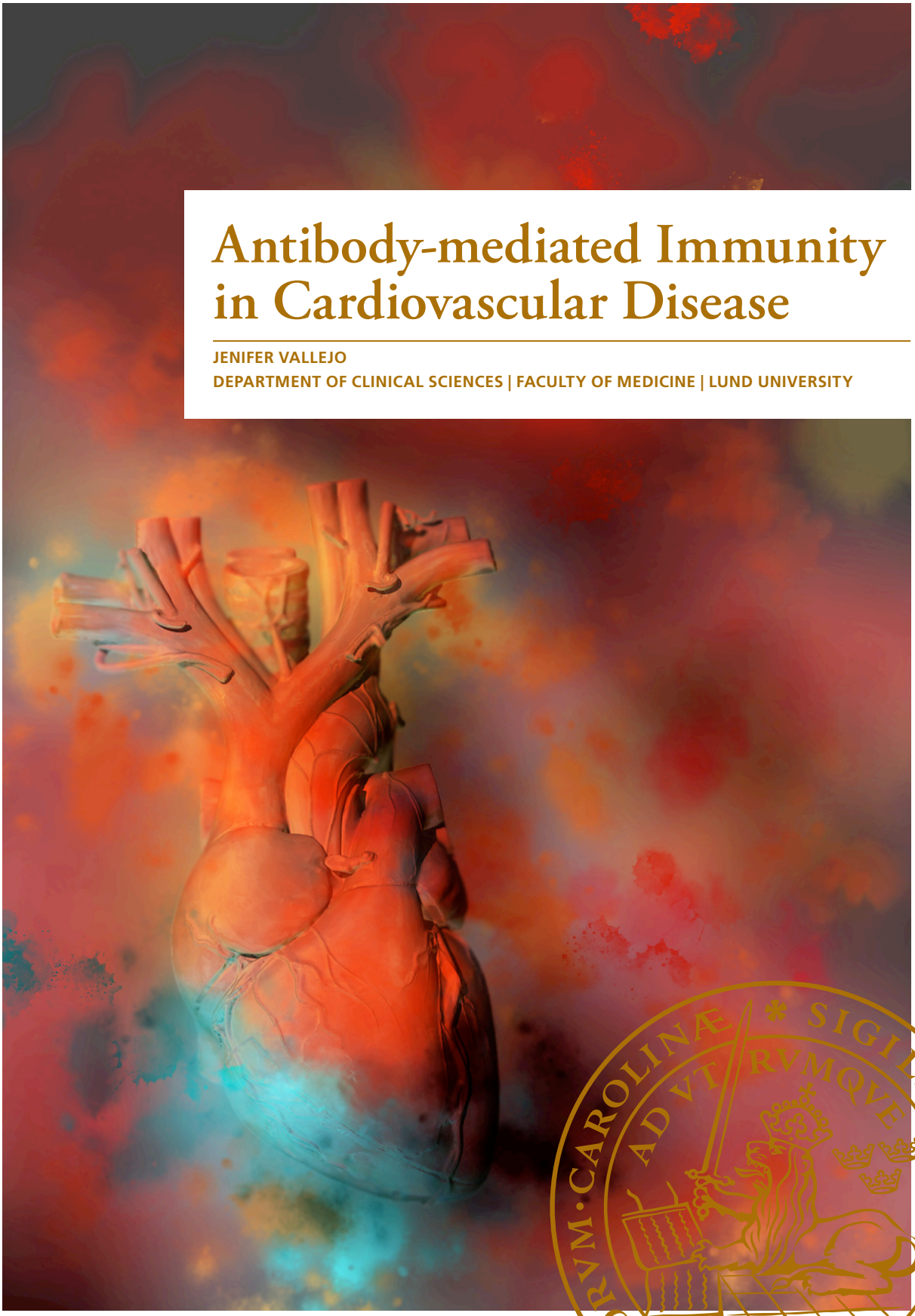

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There is no better way to finish my thesis book than with a fable that my grandma used to tell me when I was a child: *The Ant and The Grasshopper.* After all, there are many times in the scientific world when we need to work like this little insect.

One bright day in late autumn a family of Ants were bustling about in the warm sunshine, drying out the grain they had stored up during the summer, when a starving Grasshopper, his fiddle under his arm, came up and humbly begged for a bite to eat.

"What!" cried the Ants in surprise, "haven't you stored away for the winter? What in the world were you doing all last summer?"

"I didn't have time to store up any food," whined the Grasshopper; "I was so busy making music that before I knew it the summer was gone."

The Ants shrugged their shoulders in disgust.

"Making music, were you?" they cried. "Very well; now dance!"

Aesop (620-564 BCE)

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Antibody-mediated Immunity in Cardiovascular Disease

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DOCTORAL DISSERTATION
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Abstract:

Cardiovascular disease is the leading cause of death worldwide and atherosclerosis is the underlying cause of cardiovascular events. In atherosclerosis, LDL is entrapped in the vessel wall by binding to extracellular matrix proteins. There, LDL is oxidized, which is considered as an important step in the development of atherosclerotic plaques. Oxidation of LDL lead to the formation of reactive aldehydes such as malondialdehyde (MDA), which will react with the protein ApoB100 present in LDL. Immune responses against oxLDL are associated with cardiovascular disease, and immunization with oxLDL reduce atherosclerotic plaque development in animal studies. However, MDA may also react with and modify surrounding matrix proteins, generating immune responses against the modified matrix. Collagen type IV plays a crucial role in the organization and stability of basement membranes. It is located beneath endothelial cells and it is involved in smooth muscle cell behavior. MDA-collagen type IV is increased in human plaques from patients with cerebrovascular symptoms, and antibodies against MDA-collagne type IV correlate with the amount of MDA-collagen type IV in plaques. In subjects with diabetes, aldehyde-modification of collagen type IV interferes with the deposition and integrity of the protein, affecting the normal function of the basement membrane. In **paper I**, we determined whether antibodies against MDA-collagen type IV could predict the risk of development of myocardial infarction in the Malmö Diet and Cancer Cohort. We found that IgG antibodies against MDA-collagen type IV were increased in individuals with incident myocardial infarction compared to controls. These antibodies were also associated with increased carotid intima media thickness as well as elevated plasma levels of MMP-10 and MMP-12. In **paper II** we aimed to investigate if immune responses against collagen type IV contribute to the development of atherosclerotic plaques. For that, an antibody response against collagen type IV was induced in ApoE-deficient mice, a mouse model of atherosclerosis, however no effect was observed on atherosclerosis development.

Diabetes is a risk factor for CVD. Reactive aldehydes, formed from either oxidation of lipids or glucose, are increased in plasma from patients with diabetes. Autoimmune responses against aldehyde-modified proteins may therefore be of particular importance in diabetes. In **paper III** we investigated if antibodies against MDA-collagen type IV are associated with cardiovascular events in T2D in the SUMMIT cohort. We found that subjects with T2D had lower levels of IgM antibodies against MDA-collagen type IV compared to subjects without diabetes. Additionally, subjects with diabetes who suffered cardiovascular events during follow up had lower levels of IgM against MDA-collagen type IV compared to those that remained event free. Last, increased glucose levels are associated with the generation of advanced glycation end product (AGE) modifications. Methylglyoxal (MGO) is one type of reactive α-oxoaldehyde that gives rise to AGE modification. In **paper IV**, IgM and IgG antibodies against MGO-p220 were analyzed in the Malmö Diet and Cancer cohort. Low levels of IgM antibodies against an MGO-modified peptide in ApoB (MGO-p220) were associated with increased risk of cardiovascular events in subjects without diabetes. In addition, MGO-p220 IgM were produced by B-1 cells.

In summary, in this thesis antibodies against MDA modified-collagen type IV as well as antibodies against MGO modified-ApoB100 peptide and their associations with atherosclerosis, cardiovascular disease and cardiovascular complications in diabetes have been investigated.

Key words: Atherosclerosis, Cardiovascular disease,Myocardial infarction, Extracellular matrix, Collagen type IV, ApoB100, Malondialdehyde, Methylglyoxal, Antibodies, Immune responses.

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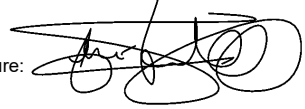
Signature: 

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Anyone can listen, you may say - what's so special about that? - but you'd be wrong. Very few people know how to listen properly, and Momo's way of listening was quite unique. She listened in a way that made slow-witted people have flashes of inspiration…Momo could listen in such a way that worried and indecisive people knew their own minds from one moment to the next, or shy people felt suddenly confident and at ease, or downhearted people felt happy and hopeful. And if someone felt that his life had been an utter failure, and that he himself was only one among millions of wholly unimportant people who could be replaced as easily as broken windowpanes, he would go and pour out his heart to Momo. And, even as he spoke, he would come to realize by some mysterious means that he was absolutely wrong: that there was only one person like himself in the whole world, and that, consequently, he mattered to the world in his own particular way.

- Momo - Michael Ende, 1973.

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Preface

Atherosclerosis is not a modern disease, and the arteries of our ancestors were not as healthy as we may think. Actually, computed tomographic findings have revealed that mummies from different geographic regions had atherosclerosis.

Several studies have investigated the evolution of atherosclerosis and possible options to reduce it, but, is it an underlying disease that can we eliminate once and for all?

In the upcoming pages I will explain, review and discuss some of the work that I have performed during the past four years. The thesis is mostly focusing on humoral immune responses against aldehyde-modified basement membrane protein collagen type IV, and its implications in atherosclerosis, cardiovascular disease and cardiovascular complications in type 2 diabetes. In addition, this thesis includes immune responses against an aldehyde-modified peptide of ApoB100, the main protein of the cholesterol carrier low density lipoprotein particle.

The reader will first be introduced, shortly, to the world of cardiovascular disease, atherosclerosis, collagen type IV, immunity and antibodies followed by some relevant methods. Last, a critical discussion of the results obtained in my studies.

With this thesis I do not pretend to answer or solve all the questions related to this worldwide affecting disease, but to raise a closer understanding, hopefully, to some of them.

- If you feel that this reading is not your cup of tea, there is a summary at the end of the book that I encourage you to read called “Science for everyone”.
- Om du känner att denna läsning inte passar dig så finns det en sammanfattning som kallas “Populärvetenskaplig sammanfattning” i slutet av boken som jag uppmanar dig till att läsa.
- Si crees que este libro (o el idioma en el que está escrito) no es muy de tu agrado, hay un capítulo al final del mismo titulado "Ciencia para todos" que te animo a leer.

Original papers

The doctoral thesis is based on the following papers and will be referred to in the text by their Roman numerals:

I. **Vallejo J**, Dunér P, Fredrikson GN, Nilsson J, Bengtsson E. Autoantibodies against aldehyde-modified collagen type IV are associated with risk of development of myocardial infarction. *J Intern Med*. 2017;282(6):496-507.

II. **Vallejo J**, To F, Engelbertsen D, Gonçalves I, Dunér P, Nilsson J, Bengtsson E. Activation of immune responses against the basement membrane component collagen type IV does not affect the development of atherosclerosis in ApoE-deficient mice. *Submitted manuscript.*

III. **Vallejo J**, To F, Rattik S, Dunér P, Björkbacka H, Nilsson J, Bengtsson E. Low levels of IgM autoantibodies against aldehyde-modified collagen type IV predict cardiovascular events in subjects with type 2 Diabetes Mellitus. *Manuscript.*

IV. Engelbertsen D*, **Vallejo J***, Quách TD, Fredrikson GN, Alm R, Hedblad B, Björkbacka H, Rothstein TL, Nilsson J, Bengtsson E. Low levels of IgM antibodies against an advanced glycation endproduct-modified apolipoprotein B100 peptide predict cardiovascular events in nondiabetic subjects. *J Immunol*. 2015;195(7):3020-3025.

*D.E and J.V contributed equally to this article.

Papers not included in this thesis

Engelbertsen D, Rattik S, Wigren M, **Vallejo J**, Marinkovic G, Schiopu A, Björkbacka H, Nilsson J, Bengtsson E. IL-1R and MyD88 signalling in CD4+ T cells promote Th17 immunity and atherosclerosis. *Cardiovasc Res*. 2018;114(1):180-187.

Mantani PT, **Vallejo J**, Ljungcrantz I, Nilsson J, Björkbacka H, Fredikson GN. Interleukin-25 reduces Th17 cells and inflammatory responses in human peripheral blood mononuclear cells. *Submitted manuscript.*

Hsiung S, Knutsson A, **Vallejo J**, Dunér P, Heinonen S.E, Jönsson-Rylander A.C, Bengtsson E, Nilsson J, Hultgårdh-Nilsson A. Hyperglycemia does not affect tissue repair responses in shear stress-induced atherosclerotic plaques in $ApoE^{-/-}$ mice. *Scientific Reports, in press.*

Abbreviations

AGE	Advanced Glycation End Products
Apo	Apolipoproteins
$ApoE^{-/-}$	Apolipoprotein E deficient
APC	Antigen Presenting Cells
BCR	B Cell Receptor
BM	Basement Membrane
BMI	Body Mass Index
C region	Constant region
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcomes Study
CEL	N ε-(carboxyethyl) lysine
CCA	Common Carotid Artery
CIRT	Cardiovascular Inflammation Reduction Trial
CVD	Cardiovascular Disease
CRP	C-reactive protein
CV events	Cardiovascular Events
CyTOF	Mass Cytometry
DAMP	Damage Associated Molecular Pattern
DDR1	Discoidin Domain Receptor type 1
ECM	Extracellular Matrix
ELISA	Enzyme Linked ImmunoSorbent Assay
ER	Endoplasmatic Reticulum
Fab	Fragment Antigen Binding
FcR	Fc Receptor

FcγR	Fc Gamma Receptor
H chain	Heavy chain
HbA1c	Glycosylated Hemoglobin
HFD	High Fat Diet
HLA	Human Leukocyte Antigen
HNE	4-Hydroxy-nonenal
HR	Hazard Ratio
HUVEC	Human Umbilical Vein Endothelial Cells
IDL	Intermediate Dense Lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMT	Intima Media Thickness
L chain	Light chain
LDL	Low Density Lipoprotein
$LDLr^{-/-}$	Low Density Lipoprotein Receptor Deficient
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MDC	Malmö Diet and Cancer
MGO	Methylglyoxal
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
mmol	Milimol
MMP	Matrix Metalloproteinase
MCP	Monocyte Chemoattractant Protein
NC	Carboxyl-terminal
OR	Odds Ratio
oxLDL	Oxidized LDL
PADRE	Pan HLA DR binding epitope

PAMP	Pathogen Associated Molecular Patterns
PDGF	Platelet Derived Growth Factor
PRR	Pattern Recognition Receptor
PUFA	Polyunsaturated fatty acids
R	Correlation Coefficient
SLE	Systemic Lupus Erythematosus
SMC	Smooth Muscle Cells
SR	Scavenger Receptor
SUMMIT	Surrogate Markers for Micro and Macro Vascular Hard Endpoints for Innovative Diabetes Tool
T2D	Type 2 Diabetes Mellitus
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T Helper Cells
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells
UKPDS	The United Kingdom Prospective Diabetes Study
V region	Variable region
VLDL	Very Low Density Lipoproteins
α chain	Alpha chain
β	Regression β coefficient
µg/mL	Micrograms per Milliliter

Background

The dominant pathologic foundation of cardiovascular disease, predominantly myocardial infarction (MI) and stroke, is atherosclerosis. Atherosclerosis is slow disease, which has one of the longest incubation processes among human diseases, and may start in early childhood. Nicholai Anichkov, discovered over 100 years ago, in 1913, the role of cholesterol accumulation in atherosclerosis and gave us the key to prevent and reverse this deadly disease.[1] For decades it was thought that atherosclerosis was a cholesterol storage disease. The studies were then focused on smooth muscle cell proliferation and its role in plaque formation. In the last part of the last century, the role of inflammation and immune system in atherosclerosis development has gained ascendency, and atherosclerosis is now considered as a chronic inflammatory disease.

Essential points including cardiovascular disease, atherosclerosis, collagen type IV and immunity will be explained in the initial pages of the book for a better comprehension and understanding of this thesis.

Cardiovascular disease

Cardiovascular disease (CVD) mortality has declined recently in many countries, but it is still the leading cause of death worldwide, accounting for one in four deaths globally.[2] In humans, early signs of arterial aging is observed in children and adolescents.[3] Even then, it will take several decades for progression into a symptomatic disease where three-fifths of deaths will be over 75 years old.[4] Usually, the clinical consequences of this disease happens from one day to another, without previous warning. CVD includes pathologies of the heart, blood vessels and vascular system of the brain.

But, which mechanisms explain the transition from stable asymptomatic atherosclerosis to acute events?

General characteristics

The definition of CVD encompasses coronary heart disease, cerebrovascular disease or peripheral arterial disease. Consequently, clinical manifestations of atherosclerosis occur foremost in coronary arteries, extra-cranial carotid arteries and lower extremities.[5] It is necessary to go back to an early detection of the disease, and even more in high-risk populations, to prevent clinical manifestations of atherosclerosis.

Risk factors

Many different risk factors for cardiovascular disease have been identified. These can be divided in two categories: non-modifiable (age, race, family history and sex) and modifiable factors, where many aspects can be included (diabetes, obesity, exercise, smoking, high blood lipids, blood pressure …).[6,7] To establish general guidelines for 10-year prediction of risk factors associated with CVD that everyone would be able to follow in their studies and take into account was initiated in the early 50's – the Framingham Heart Study.[8,9] In this study, the predictors for CVD were age, diabetes, smoking, systolic blood pressure, total cholesterol and high density lipoprotein (HDL) cholesterol, replacing lipid levels used in a simpler model.[10] Since then, these risk factors have been widely used in observational CVD studies. An inconvenience of the Framingham Heart Study is that all the scores were done in middle aged populations, and mostly white ethnic, suggesting that the risk prediction might be underestimated in elderly populations or other ethnics.[11,12] For this reason, other new markers associated to cardiovascular risk such as homocysteine and low levels of folic acid or C-reactive protein (CRP) and interleukin (IL)-6 as markers of inflammation, have been investigated in very old populations. Of these, only homocysteine seemed to be a better predictor than the Framingham risk factors in that specific population and thus, not applicable to the general society.[13]

Numerous and novel biomarkers have been described, such as inflammatory and genetic markers.[14] For example, arterial stiffness and endothelial dysfunction are mechanisms to be considered in CVD. Inflammation plays an important role in the development of arterial stiffness, and it has been shown that this process predicts an increased risk of cardiovascular (CV) events.[15] At the same time, endothelial dysfunction, being one of the first manifestations of atherosclerosis, has also been described as possible biomarker in the detection of CVD.[16] These have yet to be shown to be of value in improving risk prediction.

Taken all together, we can see that, yet having many possible risk factors to use in the study of atherosclerosis causing clinical symptoms, still no clear candidate applicable to all conditions and individuals have been found. Although studies constantly are releasing new possible "better" risk factors to have in consideration,

until nowadays, the Framingham risk factors are the ones most extensively used in research and applied in the study of CVD.

Medication

Beneficial effects for treatment of CVD have been observed for antithrombotic or antiplatelet agents, blood pressure lowering agents or vasodilatory agents which are extensively used and combined among patients with CVD. Despite this, most research in the treatment of CVD these days is focused on two major currents: decreased lipid levels and/or inflammation.

Lipid lowering treatment: Statins are the usual medication prescribed for individuals at risk of CVD. Statins (eg. Simvastatin) reduce endogenous plasma cholesterol levels by blocking the enzyme HMG-CoA reductase, an enzyme involved in cholesterol biosynthesis.[17] In addition, it has been shown in an observational study that statins reduce up to 50% of the incidence of MI or stroke among men and women with low levels of low density lipoproteins (LDL) and high levels of CRP.[18] Thus, the effect of statins may be due to LDL cholesterol reduction, due to its anti-inflammatory characteristics or due to the combination of both factors.[19] Pravastatin, another type of statin, has been shown to stabilize human plaques by decreasing lipid content, inflammation and increasing collagen.[20]

Recently, lipid lowering drugs that inhibit PCSK9, a molecule involved in elevating cholesterol levels in blood by binding to LDL receptors and promoting the degradation of these receptors in the liver, have been developed. It has been shown that when PCSK9 inhibitor was added to an already existed statin treatment, it resulted in a 60% reduction of coronary events.[21]

Anti-inflammatory treatment: Even if anti-inflammatory treatments are not used in CVD patients today, the role of inflammation as a possible treatment target for CVD has been widely studied.[22] Two different large placebo controlled trials have been designed to test the hypothesis of inflammation as a possible target for treatment of CVD: Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) and Cardiovascular Inflammation Reduction Trial (CIRT).[19] Methotrexate (folic acid antagonist) is a immunosuppressive medication that is used in humans and mice resulting in decreased atherogenesis. This compound reduces T cell proliferation, known by its role in inflammation, by increasing cAMP levels. Methotrexate is now being tested in the CIRT study as a possible anti-inflammatory treatment, measuring if low-dose methotrexate supresses CV events.[23] Additionally, in the recent CANTOS study, IL-1β inhibition with canakinumab was measured. IL-1β is produced by macrophages (via inflammasome) in response to cholesterol accumulation and it is an important cytokine in inflammation.[19] This study showed reduction of CV events when inhibiting IL-1β compared to placebo.[24] In both studies, the lipid levels were not affected, showing that lipids were not influencing

the outcome of the studies. Inhibition of other cytokines such as tumour necrosis factor (TNF)-α and IL-6 have been also studied as possible CVD treatments.[25] Further studies will be needed before as single treatment would be enough for CVD regulation.

Cardiovascular complications in type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus (T2D) (insulin resistant or non-insulin dependent), is a major risk factor for CVD[26] and among all subjects with T2D, 75% can expect to die from a cardiovascular event.[27] Metabolic syndrome, a disease associated to subsequent development of T2D, characterized by increased abdominal obesity, increased blood glucose levels, high blood pressure, low HDL levels and elevated triglycerides, is also a risk factor for CVD.[7]

It is important to reduce the CVD risk among individuals with T2D by controlling the classical CVD risk factors such as hypertension, glucose, dyslipidemia, life style or obesity.[28] Actually, several studies have shown that CVD is prevented or reduced when controlling individual risk factors in T2D.[29] For this reason, cardiovascular risk should be assessed in all patients with diabetes. As a general rule, patients diagnosed with diabetes are given lipid lowering treatment like statins. A multi center survey of 4056 individuals with T2D revealed that statin dosage should be increased among patients with CVD risk. This suggests that it is important to take risk of CVD in consideration when treating T2D patients.[30]

An important diabetic cardiovascular complication is the presence of an accelerated atherosclerosis that occurs more extensively and at younger ages and that can lead to myocardial infarction (MI). In fact, a study showed as subject with diabetes without previous MI have the same risk to get an MI that subject without diabetes with previous MI.[31] T2D and metabolic syndrome are associated to increased arterial stiffness and endothelial dysfunction.[32,33] One possible cause of the increased arterial stiffness in subjects with T2D is the breakdown of elastin fibers[34] as well as crosslinking of collagen[35] by advanced glycation end products (AGE).[15,36] Endothelial permeabilization and dysfunction is an early and critical step in the progress of atherosclerosis,[37] and its development is associated to diabetes.[38]

AGE glycation preferentially happens on hydroxylysine, and may affect wound healing in subjects with diabetes. Indeed, AGE glycation on collagen type I, which neutralizes basic charges on lysine residues, has been shown to affect proteoglycan assembly and hence, ECM formation in subjects with diabetes.[39] Additionally, AGE-glycation of collagen type IV, altering its assembly, matrix flexibility and function, has been implicated in diabetes.[40–42]

Atherosclerosis

Myocardial infarction and stroke are the most common outcomes of the same underlying disease, atherosclerosis. It is characterized by accumulation of lipids in large- and medium-sized arteries especially where the vessel divides and in curvatures.[43] Atherosclerosis is manifested throughout the entire vasculature but mostly happens at sites of low shear stress, turbulence and oscillating flow.[44] Areas with laminar flow are usually quite resistant to the development of lesions. Indeed, the degree of atherosclerosis depends on the location in the arterial tree where the measurements are assessed, as described in an autopsy-based study where atherosclerotic burden was evaluated at different locations in young individuals.[45] The common locations of atherosclerotic plaque formation in humans are: the carotid arteries, the coronary arteries, the aorta, the iliac arteries and the femoral arteries.[46]

The healthy vessel

The arterial wall consists of three layers: the tunica intima (closest to the lumen and bloodstream), the tunica media and the tunica adventitia (the outermost layer). Layers of elastin separates the intima from the media, called internal elastic lamina; and another layer of elastin, called the external elastic lamina, separates the tunica media from the adventitia[47,48] as shown in figure 1.

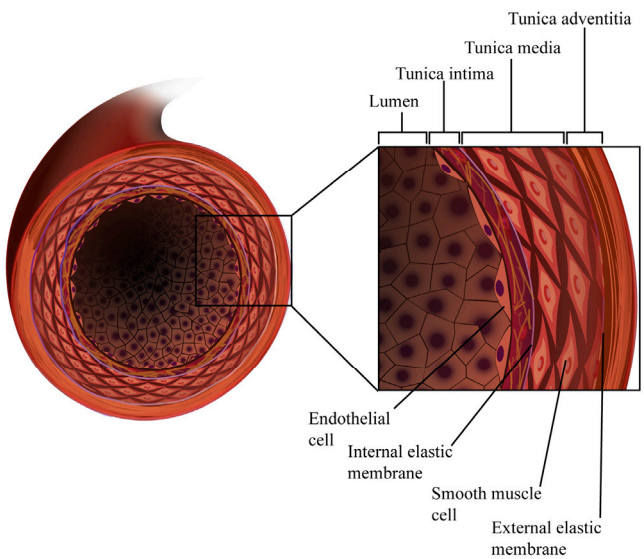

Figure 1. Cross-section of a healthy artery wall.

The tunica intima is a barrier between the blood flow and stroma of the vascular wall. It is the thinnest layer where the most predominant cell types are endothelial cells. These cells are resting on a basement membrane and subendothelial ECM composed of mainly collagen and elastin. This layer is involved in inflammation and vessel tone.[47,48]

The main components of the tunica media are the smooth muscle cells (SMC), which are in charge of keeping the integrity and structure of the media. SMC are hold together by glycoproteins and proteoglycans. It is a layer able to adapt and adjust to blood flow oscillations.[47,48]

Tunica adventitia contains predominantly fibroblasts, and neural connexions that transverse through this layer composing a network of connective tissue that is important for vascular remodelling.[48,49]

Lipoproteins

The two most important lipids in plasma are cholesterol and triglycerides. They are transported due to their hydrophobic nature as biochemical assemblies called lipoproteins through the bloodstream. Lipoproteins are composed by triglycerides and cholesterol esters in the core; a monolayer of phospholipids (and some free cholesterol) with the polar group towards the plasma, and a protein called apolipoprotein that provides structural integrity and acts as cofactor for enzymes and receptors. The different types of lipoproteins are: chylomicrons, very low density lipoproteins (VLDL), intermediate dense lipoproteins (IDL), LDL and HDL.[50]

Triglycerides are necessary for free fatty acid formation and are mostly carried by chylomicrons and VLDL in the circulation. The endogenous cholesterol (around 80% of the total) is an essential component of cellular membranes and necessary for body functions. It is involved in the regulation of processes such as immune cell activation, inflammation and SMC proliferation.[51] Cholesterol is carried by LDL and HDL.

LDL has been the major target of therapy in preventing or reducing CVD, since high levels of LDL is a known risk factor for CVD.[52] It is the most important cholesterol transporter with a hydrophobic body containing cholesterol ester molecules and triglycerides. LDL contains a monolayer of different phospholipids on the surface and apolipoprotein (Apo)B, which acts as the carrier of cholesterol. LDL has a diameter of 22 nm, it contains around 3000 lipids, has a density between 1.019 and 1.063 g/mL and a molecular weight of 1.8 to 2.8×10^6 Daltons.[50,53] Metal ions, such as Cu^{2+}, lipoxygenases, myeloperoxidase and reactive nitrogen species can be used to oxidize LDL. For instance, during *in vitro* oxidation of LDL by Cu^{2+}; reactive aldehydes are formed reacting with amino groups on lysine residues in ApoB. In

fact, it has been shown that *in vitro* oxidized LDL (oxLDL) have almost the same chemical characteristics that LDL obtained from plaques in humans and rabbits.[54] This makes LDL more negatively charged and increases its affinity for scavenger receptors (class A, class B, LOX-1) on macrophages,[55] whereas native LDL binds to LDL receptors.

HDL has a size between 8-10 nm and higher density than LDL. ApoAI is the major structural and functional protein within HDL, constituting around 70 % of the entire HDL molecule. There is an inverse association between HDL levels and cardiovascular disease, because its role in reverse cholesterol transport, that is cholesterol efflux from foam cells to HDL, followed by HDL remodelling and hepatic lipid uptake for catabolism and excretion into bile and feces.[55]

The imbalance between cholesterol uptake and efflux in immune cells leads to accumulation of cholesterol esters and an inflammatory cascade activation that may eventually initiate plaque formation.[51]

Many plasma apolipoproteins are synthesized in the liver and in the small intestine. ApoAI is also found in chylomicrons.[56] In humans, there are two different isoforms of ApoB; ApoB100, which contains 4536 aminoacids (present in LDL and VLDL, and essential for VLDL assembly in liver) and ApoB48, which contains 2152 amino acids starting from N terminal of ApoB100[57] (expressed in intestine and present in chylomicrons). In murine species, ApoB48 can be found in VLDL and LDL and it is expressed in the liver and in the intestine.[58]

There is a positive correlation between LDL and ApoB100 levels,[59] and between plasma HDL and ApoAI,[60] suggesting a possible role of these apolipoproteins as CVD markers. In fact, the ratio of ApoB and ApoAI has been suggested as a predictor of CVD risk.[52]

Atherosclerotic plaque formation – stable or vulnerable?

Progression of atherosclerosis is related to the amount of LDL particles in plasma, although patients with similar levels of LDL presented different progression on atherosclerosis, suggesting that rate of oxLDL might be of a more importance.[61] Indeed, oxLDL is important in the initial stages of atherosclerosis as well as in plaque complications.[5]

Key steps in plaque formation are summarized below and shown in figure 2:[62–64]

- Early endothelial dysfunction and vascular inflammation
- Inflammatory processes, recruitment of monocytes and foam cell formation
- SMC proliferation, migration and ECM synthesis

- Cell apoptosis and formation of the necrotic core
- Thrombus formation

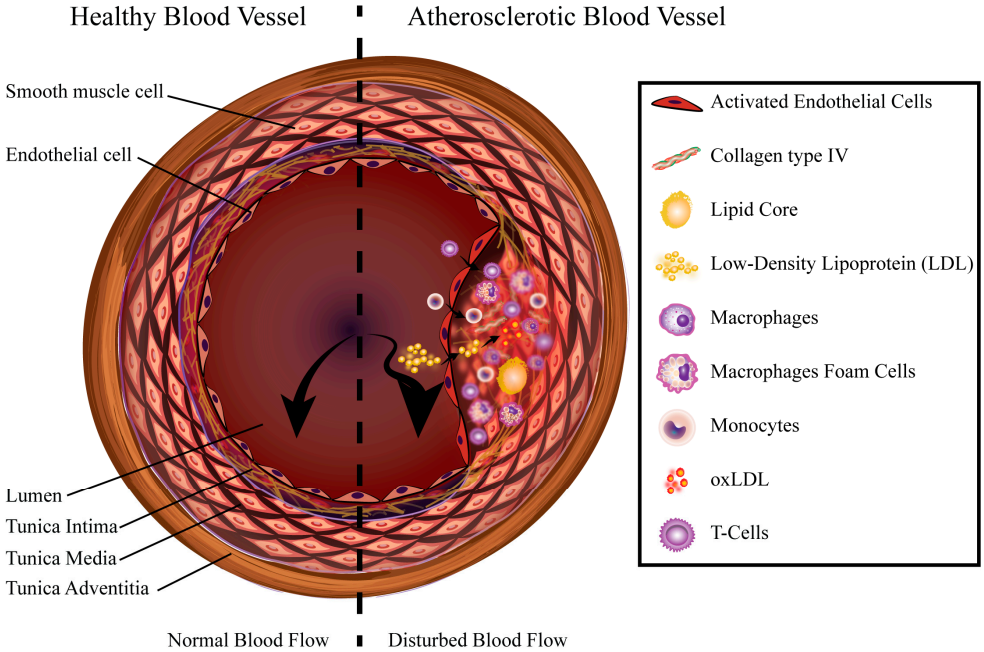

Figure 2. Comparison between a healthy blood vessel and an atherosclerotic blood vessel.

In the initial steps of atherosclerosis LDL accumulates in the intima, where it can be modified resulting in a more permeable endothelial cell layer.[47] The earliest changes happen in the endothelium with endothelial cell activation and expression of vascular adhesion molecules such as VCAM-1. These changes also include decreased production of nitric oxide or increased permeability to lipoproteins and leukocyte adhesion.[65]

Thereafter, monocytes will accumulate and migrate into the intima,[66] where activated phagocytes produce reactive species that keep oxidizing LDL.[55] Oxidized LDL particles can be taken up and degraded by scavenger receptors (SR) (mostly CD36 and SR-A) or toll like receptors (TLR) (predominantly TLR2 and TLR4) on macrophages resulting in lipid loaded foam cell formation, followed by recruitment of T cells to the artery wall.[67]

SMC migration from the media to the intima is common in the process of atherosclerosis. Inflammatory molecules would induce the switching from a contractile SMC (found in the media) to a more synthetic and migratory phenotype in the intima of the vessel wall to participate in the formation of a fibrous cap.[68,69] It is important that there is a balance in SMC content, because they have a dual role. SMC stabilize the plaque by secreting ECM or growth factors eg. transforming growth factor (TGF)-β),[70] but SMC are also able to destabilize the plaque through protease secretion (eg. MMPs, which are released in an inactive form).[71] Like macrophages, SMC also present scavenger receptors and can become foam cells in presence of lipoproteins, and accumulate cholesterol contributing to plaque formation.[72,73]

Some of the foam cells will die releasing lipids that will accumulate, and the impaired clearance of dead cells (efferocytosis) will lead to the formation of the necrotic core.[65,74]

The rupture of the plaque and subsequent thrombosis (formation of a blood clot) are critical events in the process of atherosclerosis leading to MI and stroke. Current investigations are focused on trying to identify and stabilize vulnerable plaques, and for that, progress in *in vivo* imaging techniques are needed.

Plaque rupture

The atherosclerotic process can last for decades, until the lesion breaks probably because of forces due to the blood flow generating turbulence and reduced shear stress.[43] The atherosclerotic plaque can narrow the lumen (stable plaque) compromising blood flow, but most clinical complications are because of ruptures (55% to 60%) or erosions (up to 35%).[75]

Plaques prone to rupture are known as vulnerable plaques. If these plaques rupture it could cause a thrombus, which can occlude smaller vessels and if impairing the oxygen supply in the heart or brain, could result in clinical events like myocardial infarction or stroke.[76]

Although the specific mechanisms underlying plaque vulnerability and rupture are not completely known, it is clear that a proteolytic and proinflammatory environment contribute to plaque vulnerability.[77]

The fibrous cap of the plaque has been the target of intense research since it is believed that this structure prevents the plaque from rupturing. The fibrous cap contains mostly ECM components and smooth muscle cells. In some plaques, inflammation and necrosis prevails over fibrotic processes, leading to thin fibrotic caps with increased risk of rupture, as shown in human atherosclerotic carotid plaques *in vivo*.[78] It has been identified that the best morphological indicator for a possible rupture is a fibrous cap with a thickness below 55 μm.[79] It is believed that

an impaired balance in collagen synthesis and degradation is a possible cause for a thin fibrous cap prone to rupture.[79] Collagen and other ECM proteins are degraded by matrix-metalloproteinases (MMP), and it has been shown that for example MMP-1, -3 and -9 are increased in the shoulder region of carotid plaques, and some of them correlate to inflammatory mediators such as IL-6.[77] Inhibition of MMP-12 in atherosclerotic ApoE deficient ($ApoE^{-/-}$) mice resulted in increased plaque stability, and MMP-2 is associated to disease progression and plaque instability in patients with atherosclerosis.[48]

The lipid core, usually located in the central part of the thickened intima, contains predominantly lipid depositions, foam cells and a high ratio of degraded ECM together increasing plaque vulnerability and the risk of plaque rupture.[80]

In summary, a plaque would be considered vulnerable and prone to rupture, in general terms, if having a thin fibrous cap, high lipid accumulation, a large necrotic core and high infiltration of inflammatory macrophages.[81,82] (Figure 3).

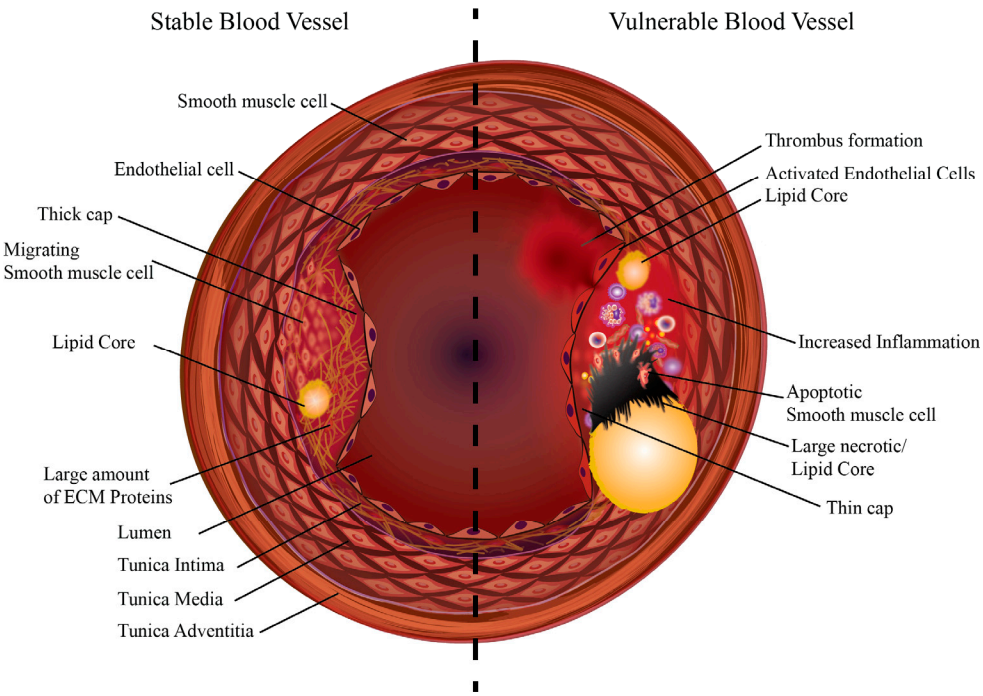

Figure 3. Comparison between a stable and vulnerable plaque.

Plaque erosion

The mechanism leading to thrombus without rupture is still an unresolved question in atherosclerosis. Plaques characterized by erosion usually have a smaller lipid core with less amount of inflammatory cells compared to ruptured plaques.[62] In fact, eroded plaques are not associated to inflammatory markers at least among women.[83] Additionally, plaques with endothelial erosion do not present an intact endothelial layer, and thereby exposing thrombogenic plaque component to the blood. These plasques also contain a well-developed media with increased SMC and proteoglycans.[84]

Retention hypothesis – ECM perspective

The response-to-retention hypothesis, proposed in 1995 by *Tabas and Williams*, postulated subendothelial ApoB100 containing lipoprotein retention in the vessel wall as the initiating event in atherosclerosis and atherosclerotic plaque formation; tightly linked to components from the extracellular matrix, mainly proteoglycans. Proteoglycans are believed to play a critical role in the subendothelial retention of LDL in the vessel wall because it has been shown that transgenic mice expressing ApoB100 with a deficient/mutated proteoglycan binding site had lower levels of atherosclerosis compared to control mice, suggesting a key role of proteoglycans in LDL retention.[85]

The ApoB binding to proteoglycans is mediated mainly by ionic interactions between the carboxy-terminal of site B (residues 3359-3369) of the ApoB100 protein or site B1b (residues 84-94) of the ApoB48,[47,86] and the negatively charged sulphate groups on proteoglycans, especially biglycan and decorin.[87,88] In fact, $ApoE^{-/-}$ mice containing either ApoB100 or ApoB48 showed atherogenesis, confirming that the binding site is different in each of them.[57,89]

Cytokines, growth factors and oxidized LDL may influence proteoglycan structure, and hence, its interactions. The interaction can be mediated via a direct interaction between proteoglycans and LDL, but in general, lipolytic and lysosomal enzymes from the ECM seem to play a role. Bridging molecules like lipoprotein lipase, an enzyme that mediates triglyceride hydrolysis into chylomicrons and VLDL,[90] enhances the adherence of LDL to proteoglycans *in vitro*. For example, human LDL does not contain ApoE, another bridging molecule, but murine LDL does. This suggests that direct binding between ApoB100 and proteoglycans (instead of through bridges) may be more important in humans than in mice.[85]

Lipoproteins are mostly delivered into the intima by transcytosis.[47] Once the LDL is retained in the intima, it is more susceptible to oxidation by metal ions, reactive oxygen species or enzymes such as myeloperoxidases or lipoxygenases,[55] getting modified. There is a large number of evidences showing that modified LDL can

promote processes that lead to atherosclerosis,[91] and that oxLDL in the intima promotes atherosclerosis development.[92–94]

Aggregated and modified LDL is taken up by scavenger receptors on macrophages and SMC to create foam cells followed by a cascade of inflammatory responses that will contribute to the formation of atherosclerotic plaque.[95,96] Likewise, modified LDL may release reactive aldehydes that can modify surrounding proteins.

Modifications during lipid oxidation

Modifications by malondialdehyde

CVD and atherosclerosis have been associated to increased oxidative damage.[97] Proteins can be modified non-enzymatically in different ways, including the formation of free radicals. The major player involved in oxidative stress is lipid peroxidation of polyunsaturated fatty acids, resulting in reactive aldehydes.[98] One of the most biologically relevant derived aldehydes is malondialdehyde (MDA), figure 4.

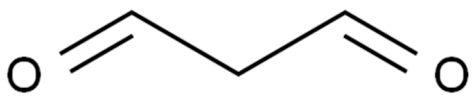

Figure 4. Chemical structure of malondialdehyde.

MDA was thought to be a marker of atherosclerosis and cardiovascular disease, but its value as a marker is not consistent, either because of its instability or because the large variation of MDA adducts.[97] It is unclear if cell stress produced by MDA modification, contributes to acceleration of atherosclerosis, and consequently, CVD.

MDA, produced in high amounts, was one of the first aldehydes that could be measured[98] and in contrast to other aldehydes, it is hydrophilic, being able to diffuse out of the oxidized LDL particle and interact with surrounding molecules. It has previously been shown that MDA is released during LDL oxidation leading to modifications on ECM proteins in vitro.[99] Furthermore, MDA has been implicated in the damage of proteins such as collagens.[100]

MDA reacts with the amino groups of the amino acids arginine, lysine or histidine and is able to cross link proteins or peptides, leading to a loss of protein function.[100–102] For example, intermolecular cross-links are observed in collagens under scanning

calorimetry and tails of rats are rigid after a short incubation with MDA.[103] Furthermore, aldehyde-mediated modifications of collagen type IV affect endothelial cell adhesion,[104,105] increase inflammation, and decrease anti-coagulant proteins on endothelial cells, which may contribute to endothelial dysfunction.

Similar to LDL, where antibodies against specific MDA epitopes have been found in human plasma and in ApoE$^{-/-}$ mice,[106] these modified matrix proteins may induce immune responses, and indeed antibodies against MDA-modified matrix proteins are found in plasma.[99,107]

MDA is also present in individuals with diabetes. Actually, it is increased in plasma from patients with diabetes and in atherosclerotic plaques, and crosslinking of collagen in diabetic vessels is suggested to increase the arterial stiffness seen in these patients.[103] In addition, initial modification of collagens by sugar adducts stimulates LDL oxidation producing MDA. This MDA will cross link the collagen, making it more susceptible to further glycation and contributing to loss of its remodelling capacity.[103]

Modifications by methylglyoxal

Methylglyoxal (MGO) (figure 5) is increased during hyperglycemia, a condition associated with diabetes.[108] MGO is a reactive aldehyde, formed from glucose and resulting in AGE modifications that in individuals with diabetes increase stiffness of the large vessels.[109] In fact, AGEs are likely to predict propensity to diabetic complications.[110]

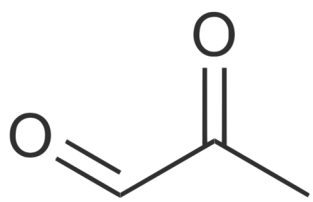

Figure 5. Chemical structure of methylglyoxal.

MGO mainly reacts with histidine, lysine or arginine residues. Arginine is present in the integrin binding RGD-sequence. Modification of these sites, may affect the interaction between integrins and proteins such as collagens, and therefore, cell-matrix interactions.[104,111] Indeed, modification of collagen type IV by MGO in hyperglycemia lead to a reduced endothelial survival and function in diabetes.[112]

Concentrations of MGO are elevated in serum and tissues in subjects with diabetes compared to subjects without diabetes[113] as well as in high fructose diet fed rats.[114]

Glyoxal and MGO may also be formed during lipid peroxidation of polyunsaturated fatty acids (PUFA), suggesting a role in individuals without diabetes.[115,116] Indeed, high levels of MGO have been detected in patients with rheumatoid arthritis without diabetes[117] and the importance of MGO in atherosclerosis progression and plaque rupture is independent on diabetes.[118,119]

Extracellular matrix – broad overview

The ECM is a mixture of macromolecules and contains in mammals around 300 different proteins (mostly collagens, elastin, fibronectin, laminin), glycoproteins, proteoglycans, sugar polymers (glycosaminoglycans and hyaluronan) as well as bound materials, cytokines, growth factors and metal ions.[120,121] Depending on their location and configuration, the ECM can be divided in the interstitial matrix, located around the cells; and the basement membrane (BM) or *basal lamina*, which separates the epithelium from the stroma (composed of connective tissue). ECM can be broken down by proteases, affecting its structure and function.[122]

Basement membrane

BM appears in mice embryos at day 4-4.5 as the primitive endoderm and will differentiate into an epithelial layer.[123] BM is a highly specialized dynamic cell-adherent ECM layer that confers tissue structure and influences cell behaviour. Around 50 different proteins are present in the BM, and collagens (mainly collagen type IV) comprise 50% of all components.[124] The core structural constituents of the BM are collagen type IV, laminin, heparan-sulphate proteoglycans (perlecan and agrin), and nidogen.[125]

Collagen type IV appears in early stages of differentiation in mammals, although it is not essential until later in the vasculogenesis. On the other hand, nidogens and perlecan are indispensable before and around birth but not later in the differentiation, and laminin is essential during all the stages.[125]

The notion that laminin is a fundamental protein for the initial formation of the basement membrane is supported by studies in *Drosophila*,[126] *Caenorhabditis elegans*[127] and mice. These proteins are responsible, in first place, for binding to cells mainly by integrins or other transmembrane receptors such as dystroglycans or sulphated glycolipids,[128,129] and secondly, of binding to self or other components from the BM. Nidogen binds to laminin and acts as a bridge for the collagen type IV network formation. Moreover, perlecan binds to nidogen and collagen type IV, and finally, collagen type IV self assembles into a network through N-terminal, lateral and C-terminal interactions.[129] (Figure 6).

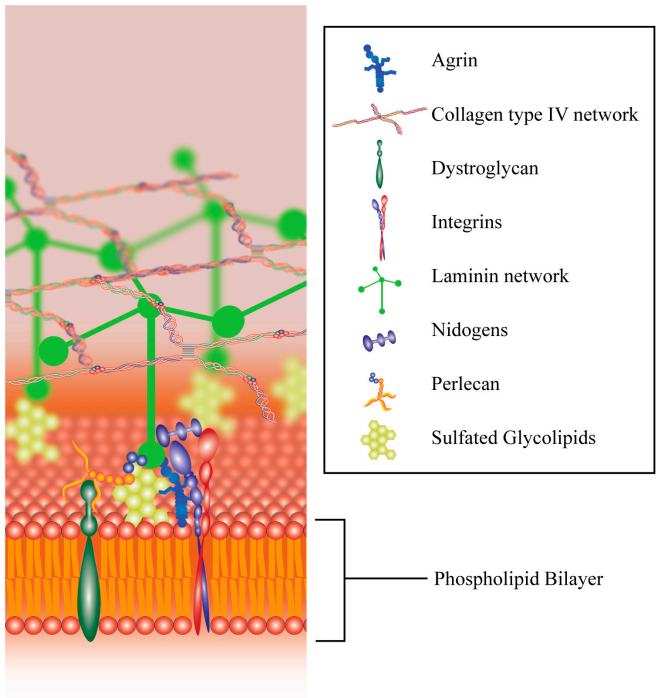

Figure 6. Interconnections between receptors and proteins in the basement membrane.

These basic components are organized in a network where laminin and collagen type IV are parallel to the surface, and differences in their assembly and receptor binding create variations in the structure and hence variations in signalling and stability.[129]

Mutations affecting some of these components are associated with impaired BM development, resulting in diseases affecting muscle, brain, nerve, skin, eye, vasculature and kidney. Because of this huge diversity of phenotypes, there is no single treatment for BM disorders.[125,130]

Collagen type IV

Collagens are the most abundant proteins in mammals (around 30% of total protein mass). Currently, there are 28 different known collagens:[131] fibrillar, collagens associated with banded fibrils, network-forming, transmembranous and endostatin precursor collagens, and collagen type XXVI and XXVIII, which do not fit in any of the previous categories.[132,133] All the collagens have in common a triple helical motif, containing the sequence Gly-X-Y, where X and Y denote any amino acid,

although it is often proline and hydroxyproline respectively, and a glycine in every third amino acid, which is affected in many human mutations.[132,134] The triple helix is flexible instead of rod-shaped because of imperfections in one to three amino acids. In the case of collagen type IV chains, these imperfections can be from 21 to 26 amino acids interruptions, of various lengths, leading to an increased flexibility in its triple helical domain.[135,136]

Collagen type IV, mainly synthesized by SMC and endothelial cells[134,137] was first discovered in 1966 by Kefalides in canine glomerular basement membrane.[138] Since then, it has been widely used in research studies in laboratories all over the world, since it is a crucial structural component of the BM.[139]

Biosynthesis of collagen type IV

Synthesis and folding of collagen type IV in the endoplasmatic reticulum (ER) is dependent of multiple and important factors where isomerases and hydroxylases are involved. Briefly, collagen type IV α chains are inserted into the ER lumen through a N-terminal peptide where the amino acid proline is essential for protomer/trimer formation.[136,140] Glycine, thanks to hydrophobic interactions, is placed in the heart of the helix, conferring structural stability.[141] Transport from the ER to the extracellular matrix via Golgi, is mediated with the help of specialized trafficking vesicles (Tango1) where an ER collagen-specific chaperon called heat shock protein (HSP47) is used as stabilizer in this transition. Without HSP47 a proper folding will not take place.[142] The binding between HSP47 and collagen type IV is pH dependent so the chaperon will dissociate from collagen type IV once the complex has reached the Golgi (which is acidic with a pH of 6.7).[143] The flexibility conferred by the interruptions present in the triple helix seems to be necessary for the supramolecular association of BMs.[144]

Structure

Collagen type IV is a network-forming molecule of approximately 400 nm in length with a terminal glomerular domain of 8-12 nm diameter.[145] Other collagens that form networks are collagen type VI, VIII and X.

Collagen type IV contains three major structures: an amino-terminal 7S domain rich in cysteine and lysine, a major collagenous triple helical, and a long carboxyl-terminal non-collagenous glomerular (NC1) domain.[146] It presents six genetically different alpha (α) chains encoded by genes COL4A1 to COL4A6,[147] oriented head to head, and located in humans in chromosome 13 (COL4A1, COL4A2),[143,148] in chromosome 2 (COL4A3, COL4A4), and in the X chromosome (COL4A5, COL4A6), whereas in mouse it is located in chromosome 8 (COL4A1, COL4A2), chromosome 1 (COL4A3, COL4A4) and in the X chromosome (COL4A5, COL4A6).[143]

Alpha (α)1(IV) and α2(IV) chains are the most common chains existing in BM in a ratio of 2:1.[149] Mouse and human α1 and α2 chains have a sequence of highly conserved amino acids. In the case of α1, there is a 83.5% identity between human and murine species whereas for α2, it is even higher, reaching 90.6%.[150,151]

The α chains are used for the formation of only three distinct trimers among 56 possible combinations: α1α1α2 (IV) located in BMs from all tissues;[134] α3α4α5(IV) present in BMs of the glomeruli of kidney, alveoli in lungs, testis, inner ear cochlea and eyes,[152–154] and α5α5α6(IV) located in BMs of bronchial epithelium, smooth muscle cells, skin, and Bowman's capsule and tubules in kidney.[154,155]

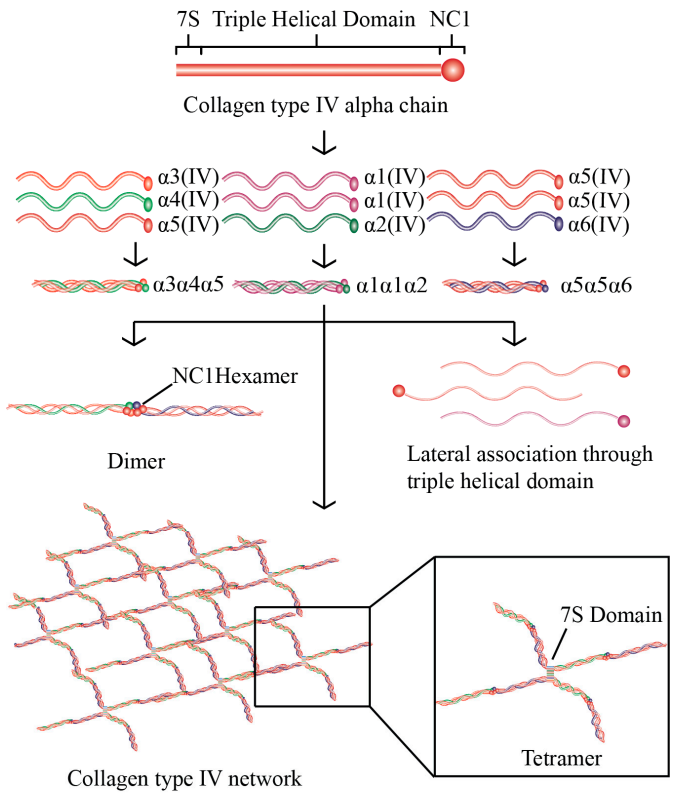

Figure 7. Simplified overview of collagen type IV biosynthesis.

The heterotrimeric molecules from collagen type IV assembly is starting with disulphide bond formation from NC1 towards the 7S domain, followed by creating networks through bindings between either N-terminal 7S domains, where cysteine and lysine are essential (forming tetramers heavily glycosylated), or NC1 domains

(to form dimers)[156] or triple helical domain (lateral association). These molecules interact further, reaching a supramolecular organization and hence a three dimensional network[143] as observed in figure 7.

Collagen type IV receptors

Besides its important role as scaffold in BMs, collagen type IV interacts with cells beneath the BMs and is essential for processes such as cell migration, survival, cell adhesion, proliferation or differentiation.[157] Collagen type IV networks bind to all cells except erythrocytes by multiple sites in the triple-helical and NC1 domain, involving integrin or non-integrin receptors.[158,159]

One of the remarkable characteristics of integrins is that they are able to recognize small peptides within the ECM. Integrins are composed by one α subunit and one β subunit forming a heterodimer[160] and vertebrates have an additional domain (αI) in the α subunit, which also can bind to collagen type IV.[161]

Integrin binding receptors α1β1 and α2β1 are the ones mainly recognized by collagen type IV.[158] In addition, α3β1, α10β1, α11β1[134] and αVβ3[162] bind to collagen type IV but to a lesser extent. In particular, α1β1 and α2β1 integrins bind three different motifs in collagen type IV: GFOGER (Gly-Phe-Pro-Gly-Glu-Arg; most common site), RGD (Arg-Gly-Asp; classical site) and non-RGD binding, where arginine is essential for recognition.[161] The RGD sequence is mostly present in the collagenous domain, but are inaccessible to cells when the protein is native. It has been shown that cell adhesion to collagen type IV is independent on the RGD motif, possibly because it is not exposed in the triple helical.[163] α1β1 and α2β1 integrins are essential for collagen type IV binding, and previous studies have reported that deletion or inhibition of these integrins resulted in decreased cell adhesion as well as migration of SMC.[164,165] Collagen type IV and collagen type I bind to the same α1β1 and α2β1 integrins, but with different affinity. α1β1 predominantly binds to network forming collagens such as collagen type IV, whereas α2β1 predominantly binds fibrillar collagens.[161,166]

Collagen type IV interaction with cells can also be mediated through non integrin receptors such as discoidin domain receptor type 1 (DDR1), mannose receptor family and glycoprotein VI (present in platelets).[158,159]

Diseases associated to collagen type IV

Mutations in genes that encode collagen type IV α1 and α2 chains, the most widespread chains, result in vascular defects such as intracerebral haemorrhages or intracranial aneurysms,[167] although most mutations in α1 or α2 chains from collagen type IV are embryonic lethal, whereas mutations in α3, α4 or α5 chains lead to renal failure and deafness in Alport's syndrome.[168,169]

Collagen type IV is the target of two important autoimmune diseases affecting the kidney where autoantibodies attack the glomerular BM, Alport's disease and Goodpasture syndrome. Alport syndrome lacks α3α4α5(IV) network in kidney and lungs, because of mutations of the alpha 5 chain on the X chromosome, whereas Goodpasture syndrome is an autoimmune disease where antibodies against NC1 domain of collagen type IV alpha 3 chain, are detected in the glomerular BM in kidney. In addition, collagen type IV alpha 5 chain is also affected, but to a lesser extent.[143,170]

Fragments released from collagen IV α chains during protein remodeling have been studied as possible markers in diseases related to ECM, such as fibrosis of the liver[171,172] or lung,[173] where plasma from these patients revealed elevated levels of 7S domain, important for collagen type IV formation. Collagen type IV fragments have been found in urine from patients with kidney disease, and subjects with T2D are associated with severe morphological alterations in kidney fibrosis, suggesting collagen type IV as a possible marker in diabetic nephropathy.[174]

Role of collagen type IV in atherosclerosis

As mentioned before, collagen type IV being one of the main proteins in BMs confers stability and affects cellular behaviour. Collagen type IV interacts with endothelial cells and it is important for their attachment and function. In addition, collagen type IV is implicated in SMC migration as well as SMC quiescence.[175] Both, endothelial cells and SMC play important roles in atherosclerosis, thus it is likely that changes in collagen type IV expression or modifications of collagen type IV will affect atherosclerotic disease.

Rekhter claimed that "too much collagen leads to arterial stenosis and not enough collagen makes atherosclerotic plaque prone to rupture".[176] Thus, a balance between synthesis and degradation is needed to keep stability. In the healthy arterial wall, the media and adventitia are rich in collagen type I and collagen type III whereas the basement membrane under the endothelial cells contain mostly collagen type IV, laminin and perlecan.[120,175] Collagens represent the 60% of all the proteins present in human atherosclerotic plaques,[177] where collagen type I is the most abundant (around 70% of all collagens) followed by collagen type III, IV (around 3% in human aorta), V and VI.[178,179]

Collagen type IV increases when the atherosclerotic lesion progresses[179] and it is found beneath endothelial cells and inside the intima in early lesions.[137] Collagen type IV is also found surrounding SMC in the intima in early atherosclerotic plaques, but in advanced atherosclerotic plaques, a thick collagen type IV multi-layer is formed and depositions of this protein are detectable in fibrous cap regions.[180] Other studies also showed that collagen type IV is present in fibrous cap regions and in advanced atherosclerotic human lesions.[137,176] Collagen type IV binds

to oxLDL, affecting LDL retention.[181] Further, MDA-collagen type IV correlated positively to oxLDL in plaques,[105] and MDA-collagen type IV was also increased in atherosclerotic plaques from patients with cerebrovascular symptoms compared to plaques from asymptomatic patients.[105]

TGF-β stimulates the synthesis of collagen type IV[182] whereas collagen type IV expression is inhibited by platelet derived growth factor (PDGF).[183] MMPs play a key role in the degradation of components from the extracellular matrix. Collagen type IV can be degraded by MMP-2, -3, -7, -9, -10, -13, -19, -25 and -26 which can be expressed by SMCs, macrophages and/or endothelial cells as cellular sources in atherosclerotic plaques.[48,180]

Finally, genome-wide association studies have identified an association between coronary heart disease risk and SNP rs4773144, present in the COL4A1-COL4A2 locus.[184,185] This SNP was shown to affect COL4A1 and COL4A2 expression, as well as atherosclerotic plaque stability.[186] Mutations in these genes have been reported to produce hemorrhagic stroke in humans.[167,187]

Immune system: Innate mechanisms – the first barrier

Atherosclerosis is a complex inflammatory disease where both innate and adaptive immunity are involved. Hypercholesterolemia is necessary for the initiation of the disease, but immune mechanisms play an important role in lesion development.

The innate immunity is a host defence that is always present in healthy individuals. It responds quickly after a foreign signal or pathogen, but also reacts against self-structures as a consequence of cellular stress. It is able to recognize hundreds of different structures without previous training, generating nonspecific immune responses in a fast and efficient way. In an atherosclerotic lesion, numerous innate immune mechanisms are playing a role simultaneously.

Molecular patterns - PAMPs and DAMPs

Recognition of invariant structures like pathogen associated molecular patterns (PAMPs) from microbes are essential in host defense. Studies showed that the immune system not only recognizes bacteria or pathogens, but also recognize other type of signals such as necrotic cells, inflammatory molecules, oxidized epitopes or ECM in the absence of microbial constituents. These endogenous signals are called damage associated molecular patterns (DAMPs). DAMPs can be nuclear and cytosolic proteins as well as nucleotides, but they do not need to be located

intracellularly and can also be generated after ECM degradation.[188] Accumulation of DAMPs in atherosclerotic plaques is an important factor in plaque progression.[189]

The most well-known example of PAMPs is lipopolysaccharide (LPS), a major cell wall component of Gram negative bacteria, which enhances atherosclerosis by increasing proinflammatory macrophages.[190] Inhibition of LPS in animal studies attenuates atherosclerosis and promote plaque stability.[191] LPS is recognized by pattern recognition receptors (PRRs) such as TLRs, expressed on innate cells initiating an inflammatory response.[192] There are ten different types of TLRs which are expressed at a low level on non-immune cells like endothelial cells, but in increased amounts on innate immune cells such as macropaghes.[192] These receptors act as a bridge between the innate and the adaptive immune system, since they are expressed on macrophages which will activate T cells.[193] TLRs (TLR-1, TLR-2 and TLR-4) are expressed in human atherosclerotic lesions[194] and may be involved in inflammatory processes during the disease.[195] Several studies in $ApoE^{-/-}$ mice lacking those TLRs have shown an important reduction in atherosclerosis[196] although an older study on TLR4 deficient mice did not alter atherosclerosis.[193] Other TLRs such as TLR-3, TLR-7 or TLR-9 may be protective in mice.[194] TLRs will activate transcription factors, including NFκB, which are needed for inflammatory gene transcription.[197]

Oxidation specific epitopes such as oxLDL can also activate immune responses. Oxidized LDL is considered a DAMP, since it shares some of its epitopes with other DAMP molecules and is recognized by PRRs like TLR or scavenger receptors. In fact, scavenger receptors (mostly SR-A and CD36) are responsible for up to 90% of the uptake of oxLDL from macrophages, at least *in vitro*.[198,199]

Complement system

The complement system is a crucial part of innate immunity and an interface between innate and adaptive responses. For example, mice lacking parts of the complement system, showed reduced antibody levels and antigen uptake as well as reduced number of T cells.[200] The complement system is activated through three main pathways, which will activate a cascade that will lead to proinflammatory mediators, cell lysis or phagocytosis of opsonized targets through C3b component. Briefly, the classical pathway through C1 complex (initiated by C1q) recognizes antigen-antibody immune complexes and binds to Fc regions in antibodies (normally IgG1 and IgM). The lectin pathway is not immunoglobulin dependent and instead, binds mostly to PRRs (specifically mannose-binding lectin). Last, the alternative pathway is activated by low levels, spontaneous hydrolysis of C3, which normally is present in high amounts in plasma.[200]

Several groups have investigated the role of the complement system in atherosclerosis experimentally or in clinical studies. Most reports underline a proatherogenic role of the complement system whereas others suggest protective effects. It seems like depending on the component of the complement studied, the outcome differs. For instance, animal studies have shown that inhibition of specific parts of the complement system cascade such as C3a or C5a reduces atherosclerosis and levels of C5a was found to be increased in patients with atherosclerosis.[194,201,202] Additionally, $ApoE^{-/-}$ mice deficient in CD55, an inhibitor of the complement component 3, developed less atherosclerosis compared to $ApoE^{-/-}$.[203] On the other hand, CD59 deficient mice were associated to an increase in plaque area.[204]

Monocytes and macrophages

Monocytes and macrophages are part of the innate immune system and play important roles in atherosclerotic disease. Once the monocytes enter the arterial wall, they differentiate into macrophages. As soon as oxLDL is captured by scavenger receptors or TLR-2 and -4 present on the macrophage surface, these cells will become lipid loaded foam cells and release pro-inflammatory (IL-1, IL-6, IL-12; IL-15; IL-18; tumor necrosis factor (TNF)-α or monocyte chemoattractant protein (MCP)-1) or anti-inflammatory cytokines (IL-10 or TGF-β) as well as growth factors.[205,206] Studies in mouse showed that oxLDL induced activation of TLR4/TLR6 heterodimer (but not of TLR2) in combination with scavenger receptor 36.[198]

Monocytes are essential for lesion formation and hypercholesterolemic mice deficient in monocyte receptor CCR2, showed a considerable reduction in atherosclerosis.[207] Monocytes are abundant in humans ($CD14^{++}$) and in mice ($Ly6C^{hi}$), and correlate with atherosclerotic lesion development in human and mouse respectively.[189]

Macrophages also drive atherosclerosis progression and can change behaviour, parallel to Th1 or Th2 lymphocyte subsets, depending on the environment.[189] For example, M1 macrophages will be formed upon classical activation in presence of interferon (IFN)-γ or LPS stimulation, whereas M2 macrophages will be formed upon alternative activation with IL-4, IL-10 or IL-13.[208]. M1 is considered mainly proatherogenic because it secrets IL-6, and this cytokine is elevated in plasma from patients with acute coronary syndrome, and M2 is considered antiatherogenic, since it releases for example TGF-β and reduce inflammation by clearance of apoptotic cells.[197] The M1 subtype will represent 40% of the total macrophages found in atherosclerotic plaques. M2, which is divided further in three subtypes depending on the stimuli, will represent 20% in mice. However, other phenotypes are also identified in plaques like Mox, Mhem or M4, but these subpopulations will not be discussed in this thesis.[209]

Immune system: Adaptive mechanisms

The adaptive immune system is a host defence stimulated by microbes that invade tissues. It is more specific but slower compared to innate immunity. There is an enormous amount of receptors that will generate the two types of adaptive immune responses, cellular (mediated by T cells) and humoral immunity (mediated by antibodies secreted by B cells). The receptors crucial for adaptive immune cell activity are T cell receptor (TCR), B cell receptor (BCR) as well as major histocompatibility complex (MHC) molecules.[51] In contrast to PRR that are more limited, there are an unlimited number of TCRs and BCRs. Lymphocytes (T and B cells) are mostly present in the adventitia of healthy arteries,[67] but in atherosclerosis they accumulate in the intima.[210] More recent investigations are suggesting the important role of the adventitia in coordinating immune responses and progression of intimal lesions.[211,212] Studies in human and mice have revealed important roles of both innate and adaptive immunity in atherosclerosis as a result of an accumulation of macrophages activating an adaptive response containing B and T (mainly $CD4^+$) cells.[210]

Initial investigations indicated that only macrophages were involved in the atherosclerotic process,[67] but later studies demonstrated that T cells were present in atherosclerotic plaques earlier than monocytes or macrophages, and actually, even before plaques were formed.[213,214] In fact, T cells are stimulated to migrate into the lesions, by the same adhesion molecules as monocytes, such as selectins or VCAM-1.[213] Notably, lymphocytes have been observed in the adventitia of children before they develop atherosclerosis.[211]

Furthermore, inhibition of early plaques has been observed due to lack of T and B cells. T- and B-cell deficiencies in murine models resulted in a reduction of atherosclerotic plaques from 40% up to 80%.[215] $ApoE^{-/-}$ or $LDLr^{-/-}$ mice lacking B and T cells (severe combined immunodeficiency) showed a reduction of lesions up to 80% when examined at 4-8 weeks of age. This difference disappeared if the lesions were observed after 16 weeks of age,[216–218] suggesting that T or B cells are not necessary for the progression of atherosclerotic lesions if the cholesterol levels are high.

Studies involving either rodent or human plaques have shown that $CD4^+$ T cells are more abundant than $CD8^+$ T cells.[219] Although not as common as monocytes and T cells, dendritic cells, mast cells, neutrophils and B cells are also located in atherosclerotic lesions.[5,211]

T cells

Lymphocytes originate from hematopoietic stem cells in the bone marrow and will migrate to thymus for further development and maturation to become T cells. Next, T cells will be released from thymus and are present in blood and lymph nodes. T cells can be categorized according to expression of surface markers, intracellular proteins and function. Although it is well known that T cells are involved in the atherosclerotic process, further investigations are needed to assess the exact role of different T cell subtypes in this worldwide disease.

T cells are present in plaques from humans and from mice. In advanced human plaques, T cells constitute 10-20% of the total cell population.[213] Seventy percent of the T cells found in plaques are $CD4^+$, of which the majority is Th1 cells known to release proatherogenic cytokines or cell-signaling proteins, followed by $CD8^+$ cells.[220] Actually, transfer of $CD4^+$ T cells from oxLDL immunized mice, increased atherosclerosis development, suggesting a role in specific antigen-driven responses.[221] The role of $CD8^+$ in atherosclerosis is not clear; deficiency of $CD8^+$ in mouse models did not show an effect on plaque, although $CD8^+$ cells are present in lesions, and transfer of $CD8^+$ from immunized mice was atheroprotective.[220]

Initial experiments on $ApoE^{-/-}$ mice showed that T-cell deficient mice developed atherosclerotic plaques, but those plaques were smaller, suggesting a possible role in atherosclerosis.[214] Indeed, transfer of $CD4^+$ cells into $ApoE^{-/-}$ worsened atherosclerosis.[222]

$CD4^+$T helper (Th) cells can be distinguished based on their cytokine production; Th1 cells secrete INF-γ, TNF-α, IL-2, IL-12, Th2 cells secrete IL-4, IL-5, IL-13, and Th17 cells secrete IL-17. Th1 cells are believed to be proatherogenic and are found in large amounts in atherosclerotic plaques in both humans and mice whereas Th2 cells, known for their B cell help, have been shown to be antiatherogenic by releasing IL-5 which will promote B-1 cells and antibody production. The role of Th17 in atherosclerosis is inconsistent.[67,205,223] Regulatory T cells (Tregs) keep the balance between Th1 and Th2 cells and lack of Tregs increases inflammatory responses in atherosclerosis. In fact, high levels of IL-10 (produced by Tregs) reduced LDL levels in LDL receptor deficient ($LDLr^{-/-}$) mice, and transfer of $CD4^+CD25^+$ Treg-deficient bone marrow into $LDLr^{-/-}$ mice increased lesion size.[67,205]

In mammals CD3 will connect to TCR to enable intracellular signaling in T cells through epitopes/peptides bound to MHC or human leukocyte antigen (HLA) in humans. $CD4^+$ cells bind to MHC class II on antigen presenting cells (APC) such as dendritic cells generating signals through the CD80/CD86-CD28 pathway (CD28 on the T cell interacts with CD80 or CD86 on the APC), whereas $CD8^+$ cells interact with MHC class I present on any cell type.[220,224]

B cells

The role of B cells in atherosclerosis has been less studied compared to T cells, but has gained more and more attention during the last years. In fact, murine studies have reported that B cells regulate atherosclerosis, but the effects are subset dependent. B cells are mainly found in spleen and lymph nodes (secondary lymphoid organs), as well as in intestine, lungs, adipose tissue, sites of inflammation, aortic adventitia and blood.[211,225] In particular, the spleen plays an important role in development of humoral immunity because it contains approximately 25% of all leukocytes and memory B cells.[226]

B cells can influence atherosclerosis with release of antibodies that will activate macrophages, or by binding to Th cells by recognition of endocytosed MHC II on B cells or by production of cytokines.[225] Functional studies in splenectomized mice showed an increase in atherosclerosis compared to controls, whereas transfer of B cells into the same mice were able to decrease the process.[227] A follow up study of splenectomized human subjects, showed an increased risk for mortality in ischemic heart disease.[228] On the other hand, several studies reported a decrease in murine plaque formation when $CD20^{+}$B cells were depleted,[229] supporting the controversial role of some B cell populations.

There are two main B subsets in mice based on their developmental origin: B-1 cells and B-2 cells. Studies on different B cell populations and their function in the development of atherosclerotic plaque seem to agree that B-1 cells (innate B cell population) prevent lesion formation, whereas the role of B-2 cells are still conflicting, yet most studies suggest a proatherogenic role.[230] B cells are present in healthy vessels and they are mostly found in the adventitia. B-2 cells are found in murine plaques and at a higher concentration than B-1 cells, supporting the proatherogenic role of B-2 cells.[231]

B-1 cells – innate B cell population

The B-1 repertoire is already set by birth (develops primarily from fetal liver) or within few weeks after birth, and they will not be produced *de novo* once they have been generated, but will be maintained by self-renewal.[225,232] B-1 cells in mice produce predominantly natural IgM, but also IgA and to lesser extent IgG3 antibodies.[230,233] B-1 cells release natural antibodies without any type of antigen stimulation. Mice that have not been infected will still have IgM in plasma, which almost exclusively come from B-1 cells in spleens.[198]

In mice, B-1 cells can be divided in B-1a and B-1b, but this division is not clear in humans. Mouse B-1a ($CD19^{+}B220^{low}IgM^{high}CD5^{+}CD43^{+}CD23^{-}$) cells release natural antibodies and respond independently to the presence of antigens. They are located mainly in spleen and peritoneum and around 2% of splenic B cells are from the B-1a subclass. B-1a cells are the smaller subset of B cells and they have been

shown to be atheroprotective.[232,234] In addition, mouse B-1b ($CD19^{+}B220^{low}IgM^{high}CD5^{-}CD43^{+}CD23^{-}$) cells are also located in spleen and peritoneum, and constitutes less than 1% of the splenic B cells.[230] B-1b cells are a specific subset that responds in presence or absence of antigens and although their role was unclear in the beginning, it is now believed that they have atheroprotective characteristics.[233]

The role of B-1 cells in humans is poorly understood since most of the studies have been done in mouse models. The human equivalent to murine B-1 cells have been described as $CD20^{+}CD3^{-}CD27^{+}CD43^{+}CD70^{+}$ cells with the same functions as B-1 murine cells, including spontaneous production of IgM antibodies.[235,236] However, a later study have questioned if this population represents a phenotype closer to other B cell populations than B-1 cells.[237]

Natural antibodies, mainly IgM immunoglobulins[238] but also IgA[239] and IgG (IgG3 in mouse),[240] are essential in innate immunity. They are released by B-1 cells in the bone marrow (in small amounts) and in the spleen,[232] but not in the peritoneal cavity,[241] and a least 80% of natural IgM found in plasma comes from B-1 cell type.[232] They are conserved by natural selection with a genetically stabilized repertoire independent of external stimuli, and are already present from birth or shortly thereafter.[242,243] IgM binds with much higher affinity to the complement component C1q than for example IgG, probably because its pentameric structure.[244] The production of these antibodies is stable in healthy individuals and uninfected mice contain mostly natural antibodies.[239]

Mice deficient in IgM and mice with reduced levels of IgM after splenectomy have shown increased number of apoptotic cells suggesting the importance of IgM natural antibodies to reduce atherosclerosis[245,246] by clearance of apoptotic cells.[244] Further, IgM natural antibodies bind to oxidized phospholipids of the oxLDL and it has been shown that oxidized epitopes, especially MDA epitopes, are the predominant targets for natural antibodies in both humans and mice.[247]

B-2 cells – adaptive B cell population

B-2 cells develop from the adult bone marrow[225] and later in evolution than B-1 cells. They can be divided into marginal (considered part of the innate system)[234] and follicular B cells, and the atherogenic effects of for example follicular B cells, are highly dependent on MHC class II and CD40.[248] In mice, B-2 cells produce adaptive IgM, high affinity IgG (IgG1, IgG2a/c, IgG3), IgA and IgE in response to T cells,[230] although it is mainly secreted IgG antibodies, which activate specific humoral immune responses. It is known that B-2 cells produce both pro and anti-nflammatory cytokines. For example, 50% of the TNF-α found in spleen was shown to come from B-2 cells.[241]

Depletion of CD20+ B cells reduced atherosclerosis in $ApoE^{-/-}$ and $LDLr^{-/-}$ mice[229] and transfer of splenic B-2 cells into T- and B-deficient $ApoE^{-/-}Rag^{-/-}$ mice induced atherosclerosis compared to control.[249] In addition, disruption of B cell activating factor, BAFF (necessary for B-2 cell survival), in mice resulted in a reduced plaque formation.[250,251] This data supports the role of B-2 cells as proatherogenic. Another example of the proatherogenic role of B-2 cells are: production of IgG2 antibodies and activation of macrophages through Fc receptors or activation of CD4+T cells.[225] However, in another study the opposite results were obtained when transfer of B-2 cells resulted in reduced atherosclerosis.[230]

Antibodies

The production of antibodies is the main function of B cells; thus it is reasonable that antibodies have an important role in atherosclerosis. Furthermore, the presence of IgM and IgG antibodies are much more abundant than B cells in both mouse and human plaques.[225]

An antibody molecule contains two identical heavy (H) chains and two identical light (L) chains linked together by disulphide bonds. Each H and L chain present an amino terminal variable (V) region and a carboxyl terminal constant (C) region. The four chains form a Y-shaped molecule. A light chain contains one V and one C domain whereas a heavy chain has one V and three or four C domains. The domains are folded in a three dimensional shape called Ig domain. The antigen binding fragment (Fab) is the portion of the antibody required for antigen recognition. It contains the entire light chain attached to the V and first C domain from the heavy chain. The remaining C domains from the heavy chain form the Fc region, to which Fc receptors on cells and complement bind. In general terms, Fab regions are used to block microbes and toxins, but antibodies will use their Fc region to activate diverse effector mechanisms that will eliminate these microbes and toxins. Each antibody has two identical Fab regions that bind antigens and one Fc region. responsible for most of the antibody functions.[252] (Figure 8).

The affinity of the antibodies increases with repeated stimulations. IgM is secreted as pentamers and contains ten binding sites, whereas IgG, IgD and IgE are monomers and contain only two antigen binding sites. Secreted IgA is a dimer, which contains four antigen binding sites. Therefore, each antibody can bind from 2 to 10 epitopes of an antigen if the identical epitopes (parts of the antigens recognized by the antibody) are situated close enough.

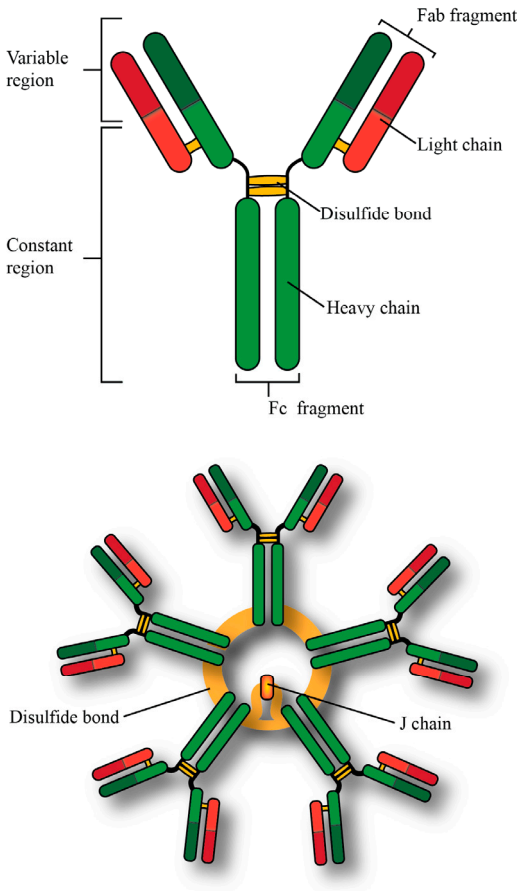

Figure 8. Structure of Immunoglobulin G (upper figure) and Immunoglobulin M (lower figure).

Type of antibodies

There are five immunoglobulin (Ig) classes or isotypes based on their different heavy chains: IgG, IgD (which will not be described), IgM, IgE and IgA. They are composed of 82-96% protein and 4-18% carbohydrates and have different effector functions.[253]

IgG is the most common and major immunoglobulin in human plasma (10-20% of plasma protein). IgG can be further divided in four subclasses in humans (IgG1, IgG2, IgG3, IgG4) and in mice (IgG1, IgG2a/c, IgG2b, IgG3), which differ in their constant regions and in their affinity to Fcγ receptors or to C1q from the complement system as well as half-life or immune complex formation.[230,253]

The equivalent of human IgG1 in mouse is IgG2a (both have the ability to fix complement and bind to protein antigens); the equivalent of human IgG2 in mouse is IgG3 (both can bind to mast cells); the equivalent of human IgG3 is murine IgG2b/c (which have the ability to fix complement and bind protein antigens) and finally the equivalent to human IgG4 is murine IgG1 because both can recognize carbohydrate epitopes.[254]

IgG is the only antibody that can cross the placenta and give protection to the fetus. IgG is found in mouse and human plaques,[255] and immunizations with specific ApoB100 peptides induced B- and T-cell responses, decreased atherosclerosis and increased IgG1 depositions in plaque from immunized mice.[256] In fact, IgG1 and IgG2a have been studied as markers for Th2 and Th1 lymphocytes, respectively, and mice infected with *Schistosoma mansoni* (used for polarization of Th cells in mice) exhibited an increase of IgG1 in plasma compared to IgG2a, suggesting that Th2 responses are predominant after bacterial infection.[257]

Adaptive IgM is the first antibody to appear when an immune reaction is needed and the first class produced before the isotype switch. The amount of IgM decreases after class switch from IgM to IgG, usually towards IgG1 or IgG3 and CD40 expression on B cells is required for isotype switch.[253] Adaptive IgM antibodies represent a small fraction but have higher affinity than natural IgM antibodies.[258] These IgM antibodies differ from the natural antibodies in the binding centers, repertoire and functions.[259–261] Natural IgM antibodies have been described previously in this thesis. Briefly, splenectomy in mice and humans caused reduced IgM plasma levels, increased atherosclerosis and was associated to increased risk of development of myocardial infarction,[230,245,262] and increased IgE antibodies.[263] A recent study confirmed the increase of IgE in plasma in mice lacking IgM.[264]

IgE has been broadly studied in asthma or allergy, but not to a large extent in atherosclerosis,[230] although it has been reported that IgE is increased in high fat diet fed mice[265] and correlates to CVD.[266] Mice lacking high affinity IgE receptor (FcεRI), display decreased atherosclerosis as well as macrophage content.[230]

IgA is mostly produced in the mucosal immune system by B-1 cells and it is the first barrier of defence in gut mucosa and in respiratory tract.[267] IgA is believed to be expressed continuously to maintain the microbiota in the gastrointestinal tract, although germ-free mouse experiments increased B-2 cell responses instead of production of IgA from B-1 cells, suggesting a disturbed balance in the intestines in response to pathogen stimuli, possibly, food allergies.[232] Correlations between IgA and atherosclerosis have been proposed, but its implications in CVD are not as clear as for IgG.[230,268] Recent findings revealed that IgA levels binding to phosphocholine, a molecule present in many microorganisms, correlated to cardiovascular disease.[269]

Fc receptors

The main receptor binding to antibodies is the Fc receptor (FcR). In addition, there are other receptors that bind to for example IgG immunoglobulins such as Fc receptor-like proteins or tripartite motif-containing protein 21, but these will not be discussed in here.[253] Fc receptors can be found on a number of different cells, including B cells, T cells, NK cells, monocytes, macrophages, neutrophils, dendritic cells, basophils, mast cells, eosinophils and endothelial cells.

Depending on the immunoglobulin binding to the Fc receptors, the function of the FcR (either activation or inhibition of cells) as well as the name used will be different. In general terms, Fc gamma receptor (FcγR) binds IgG, FcεR binds IgE, FcαR binds IgA and Fcα/μR binds IgA and IgM.[259,270]

The most important Fc receptors for phagocytosis of opsonized particles and immune reactions are receptors for the heavy chain of the IgG, FcγR. The majority of IgG receptors in humans are activating receptors (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA), but there is also an inhibitory receptor (FcγRIIB). Likewise, there is a receptor which function is unknown called FcγRIIIB, as well as FcRn involved in recycling and IgG transportation. Most of them will bind to all IgG subclasses, but it has been reviewed that high affinity binding will be between FcγRI and IgG1, IgG3 and IgG4 subclasses, and between FcγRIIIA and IgG3.[271,272]

In mice and primates (except humans) another type of receptor that will induce activation exists: FcγRIV.[273] Besides this one, these species contain other activating receptors (FcγRI, FcγRIII), as well as an inhibitory receptor (FcγRIIB) and a receptor involved in recycling. In mice, high affinity receptors will be FcγRI and FcγRIV.[271] Experiments performed on mice lacking FcγR, such as FcγRI and FcγRIII, have reduced atherosclerotic lesions, suggesting a possible atherogenic role.[274,275] Further, mice without the inhibitory receptor FcγRIIB displayed exacerbated atherosclerosis.[276]

IgE does not bind FcγR in humans, but it does in mice, where it binds to FcγRIIB, FcγRIII and FcγRIV.[271] In humans it binds to FcεR1 and to FcεR2 (also known as CD23).[266]

How do antibodies affect atherosclerosis? – antigen variability

Humoral responses against antigens present in plaques have been described to play a key role in the modulation of atherosclerosis. Multiple oxidative mechanisms are present in the vessel wall leading to reactive lipid species which will increase the development of the disease.[277] Antibodies can recognize inflammatory molecules or oxidized epitopes associated with processes that happen during atherosclerotic plaque formation like for example apoptosis[278] influencing the disease outcome.

Several autoantigens have been proposed to trigger immune responses, where oxLDL, ApoB100 and HSP60/56 are the three main and most studied antigens. In addition, oxidation of LDL results in aldehydes, MDA and HNE (4-hydroxy-nonenal), which further modify ApoB100 in LDL. As already mentioned, MDA is present in atherosclerotic plaques and its role in atherosclerosis has been already described in section "Modifications by malondialdehyde".

Heat shock proteins (HSPs)

An interesting cause of inflammation in atherosclerosis comes from HSPs, especially HSP60 (in humans, which mimicry with mycobacterial HSP65), although other subtypes such as HSP70 and HSP90 are also involved.[5] HSPs are proteins released by cells under specific conditions such as infections, stress or elevated temperature to protect themselves.[279] HSPs have been shown to behave as DAMPs, and all humans get immunity against HSPs once they have been infected or vaccinated for the first time.[188] APC can take up HSPs and present them with MHC class I to activate T cells. Actually, T cells recognizing HSP60 are present in early and developed atherosclerotic lesions[5] and it has been shown that immunizations with HSP60/65 aggravates atherosclerosis.[280] In addition, administration of IgG from HSP65 immunized mice, enhanced fatty streak formation. Last, antibodies against HSP60/65 have been shown to be increased in subjects with atherosclerosis[281,282] and transfer of antibodies against HSP60 from subjects with coronary artery disease into ApoE$^{-/-}$ mice also increased atherosclerosis.[214]

Oxidized LDL, MDA-LDL and MDA-ApoB100 peptides

Antibody responses against oxLDL have been studied extensively in both human and mice.[47] Generally, there is an inverse association between IgM against oxLDL and cardiovascular disease. IgM binds to and facilitates removal of apoptotic cells and can also prevent uptake of oxLDL by scavenger receptors and thus, inhibit foam cell formation.[230] The association of IgG against oxLDL with CVD is less consistent. Some studies have shown a positive association between IgG against oxLDL and CVD,[283–286] whereas other studies have not been able to establish an association.[284] Still some controversy is present regarding the role of IgG in atherosclerosis. Studies in rabbits reported a decrease in atherosclerotic levels when immunized with oxLDL as well as an increment in titers against oxLDL, suggesting an atheroprotective role of these antibodies.[287] On the other hand, other studies reported that plasma antibodies against oxLDL in LDLr$^{-/-}$ mice increased and correlated to lesion size and cholesterol levels in blood,[288] as well as to atherosclerotic surface area, aortic weight and content of oxLDL in aorta.[289]

Several observational studies have shown elevated levels of antibodies against modified LDL in subjects with atherosclerosis whereas other studies have seen an inverse correlation or no association. High levels of MDA-LDL IgG antibodies were

associated to cardiovascular events,[290] and MI,[291] and high levels of MDA-LDL immune complexes were observed in patients with T2D.[292] MDA-LDL is produced in vivo[293] and studies in LDLR$^{-/-}$ rabbits and mice have shown a considerable reduction of atherosclerotic lesions when immunized with MDA-LDL.[294–297] In fact, IgM and IgG antibodies against MDA-LDL in plasma from the same mouse model were associated to plaque surface area, aortic weight and modified LDL in the aorta.[289] Moreover, bone marrow transferred from BAFF (cytokine that belongs to the TNF family) receptor deficient mice into LDLr$^{-/-}$, showed decreased plaque formation and decreased levels of IgG1 and IgG2c against MDA-LDL.[251] Additionally, immunization of ApoE$^{-/-}$ mice with MDA-LDL also resulted in a reduction in atherosclerotic plaques[298] and formation of IgG antibodies.[299] All these studies support the notion of immune responses against MDA-LDL as atheroprotective.

In most studies immunizations with ApoB100 peptides have resulted in an increased regulatory T-cell response, antibody production and/or a shift from Th1 to Th2 responses. Immunizations with ApoB100 peptides in presence of an adjuvant or not, induced Tregs and reduced atherosclerosis.[225,300,301] Furthermore, passive immunizations with IgG1 against MDA-ApoB100 human peptides reduced plaque formation and increased collagen content.[302] In addition, mice immunized with human IgG1 against peptide 45 from ApoB100 protein presented regression of atherosclerosis.[303] Similar results were show in immunizations with a combination of peptide 2, 143 or 210 from ApoB100.[304,305] In addition, antibodies and peptides show an inverse correlation to CVD. In humans, antibodies against peptide 45 was associated to less MI[306] and high levels of IgG antibodies against peptide 45 correlated to less inflamed atherosclerotic plaques from endarterectomy patients.[307] Finally, a study population showed that higher levels of MDA-ApoB100 peptides IgG were associated to reduced risk of CVD.[308]

MDA-ECM proteins

Most oxidants diffuse a small distance from their formation site.[120] Damage on the ECM can be manifested by functional inactivation, increased permeability, protein unfolding, cross-linking, etc.[309] For instance, glycoxidation and MDA lipoxidation cause damage in collagen of hearts from hemodialysis patients and it is associated with cardiac damage.[310] MDA epitopes can modify LDL as well as surrounding proteins from the ECM generating immune responses against them. It has previously been shown that MDA is released during LDL oxidation leading to modifications on ECM proteins *in vitro*.[99] In addition, high glucose levels, and reactive aldehydes formed such as MGO, can also result in modification of the ECM.[120]

MDA modified proteins have been studied in the context of atherosclerosis in human and murine models,[107,311] but information of oxidized ECM *in vivo* is limited.

IgM and IgG antibodies against collagen type I, collagen type III, fibronectin and laminin have been found in human plasma[99,311] and the presence of MDA modified ECM proteins in atherosclerotic lesions have been demonstrated in humans.[99] Moreover, immune responses against modified ECM proteins have been investigated in mouse. Development of atherosclerosis in ApoE$^{-/-}$ mice was inhibited upon immunization with MDA-fibronectin[107] whereas an acceleration of atherosclerosis was reported after MDA-laminin immunization.[311] Last, antibodies against MDA-collagen type IV in plasma correlated to the amount of MDA-collagen type IV in lesions.[105]

Aims

Overall aim

The aim of my PhD studies was to study immune reactions against aldehyde-modified collagen type IV and aldehyde modified ApoB100 in cardiovascular disease and cardiovascular complications of diabetes.

Specific aims

- Are immune responses against aldehyde modified collagen type IV associated with myocardial infarction? (Paper I).
- Do antibodies against collagen type IV induced by immunization affect atherosclerosis in ApoE-deficient mice? (Paper II).
- Are immune responses against aldehyde modified collagen type IV associated with cardiovascular disease in subjects with diabetes? (Paper III).
- Are immune responses against aldehyde modified ApoB100 peptide associated with cardiovascular events? (Paper IV).

To be able to address these questions, I have performed analysis on material from human cohorts, *in vivo* studies in mice and *in vitro* studies on cells.

Methods

Cell culture studies

Cell culture studies provide a valuable insight that allows one to better understand and investigate mechanisms of both *in vivo* experiments and studies on human cohorts. Cell culture experiments are cheaper and faster compared to *in vivo* experiments, and can be performed in a controlled environment that can be reproduced several times. The main disadvantage of this technique is that cells or tissues are removed from their natural environment, eliminating or reducing interactions that exist in real life.

Cell culture studies were performed in paper I where human umbilical vein endothelial cells (HUVEC) were stimulated with anti-MDA bound to MDA-collagen type IV, and MMP secretion was measured. In paper II splenocytes were isolated from mice and stimulated with anti-CD3/CD28 beads to measure cytokines release. Cell culture studies were also used in paper IV, where B cells were isolated from peripheral blood mononuclear cells from healthy donors, and different B cell populations were sorted, and secreted antibodies were analyzed. Thus, cell studies have provided important data to most of the papers in this thesis.

Mouse models used in atherosclerosis

The most critical advancement in the study of atherogenesis has been the development of mouse models of atherosclerosis. Mice are small, easy to house, inexpensive and can generate offspring in a short period of time.

Contrary to rabbits, rats and mice are relatively resistant to cholesterol-rich diet (also known as Paigen diet, which contains 5% fat, 1.25% cholesterol and 0.5% cholic acid[312]) possibly because of their capacity to transform cholesterol into bile acids in the liver. Mice lack the cholesteryl ester transferase enzyme that transfers cholesterol esters from HDL to LDL,[313] and this factor is strain dependent. C57BL/6 is the most susceptible strain,[312] and therefore the most used strain in the study of atherosclerosis. However, to generate advanced atherosclerotic lesions, the mice need to be modified genetically. In experimental atherosclerosis, $ApoE^{-/-}$ and $LDLr^{-}$

$^{/-}$ mice are the most commonly used mouse models. Contrary to humans, mice develop readily lesions in the aortic sinus, probably due to a rapid heartbeat and disordered flow around the aortic valves.

ApoE$^{-/-}$ mice have been widely used since its inception in 1992[314,315] and has become the most used *in vivo* model for experimental atherosclerosis. ApoE is a protein mostly expressed in the liver, although it is also expressed in other tissues such as brain, spleen or kidney. ApoE$^{-/-}$ mice develop atherosclerotic lesions because an impaired clearance of chylomicrons and VLDLs by the LDL receptor that leads to increased plasma cholesterol levels. In mice, atherosclerotic plaques are quite stable, and there is no clear evidence that plaque rupture occurs in old (24 and 60 weeks of age) ApoE$^{-/-}$ mice fed with high fat diet.[81] LDLr$^{-/-}$ mice have a defective uptake of LDL[81] and thus elevated levels of plasma LDL. However, these mice develop no or very small lesions on normal chow diet, and cholesterol-rich diet is needed for these mice to develop large lesions.[316]

In paper II, female ApoE$^{-/-}$ mice on C57BL/6 strain background were fed with high fat diet (HFD) (0.15% cholesterol, 21% fat) for 11 weeks. ApoE$^{-/-}$ mice are severely hypercholesterolemic from birth and will develop quantifiable aortic atherosclerosis by approximately 6 weeks of age even if fed a standard chow diet. ApoE$^{-/-}$ mice fed cholesterol-containing diet become even more hypercholesterolemic than chow fed mice, and will develop larger lesions in a shorter time.[317]

The gender may also influence the development of atherosclerosis, and hence, the output of the experiment. Administration of estrogens has been shown to have atheroprotective effects in murine models,[318] arguing for the use of male mice, but on the other hand, ApoE$^{-/-}$ and LDLr$^{-/-}$ female mice develop larger lesions than the corresponding male mice.[319]

Each of these models has its unique advantages and limitations. None of them fully duplicates the human condition but the development of new genetic technologies is allowing the generation of new mouse models with closer characteristics to human atherosclerosis and complications.

Immunizations

The most important elements of the immune system that are affected by immunization in animals are the T cells, B cells and the antibodies that the B cells produce. In paper II, ApoE$^{-/-}$ mice were immunized at 12 weeks of age, followed by three boosters at 14, 17 and 22 weeks of age, to enhance the antibody response. The time of the immunizations may affect the outcome, and late immunizations may therefore be less effective if the induced antibodies play a role in early plaque processes. For example, immunizations using LDL modulated cellular and humoral immune responses as well as plaque size when the immunization was done at an

early age, whereas no effect was observed when the immunizations were performed at 20 weeks of age.[320]

Adjuvants are non-specific agents usually used to enhance immune responses against immunogenic antigens. Adjuvants may be added to a vaccine to modify the immune response by boosting it such as to give a higher amount of antibodies and a longer-lasting protection, thus minimizing the amount of injected foreign material. When using small antigens, the antigen needs to be conjugated to a carrier to enhance the immune response. Different adjuvants have been shown to have potent atheromodulating capacities and help the antigen to attract immune cells, thus, the choice of these adjuvants in mouse studies can affect the results.[321] Aluminium salts (aluminium hydroxide or aluminium phosphate), Freund's incomplete (emulsion) and Freund's complete (emulsion that contains killed cells of *Mycobacterium tuberculosis*) are the most widely used adjuvants. Freund's adjuvants have stronger and longer lasting response. Freud's incomplete will mostly activate Th2 responses, whereas Freud's complete will activate Th1 immune responses.[322] On the other hand, Aluminium adjuvants, usually known as Alum, have less adverse effects compared to Freund's adjuvants, and that is why is the most common used adjuvant in human vaccinations. It adsorbs soluble antigens and present them to APC, facilitating its uptake resulting in a Th2 response.[321,323,324] In paper II, peptides from the α1 and α2 chains from collagen type IV coupled to PADRE, PADRE alone or PBS were injected subcutaneously using Alum as adjuvant.

Immunizations were carried out after a brief sedation with isoflurane to immobilize the animals. It has been suggested that subcutaneous immunization of an antigen is more effective than intranasal delivery to obtain immune responses because possible degradation of the antigen as well as immune tolerance at mucosal sites.[325] Additionally, immunizations with adjuvants like Alum might create plugs that would cause obstruction of breathing in the case of nasal or oral immunization. However, to develop mucosal vaccines there is a need to find systems for antigen delivery and adjuvant, that results in effective ways to present vaccine or immunotherapy antigens to the mucosal immune system.[326]

PADRE as enhancer of immune responses

T cell receptor recognition by MHC molecule through peptide binding is needed for T cell activation. HLA-DR is an MHC class II cell surface receptor in humans and it has been reported that peptides can bind specifically to more than one DR type in the MHC, which may be a problem in the design of vaccines.[327]

Pan HLA DR binding epitope (PADRE) is a 13 amino acid long peptide designed to bind with high affinity to DR alleles, and binds to 15 to 16 of the most common HLA-DR receptors.[328] It is commonly used as carrier to induce CD4+ T helper responses in vaccines designed for human use,[327,328] since PADRE peptide binds to

the MHC class II molecule on the surface of antigen presenting cells for recognition by the CD4+T cells. PADRE peptides also bind to mouse MHC molecules, making it suitable also for *in vivo* studies in mice.[329]

PADRE has showed to be more potent than other universal T helper epitopes such as the tetanus toxin derived universal epitope[328] and it is safer and well tolerated in human clinical studies.[330] It is used as enhancer of antibody immune responses in some diseases[329,331–333] including the study of atherosclerosis in for example LDLr$^{-/-}$ mouse.[334] In our experiment, PADRE was coupled to collagen IV peptides via a cysteine residue located in the C-terminal of PADRE to enhance antibody responses against collagen type IV peptide.

Study populations

In this thesis I have had the opportunity to work with human plasma from two different cohorts approved by the Ethics Committees.

Malmö Diet and Cancer (MDC) Study

A total of 70000 subjects (men and women) 45-73 years old living in Malmö, Sweden were recruited for the Malmö Diet and Cancer (MDC) study. It is a population-based, prospective cohort examining the relationship between diet and certain forms of cancer. A baseline clinical examination and questionnaire was done on 28449 subjects.[335] In a substudy focusing on cardiovascular disease (MDC Cardiovascular Cohort), 6103 subjects were enrolled between 1992 and 1994, and out of those, 5540 subjects had a second visit (approximately 8 months after the first one) with blood sampling after the initial health examination.[336] Plasma samples were taken and stored at -80°C until analyzed.

In paper IV, MGO-p220 IgM and IgG were measured in plasma samples from 700 randomly selected individuals from the MDC Cardiovascular Cohort. These individuals were followed for 15 years to evaluate CV events.

In paper I, MDA-collagen type IV IgM and IgG were analyzed in plasma from 795 individuals from the MDC Cardiovascular Cohort in a case-control prospective study followed for 13 years to evaluate CV events. Individuals with a prevalent nonfatal myocardial infarction were excluded. The cohort contained 402 incident cases with fatal or nonfatal MI and 402 age- and sex-matched controls.

Statistical analysis

Spearman (non-normally distributed data) or Pearson's (normally distributed data) correlation was used to analyze associations between laboratory parameters or cytokines and antibody levels. Kaplan Meier analysis and Cox proportional Hazard regression were used for time to event analysis and to determine HR and 95% confident intervals between antibody levels in tertiles and the incident CV events during the follow-up. The analyses were adjusted for cardiovascular risk factors.

SUMMIT study

In paper III, MDA-collagen type IV IgM and IgG were analyzed in the Surrogate markers for Micro and Macro vascular hard endpoints for Innovative Diabetes Tool (SUMMIT) cohort. SUMMIT is a multicentre study which investigates cardiovascular complications in diabetes in subjects from Malmö (Sweden), Pisa (Italy), and Exeter and Dundee (UK) between 2012 and 2013. It contains 1500 subjects divided in four different groups: subjects with T2D and clinically manifest CVD (n=458); subjects with T2D but without clinical signs of CVD (n=527); subjects with CVD but without T2D (n=245) and subjects without either CVD or T2D (n=270). Clinically manifest CVD includes non-fatal acute MI, unstable angina, resuscitated cardiac arrest, coronary revascularization (bypass or percutaneous coronary intervention), non-fatal stroke, transient ischemic attack and lower extremity arterial disease.[337] At the basal examination additional measurements of clinical parameters such as carotid intima media thickness (IMT) (marker of carotid atherosclerosis), pulse wave velocity (arterial stiffness measurement) or reactive hypermedia index (endothelial function measurement) were performed.[338] Information of incident CVD events were recorded after 36 months follow-up. Vascular measurements on follow-up were assessed as regression, progression or no change in IMT as well as change in pulse wave velocity and change in reactive hyperemia index.

Statistical analysis

Spearman correlation was used for continuous variables. Linear regression was used to analyze correlations between vascular measurements and antibodies, whereas binary logistic regression was used to calculate odds ratios in regression of IMT. Additionally, binary logistic regression was used to measure associations between antibodies and CV events adjusted by UKPDS risk factors and study center. In this study, where T2D subjects was the group of interest, UKPDS risk factors adjustments instead of Framingham or traditional cardiovascular risk factors.

Statistics – more than a p value

In statistics analysis a P value of less than 0.05 is generally considered statistically significant. This means that there is only 5% chance that the distribution of the samples was as extreme as in the current test, but there is still a 5% chance that the result is a false positive. The more tests you run in a sample, the greater is the likelihood to find a positive result. For example, when performing 100 variables, 5 of those tests will have a p value below 0.05, and hence, be significant. In these cases, corrections for multiple testing should be done, for example by reducing the critical value from 0.05 to 0.00005 (dividing the p value by the number of hypotheses tested) to avoid false positive results.

There is conflicting literature regarding when multiple testing correction should be used. It is a statistical method of adjusting the significant level used for testing each hypothesis, so the chances to generate false positives are controlled. Some authors claim that multiple test corrections should never be done in scientific experiments,[339] whereas others have claimed the opposite.[340] There is no unanimous view on the issue of multiple testing correction. Some statisticians recommend not doing corrections when the study focuses on a few comparisons pre-specified before analysing the data.

Other relevant techniques

In this section I will briefly mention some of the techniques that are used in the papers included in this thesis.

ELISA

Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to measure antibody levels in human plasma (papers I, III and IV), in human B cell populations (paper IV) and in $ApoE^{-/-}$ mice (paper II). ELISA was also used in MMP measurements in supernatants in paper I.

ELISA is a colorimetric technique widely used because it is affordable, fast and sensitive. Antibodies, from mouse or human plasma; binding to for example collagen type IV peptides, MDA-collagen type IV, or MGO-p220 were detected by a secondary antibody either conjugated to an enzyme or conjugated to biotin followed by streptavidin conjugated to an enzyme. After addition of a substrate to the enzyme, the absorbance of the developed color is measured, and used as indirect measurement of the specific antibody concentration.

Multiplex Technology

Multiplex technology is a bead-based multiplex assay designed to measure multiple analytes in a small volume of serum, plasma or tissue culture supernatant. The theory behind the technique is similar to an ELISA. The analyte of interest, in our case cytokines or growth factors, is captured by an antibody which is covalently coupled to beads. Next, a biotinylated detection antibody is added, and finally, the bound components react with a complex formed by streptavidin and phycoerythrin giving a detectable fluorescence. This is the technique used in paper I when analyzing MMPs in supernatant from cultured HUVECs and in paper II when analyzing cytokines in supernatant and growth factors in plasma.

Aldehyde modification of proteins

Human collagen type IV was MDA-modified by addition of 50 mmol/L MDA in paper I and III. MDA-modification was verified by TBARS assay, which is based on the reaction with thiobarbituric acid, where two molecules of the acid react with one molecule of MDA which give a pink pigment measured at 535 nm.[341] This method is used to measure lipid oxidation and aldehyde breakdown products.[342] In addition, MDA-modification was verified by Western blots using anti-MDA antibody.

MGO modification was performed on ApoB100 peptides (paper IV) by addition of 100 mmol/L MGO. MGO is highly reactive and can generate adducts such as Nε-(carboxyethyl) lysine (CEL)[343] when modifying the lysine residues. To verify MGO-modification, CEL-epitopes were measured in ELISA using an anti-CEL antibody.

Immunohistochemistry

Immunohistochemistry is used to visualize cells or molecules present in tissue sections. In this technique embedded tissues are cut in 5-10 µm thick sections in a cryostat or by paraffin-sectioning. The sections are then incubated with a primary antibody recognizing the antigen, followed by a biotin-linked secondary antibody binding to the primary antibody. Bound antibodies are detected by enzyme-linked streptavidin and color is developed by the addition of a substrate to the enzyme. In paper II, immunohistochemistry was used to stain murine subvalvular plaques to detect smooth muscle cells and CD68-positive macrophages.

Flow cytometry

Flow cytometry can be used to distinguish different cell types based on their expression of markers, either on the surface or inside the cell (if the cell is permeabilized). Antibodies labelled with fluorochromes are bound to markers on the cells. Cells are then run one-by-one through the flow cytometer, where they are hit by laser beams, and light emitted from each fluorochrome is detected. By this method you can measure the expression of several different markers on a single cell, which can be used for identification and quantification of several different cell types in the same sample or for sorting out specific cell types.

Flow cytometry has been utilized in the paper II to quantify Tregs, $CD3^+CD8^+$ and $CD3^+CD4^+$, and in paper IV to sort out different B cell populations.

Proximity extension ligation

Proximity extension ligation is a technique developed by Olink Bioscience, and allows quantification of 92 proteins using only 1μL of biological material. A pair of antibodies binding to the target molecule are linked to DNA oligonucleotides. When the two antibodies bind close enough a DNA duplex will be formed, and quantification is performed using real time PCR.[344]

Inflammatory cytokines, smooth muscle cell growth factors and endothelial cell growth factors in paper I were measured by Olink (www.olink.com) using this method.

Objectives and Key findings

Paper I

Objective: To determine whether antibodies against MDA-collagen type IV predict risk of myocardial infarction.

Key findings: Individuals with high plasma levels of IgG antibodies against MDA-collagen type IV had an elevated risk of development of MI during a 13-year follow-up period (figure 9) after adjusting for Framingham risk factors and diabetes. Furthermore, MDA-collagen type IV IgG was associated with intima media thickness in the common carotid artery (CCA) as well as to MMP-10 and MMP-12.

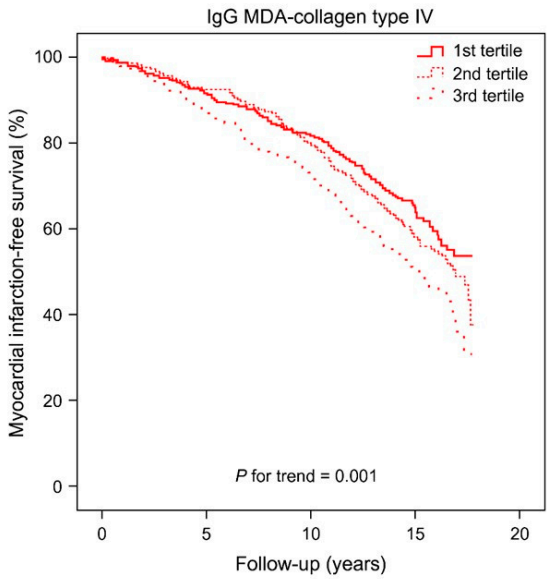

Figure 9. Kaplan-Meier curves for tertiles of MDA-collagen type IV IgG antibodies and myocardial infarction-free survival analyzed with long rank test for trend.

Paper II

Objective: To elucidate if immune responses against collagen type IV could contribute to vascular injury affecting the development of atherosclerosis in ApoE-deficient mice.

Key findings: ApoE-deficient mice immunized with collagen type IV α1α2 peptides developed IgG1 antibodies against collagen type IV (figure 10A, 10B), however atherosclerosis development was not affected (figure 10C).

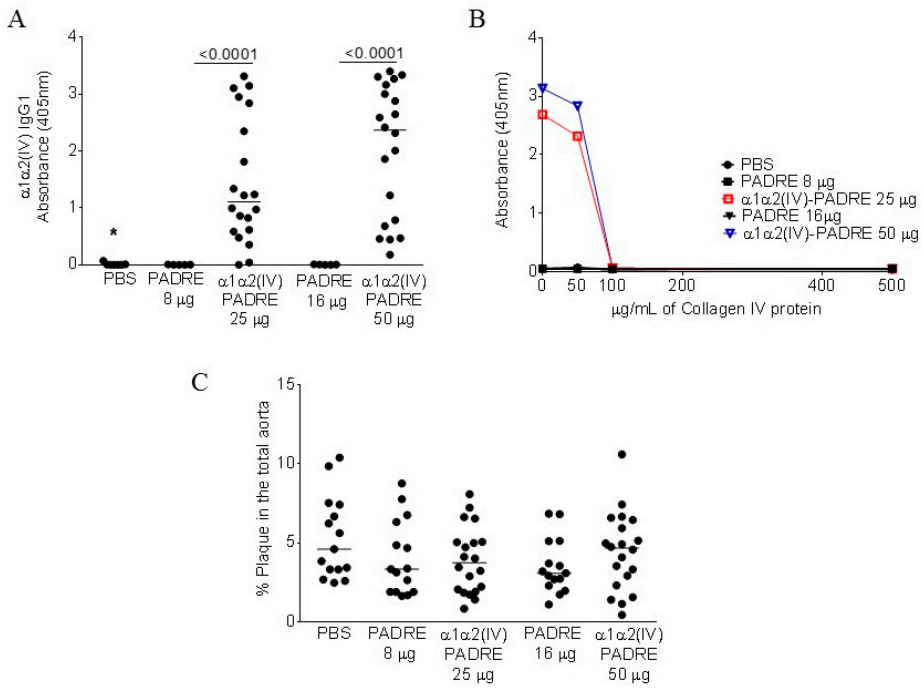

Figure 10. Immunization of ApoE$^{-/-}$ mice with collagen type IV α1α2 peptides coupled to PADRE induced an IgG1 response against α1α2 peptides(A). IgG1 binding to collagen type IV α1α2 peptides was competed with collagen type IV protein (B). Immunizations with collagen type IV α1α2 peptides did not affect atherosclerotic plaque area in the aorta (C).

Paper III

Objective: To investigate if antibodies against MDA-collagen type IV are related to development of cardiovascular events in subjects with T2D.

Key findings: Subjects with T2D had lower levels of IgM antibodies against MDA-collagen type IV compared to non-T2D subjects. Additionally, T2D subjects without clinically manifested CVD at baseline who developed a CV event during 36 months of follow-up had decreased levels of MDA-collagen IV IgM antibodies (figure 11). These antibodies were associated with IMT regression in the CCA.

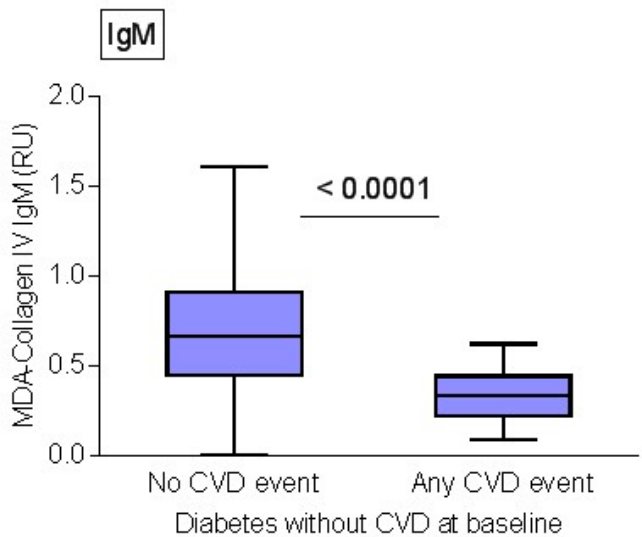

Figure 11. Subjects with T2D who developed CV events during follow-up, had lower levels of IgM antibodies against MDA-collagen type IV.

Paper IV

Objective: To analyze whether antibodies against MGO-modified epitopes of ApoB100 predict cardiovascular events.

Key Findings: MGO-modified peptide 220 in ApoB100 was identified as one of the major targets for antibodies. Subjects with low levels of MGO-p220 IgM had an increased risk to develop cardiovascular events during a 15-year-follow up period (figure 12A). Finally, IgM antibodies against MGO-p220 were produced by B-1 cells (figure 12B).

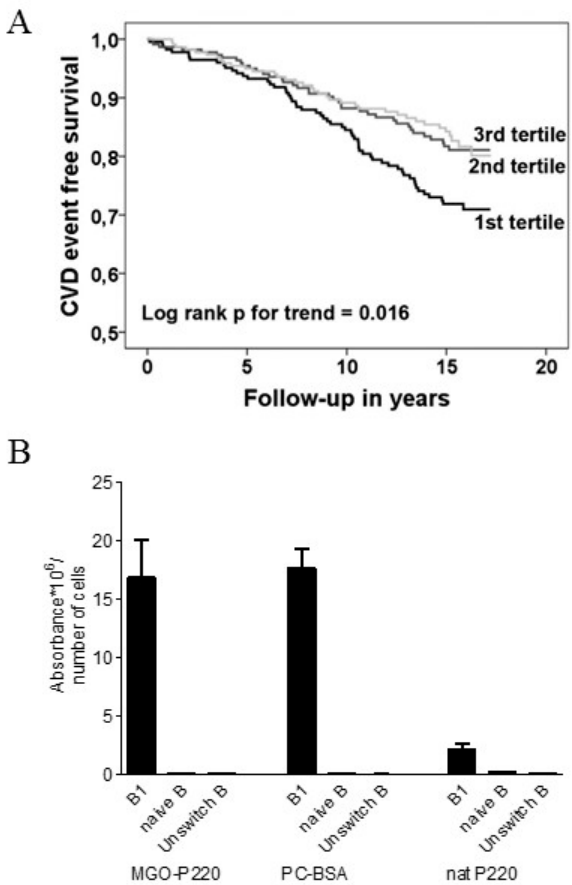

Figure 12. Kaplan-Meier survival curves for tertiles of MGO-p220 IgM and cardiovascular event-free survival (A). B-1 cells secrete IgM binding to MGO-p220 (B).

Results and Discussion

More and more evidences show that humoral and cellular immune responses are key factors in human atherosclerosis development. Antibodies against native or modified antigens such as phospholipids, heat shock protein, ApoA1, ApoB100 and many other antigens have been tested in human cohorts and animal studies. Some of them present protective effects whereas others are proatherogenic.[345]

Hereunder, I will discuss the results obtained in my four papers, where I have studied immune responses against two different aldehyde modified antigens; collagen type IV and ApoB100 peptide 220 and their possible implications in CVD with or without T2D and atherosclerotic plaque formation.

Immune responses against modified collagen type IV

Modifications of amino acids can alter protein structure, which in turn can affect both interactions between ECM proteins as well as protein-cell communication and biological processes such as cell growth, proliferation, migration, signalling or survival.[97] The ECM does not repair or prevent oxidative damage as good as cells do because it contains less antioxidant defenses and repair mechanisms. Although the consequences of this damage is not totally understood, it is believed to play a role in human pathologies.[120] ECM it is closely involved in CVD, and its damage can result in protein unfolding, increased permeability of the ECM as well as crosslinking of proteins form the ECM. Additionally, vascular oxidative stress was increased in patients from heart failure and is associated to lesion rupture.[309]

Antibodies against MDA-collagen type IV and cardiovascular events

Acute myocardial infarction is the most severe manifestation of coronary artery disease, and although lifestyle and therapies have reduced mortality, it is still affecting more than seven million individuals globally every year.[346]

During LDL oxidation, reactive aldehydes such as MDA, may be released into the intima modifying not only ApoB100 but also surrounding extracellular matrix proteins, as observed *in vitro*[99] (figure 13). Similar to oxLDL, these modified matrix

proteins may induce immune responses, and indeed, antibodies against MDA-matrix proteins are found in plasma.[99,311]

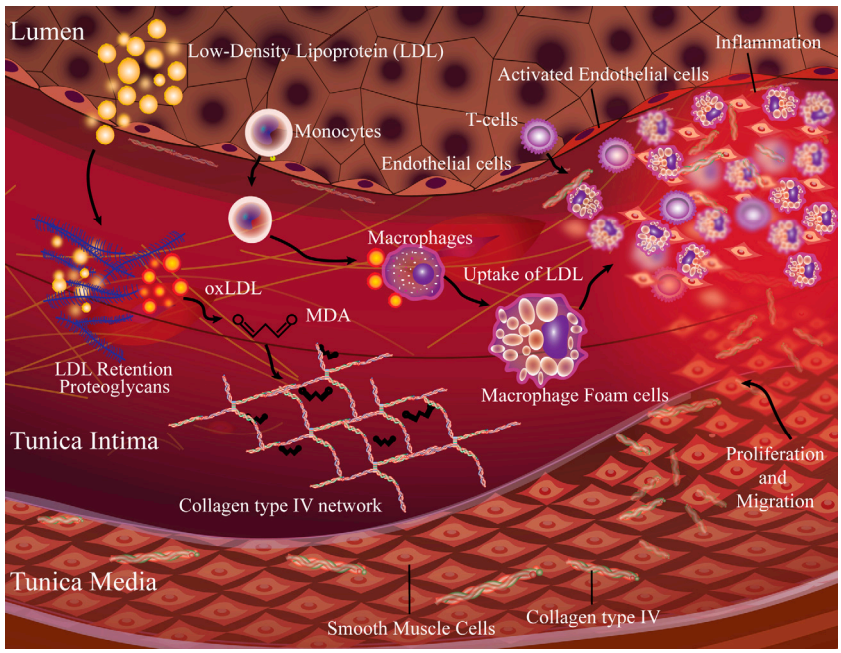

Figure 13. Schematic illustration of the interaction of MDA epitopes released from oxLDL with surrounding extracellular matrix components during atherosclerotic plaque formation.

MDA-collagen type IV is present in lipid rich regions of human plaques supporting the notion that LDL oxidation induces MDA modification of ECM proteins. Indeed, MDA-collagen type IV levels correlated to oxLDL in lesions.[105] Furthermore, MDA-collagen type IV was increased in plaques from patients with cerebrovascular symptoms compared to plaques from asymptomatic patients. Interestingly, antibodies against MDA-collagen type IV in plasma correlated to the amount of MDA-collagen type IV in lesions,[105] suggesting a possible association between antibodies MDA-collagen type IV and CV events.

In **paper I**, IgM and IgG antibodies against MDA-collagen type IV were measured in 795 subjects (385 MI cases and 410 controls) from the prospective Malmö Diet and Cancer (MDC) study to examine if these antibodies were associated to future MI during a 13-year-follow up period. Indeed, individuals with high levels of IgG antibodies against MDA-collagen type IV (third tertile) showed elevated risk to develop an MI over time compared to individuals with low levels of antibodies (first tertile) (HR 1.56 (1.22-2.00, P=0.0004). No associations were found for IgM. The association found between MDA-collagen type IV IgG and MI remained significant

after adjusting for Framingham risk factors (age, sex, total cholesterol, smoking, HDL, systolic blood pressure) and diabetes (HR 1.47 (1.12-1.93), P=0.005)

Next, antibodies against MDA-collagen type IV were correlated to atherosclerotic burden. In particular, IgG correlated positively to mean IMT in the common carotid artery (r=0.11, P=0.002), as well as to maximal IMT in the carotid bifurcation (r=0.12, P=0.01). In addition, IgM correlated positively to mean IMT in the common carotid artery (r=0.12, P=0.004), however no associations between IgM antibodies and future MI were found. IMT has previously been shown to predict CV events, including stroke.[347] Thus, it is surprising that both IgM and IgG correlated positively with IMT, but only IgG was associated with MI. The association of MDA-collagen type IV IgG with future MI may therefore not be explained by the correlation between MDA-collagen type IV IgG and IMT.

Collagen type IV interactions with cells are essential for cellular processes such as migration, survival, adhesion, proliferation or differentiation.[157] In fact, collagen type IV is found surrounding smooth muscle cells in the media and it is involved in their migration.[175] Collagen type IV is also found beneath endothelial cells. Moreover, aldehyde-modified collagen type IV reduces adhesion and anticoagulant properties on endothelial cells and also inhibits collagen type IV-cell interactions in diabetes.[104,105] Furthermore, antibodies against MDA-collagen type IV correlated to MMPs in plasma.[348] Based on this knowledge, in **paper I** associations between antibodies against MDA-collagen type IV and markers of inflammation, smooth muscle cell growth factors, endothelial cell growth factors, MMPs or protease inhibitors were analyzed. The result showed that IL-6 was associated to MDA-collagen type IV IgG (r=0.10, P=0.01) but not to any other inflammatory marker, suggesting that these antibodies do not reflect inflammation. Additionally, IgG antibodies were not correlated to smooth muscle cells or endothelial cells growth factors, but positive correlation was observed with MMP-10 (r=0.11, P=0.003) and MMP-12 (r=0.09, P=0.01). Overexpression of MMP-12 has previously been shown to promote inflammation and reduced collagen in hypercholesterolemic rabbits.[349] Additionally, MMP-12 deletion in mice is linked to more stable and smaller plaques as well as reduced elastin degradation[350] supporting the atherogenic role of this MMP. MMP-10 has also been proposed to be proatherogenic[48] due to its role in degradation of several ECM molecules.[351] Consequently, the positive correlation between IgG antibodies against MDA-collagen type IV and MMP-10 and MMP-12 may reflect a possible response to increased modification of collagen type IV, resulting in degradation of ECM and hence, plaque instability.

Some MMPs have dual effects, being able to either stabilize plaques or promote progression and development of atherosclerosis by degradation of matrix components.[350] For example MMP-3 is associated to CV events,[352,353] but has also been reported to stabilize plaques.[350,354] Likewise, MMP-1 is mostly present in stable

plaque phenotypes with thicker fibrous caps, and it is known to facilitate smooth muscle cell migration,[48,355] but has also been found to associate with CV events.[353] In the present study, no associations between MMP-1 and MDA-collagen type IV IgG were found, which is in accordance with a proatherogenic role of MDA-collagen type IV IgG.

Last, cathepsin D (r=0.11, P=0.002) and the cathepsin inhibitor cystatin B (r=0.13, P=0.0003) were associated to MDA-collagen type IV IgG. Cathepsin D is released by macrophages and it is found in elevated levels in atherosclerotic lesions compared to healthy vessels[356] increasing plaque vulnerability.[357]

The majority of modified LDL in the circulation produce immune complexes with antibodies, and they are associated to CV events[290] including MI.[291] These immune complexes present pro-inflammatory properties by binding to FCγ receptors and resulting in MMP release.[358,359] MDA-modification of the collagen type IV protein affects endothelial cell inflammation,[105] and in **paper I**, IgG antibodies against MDA-collagen type IV were correlated to MMP-10 and MMP-12. In order to investigate a possible functional role of complexes formed between MDA-collagen type IV and IgG antibodies, MMP secretion was measured from endothelial cells stimulated with different concentrations of anti-MDA bound to MDA-collagen type IV. MMP-1, MMP-2 and MMP-10 secretion were increased by stimulation with MDA-collagen type IV. Furthermore, MMP-1 was inhibited in a dose-dependent manner in the presence of anti-MDA antibody, whereas MMP-2 secretion increased with higher concentrations of anti-MDA. No differences were observed for MMP-10. These findings suggest that complexes formed between MDA-collagen type IV and antibodies can influence MMP secretion.

Do antibodies against collagen type IV have a functional role in atherosclerosis?

Based on the results obtained in **paper I**, we aimed to elucidate a possible functional role of antibodies targeting the basement membrane collagen type IV in atherosclerotic disease.

Immunizations of rats with peptides from collagen type IV α1 α2, present in the NC1 terminal of collagen type IV, were previously shown to generate antibodies against collagen type IV.[360] In **paper II**, these peptides were used for immunizations of atherosclerotic ApoE-deficient mice to assess the effect of an immune response against collagen type IV in atherosclerotic plaque development. $ApoE^{-/-}$ mice were immunized with two different concentrations (25 and 50μg/mL) of collagen type IV peptides coupled to PADRE to enhance immune responses.[328,361] Control mice were immunized with the corresponding concentrations (8 and 16μg/mL) of PADRE or PBS alone.

To measure humoral immune responses in mouse models, it is of a special interest to measure IgG immunoglobulins because isotype switching is a common rule for booster immunizations with protein antigens. In **paper II**, IgG1 was measured to analyze Th2 immune responses and IgG2c was used to analyze Th1 immune responses. The ApoE$^{-/-}$ mice used in our study have a C57BL/6 background, and it has been reported that some mice strains including the C57BL/6 strain, contain the IgG2c isotype gene rather than IgG2a, which is present in most mouse strains.[362] For this reason, IgG2c as indirect method to measure Th1 immune responses was used instead of IgG2a. In fact, there is a difference in the amino sequence of IgG2a and IgG2c of 16%, and studies have shown that levels of anti IgG2a do not correlate with levels of IgG2c in our strain mouse model, suggesting the results of the study could be influenced by using the correct isotype depending on the mouse strain.[362]

High IgG1 immune responses against collagen IV peptides were obtained in plasma from mice immunized with collagen type IV α1α2 peptides ($P<0.0001$). Furthermore, IgG1 antibodies against collagen type IV α1α2 peptides could be competed with collagen type IV protein. IgG1 immune responses against PADRE were observed in mice immunized with PADRE 16μg/mL ($P<0.0001$).

Collagen type IV is located in basement membranes, and oxidation or other types of modification of the ECM proteins in the basement membrane may impair endothelial cell attachment.[363,364] Therefore, it is possible that the antibodies against collagen α1α2 peptides produced upon immunization of ApoE$^{-/-}$ mice, would have an effect on endothelial cells apoptosis. In **paper II** we were not able to quantify endothelial cell apoptosis in the mice because most samples had lost the endothelial cell layer. In those samples where the layer was intact, we could not observe apoptosis (measured by TUNEL). However, we did not observe any differences in endothelial growth factors levels between the groups.

Surprisingly, mice immunized with PADRE presented an increase in the Th2 cytokine IL-4 compared to mice immunized with collagen type IV α1α2 peptides, suggesting an increased Th2 response in PADRE immunized mice.

In atherosclerotic mice, lesions develop at the different locations at various time points, e.g. the aortic root is the first site where lesions develop.[365] Experimental interventions may have different effects depending on the stage of the atherosclerotic plaque. Thus, an intervention that has its primary effect on early atherosclerotic processes may affect plaque size and contents in early lesions, but at later stages of the disease these effects may not be present. For this reason, it is important to measure atherosclerosis at different sites, as done in **paper II**. In our study, atherosclerotic development was measured in aortic root, aortic arch and descending aorta, although no differences were found at any of these sites.

In **paper I** we found an association between IgG immune responses against MDA-collagen type IV and myocardial infarction whereas in **paper II** generation of an IgG immune response against collagen type IV did not affect atherosclerosis development. Thus, the associations between antibodies against collagen type IV and MI found in humans do not seem to reflect a pathogenic role of these antibodies. However, we cannot exclude that antibodies generated against other epitopes of collagen type IV, native or MDA-modified, can affect atherosclerosis disease.

Antibodies against MDA modified collagen type IV and cardiovascular events in T2D

In **paper I** we showed that subjects with high levels of IgG antibodies against MDA-collagen type IV had an increased risk to develop MI. T2D is associated with increased arterial stiffness[366] and endothelial dysfunction.[367] MDA epitopes are elevated in subjects with diabetes and MDA has been proposed to play a role in arterial stiffness due to crosslinking of collagen.[103] In paper II, we therefore asked whether antibodies against MDA-collagen type IV could be of particular importance in T2D and associate with measurements of vascular changes observed in T2D.

In **paper III**, IgM and IgG antibodies against MDA-collagen type IV were measured in plasma from 1451 subjects from the SUMMIT cohort consisting of four groups: T2D with clinically manifest CVD, T2D without CVD, non-T2D with CVD and non-T2D without CVD. We observed that subjects with T2D had lower levels of IgM antibodies against MDA-collagen type IV ($P<0.000001$) compared to subjects without T2D, and MDA-collagen type IV IgM were also negatively associated with glucose, whereas no difference was found for IgG. These observations are supported by the results described in **paper I,** where MDA-collagen type IV IgM antibodies were inversely associated to body mass index (BMI) ($r=-0.09$, $P=0.01$), waist circumference ($r=-0.09$, $P=0.01$) and fasting blood glucose ($r=-0.13$, $P=0.0003$) in the MDC cohort, and decreased in individuals with metabolic syndrome ($P=0.001$).

Next, in **paper III** we investigated if subjects with T2D have an increased risk to develop a fatal or non-fatal CV event during 36 months of follow-up. A total of 154 CV events were recorded. Subjects with T2D and no CVD at baseline, that developed a CV event during the follow-up, had decreased levels of IgM antibodies against MDA-collagen type IV ($P<0.0001$) compared to individuals that did not suffer any CV event. The association remained significant ($P=0.005$) after adjusting for the United Kingdom Prospective Diabetes Study (UKPDS) risk factors (age, sex, duration of diabetes, current smokers, total cholesterol, HDL, HbA1c, systolic blood pressure and ethnicity[290]) and study center.

No correlations between measurements of vascular function and MDA collagen type IV IgM antibodies were found among subjects. However, in non-T2D subjects IgM antibodies were associated to pulse wave velocity (β=0.11, P=0.01) at baseline as well as IMT CCA regression (OR 2.30(1.22-4.33), P=0.01) during follow-up.

The main cause of arterial stiffness, assessed by pulse wave velocity, is thought to be due to structural changes in the extracellular matrix, such as degradation of elastin and crosslinking of fibrillar collagens.[368] Even though collagen type IV is a network forming collagen, it has been implicated in arterial stiffness. Collagen type IV α1 expression was associated to progression of pulse wave velocity in a cohort consisting of 121 women,[369] and the expression of collagen type IV α1 in arteries was increased in patients who suffered from high pulse wave velocity.[370] Further support for a role of collagen type IV in arterial stiffness came from a GWAS including 4221 subjects, where the SNP rs3742207 in COL4A1 gene was associated with pulse wave velocity.[35] The functional role of collagen type IV and arterial stiffness remains to be determined, although it has been speculated that the mutation in rs3742207 could result in increased MMP-2 mediated collagen type IV degradation, followed by increased SMC migration and deposition of ECM components in the intima, which may contribute to arterial stiffness.[35] Furthermore, the secretion of MMP-2 is regulated by collagen type IV *in vivo* and *in vitro*.[371] Interestingly, in **paper I** we found that endothelial cells stimulated with anti-MDA bound to MDA-collagen type IV increased their secretion of MMP-2. It may be that modifications of collagen type IV or immune responses against it affect arterial stiffness through MMP expression.

Altogether, the results from **paper III** show an association between MDA-collagen type IV IgM and T2D and suggest that these antibodies may have a protective role. These findings are in agreement with previous studies that showed that IgM antibodies against MDA-LDL or oxLDL are associated to less severe atherosclerosis.[290,372]

Contrary to MDA-collagen type IV IgM results, IgG against MDA-collagen type IV in non-T2D were positively associated to total plaque area (β=0.17, P=0.0001) and negatively associated to reactive hyperemia index (β=-0.16, P=0.0003) at baseline, indicating decreased endothelial function. It has been reported that aldehyde modifications of collagen type IV inhibit endothelial cell adhesion[104] and also that high levels of MDA-collagen type IV are present in symptomatic plaques, and may play a role in endothelial dysfunction.[105] It is possible that the association observed between MDA-collagen type IV IgG to endothelial dysfunction is indirectly reflecting the presence of MDA-collagen type IV in lesions.

To the same extent, subjects with T2D and elevated levels of MDA-collagen IV IgG, showed decreased regression of IMT in the bulb (OR 0.49(0.29-0.83), P=0.007) measured during the disease progression, indicating a possible atherogenic

role of these antibodies. This is in line with the results obtained in **paper I**, where elevated levels of IgG were associated to increased risk of future MI.

Immune responses against ApoB100 peptide

Antibodies against several native and modified ApoB100 peptides and their role in atherosclerosis have been studied previously.[47] Immune responses against advanced glycation end products (AGE)-modified LDL are associated to cardiovascular disease in subjects with diabetes[373–375] and AGE-LDL is increased in patients with diabetes.[376] As mentioned before, MGO is a reactive α-oxo-aldehyde, which is formed from glucose and give rise to AGE. However, α-oxo-aldehyde metabolites may be also formed during peroxidation of polyunsaturated fatty acids, suggesting that these aldehyde epitopes could be important also in subjects without T2D.[115,116]

In **paper IV**, we aimed to test if immune responses against MGO modified epitopes of the LDL-protein ApoB100 could predict cardiovascular events. In this study, we investigated baseline levels of anti-MGO p220 in 700 randomly selected individuals from the MDC cohort with a mean age of 65 years, and with a 15-year follow-up of CV events. A library constituted of 302 peptides, comprising the entire ApoB100, was screened for immune responses against MGO epitopes. Peptide 220 was chosen for further analysis based on two selection criteria: 1) at least a two-fold increased absorbance value compared to a peptide control and 2) an antibody response against modified to native peptide ratio above 2. In addition to peptide 220, peptide 211 in ApoB100 also fulfilled these criteria, but since p211 shares 5 amino acids with p210, which has been extensively studied (in native and modified form) in atherosclerosis and cardiovascular disease,[377–382] this was not included.

Subjects with CV events (n=139) during the follow-up had lower levels of MGO-p220 IgM antibodies, whereas no association was found for IgG. Individuals with low levels of IgM antibodies against MGO-p220 (first tertile) had increased risk to develop a CV event (HR 1.99 (1.17-2.57), P=0.010) during the follow-up compared to individuals with high levels of MGO-p220 IgM. This association remained significant after adjusting for cardiovascular risk factors (age, sex, diabetes, smoking, systolic blood pressure, LDL/HDL ratio, CRP, fasting glucose), treatment with blood pressure-lowering, lipid-lowering or anti-diabetic medication and prevalent CV events (HR 2.07 (1.22-3.50), P=0.004). High levels of MGO-ApoB100 IgM were previously shown to be associated with less coronary artery calcification in T2D.[383] To determine if the observations found in the present study were due to the subjects with diabetes (13% of the cohort), subjects with prevalent diabetes were excluded in the analysis. However, the associations between low

levels of MGO-p220 IgM and high risk for incident CV events remained significant (HR 2.51(1.37-4.61, P=0.002) even after removing subjects with prevalent diabetes.

Interestingly, the levels of IgM antibodies against MGO-p220 were inversely correlated to blood glucose (r=0.121, P<0.01), suggesting a possible relation to the amount of glycemic stress and MGO generation. It is possible that MGO present in lesions react with LDL generating MGO-p220 epitopes, and that antibodies against MGO-p220 therefore accumulate in lesions, and reduce the amount of antibodies available in plasma. This is supported by studies showing that AGE products are increased in subjects with diabetes.[384,385]

No association was found between MGO-p220 IgM and oxLDL, which indicate that the antibodies are primarily binding to structures arising from glycoxidation rather than lipid peroxidation.

MGO and MDA are two different type of aldehydes reacting with amino groups, and both can affect ApoB100 as well as other proteins in individuals with or without T2D, since it has been reported that MDA is higher in plasma from subjects with diabetes,[103] and MGO is involved in progression of atherosclerosis and plaque rupture also in subjects without T2D.[119] In **paper III** we studied MDA modification on the basement membrane protein collagen type IV, whereas in **paper IV**, we investigated the role of MGO-ApoB100 epitope. Even if the antigens were different, in both cases IgM antibodies were protective, predicting the risk of developing CV events. A possible explanation is that these modified antigens will be present in the lesion, influencing atherosclerosis formation, and IgM antibodies, generally believed to be antiatherogenic, will target these autoantigens trying to improve or recover an inflammatory situation. Antibodies binding to oxLDL inhibit uptake of oxLDL by scavenger receptors.[386,387] MGO-LDL have been shown to induce foam cell *in vitro* and it is possible that antibodies against MGO-p220 may reduce foam cell formation. [388,389]

Are MGO-p220 IgM natural antibodies?

A possible relationship between IL-5 secretion and natural antibodies has been described previously and it has been reported that IL-5 stimulates natural antibody secretion from mice. In fact, immunizations with MDA-LDL produced expansion of Th2 cells, which also resulted in an increase in IL-5. Additionally, IL-5 deficiency in mice accelerated atherosclerosis.[390] Human studies have also reported that high levels of IL-5 are related to reduced atherosclerosis, supporting the findings from mouse studies.[391]

In **paper IV** IgM antibodies against MGO-p220 were strongly associated to IL-5 indicating that these antibodies could be natural antibodies recognizing epitopes in modified LDL. To test if IgM against MGO-p220 were natural antibodies secreted

by B-1 cells, we purified different B cell populations from healthy donors and measured binding of secreted antibodies to MGO-p220. B-1 cells from three out of four donors presented elevated levels of IgM binding to MGO-p220, whereas antibodies from other B cell populations bound to MGO-p220 to a lesser extent or not at all. Although, we cannot say if antibodies against MGO-p220 have a protective role in cardiovascular disease or if they only are markers of disease, the results are in line with previous studies showing that depositions of IgM antibodies from B-1a cells in atherosclerotic plaques reduced the necrotic core.[245]

Different roles of IgM and IgG in atherosclerosis

Many studies have reported associations between IgM and IgG against different antigens and CVD, but the results are still conflicting. Different antibodies against the same antigen have conferred diverse results, suggesting the importance of, not just the target analyzed, but also the type of antibody investigated.

For example, there are disparity of results concerning the role of oxLDL as marker for CVD. Some studies have reported a positive association of oxLDL IgG to CVD[283,285,392] while others have not. Moreover, most reports agree that oxLDL IgM antibodies are antiatherogenic, but some studies have challenged these results with either opposite findings associating oxLDL IgM with carotid disease progression,[377] or no associations.[393]

These differences between studies may be due to that the oxidation of LDL generates several different epitopes binding to different antibodies. Indeed, variations in the levels of oxidation affect MDA-LDL preparations which may affect antibody measurements[97,394,395] and thus variations in results. In addition, differences may also be explained by the heterogeneity in the antigen binding site and the constant region of the immunoglobulins[345] as well as the specificity of the antigen. All these factors will influence or affect the binding or behaviour of the antibody when interacting with an antigen.

In spite of these conflicting data the general idea is that IgM antibodies against oxLDL or modified LDL are associated to less CVD,[283,285,372,392] but the role of IgG is not as clear, although most studies suggest a positive association with CVD. This is in line with the present findings, where IgM against MDA-collagen type IV (**paper III**) and IgM against MGO-p220 (**paper IV**) showed inverse associations to CVD risk, whereas IgG against MDA-collagen type IV was linked to increased risk of MI (**paper I**). In addition, high levels of collagen type IV IgG have been associated to complications in diabetes such as retinopathy,[396] which is also in line with our finding for MDA-collagen type IV IgG (**paper III**).

The possible functional role of antibodies in atherosclerosis has been extensively investigated in mouse models such as ApoE$^{-/-}$ or LDLr$^{-/-}$. Researchers have been looking for agreements or discrepancies of already published data on human studies where associations between IgG and IgM levels and CVD were found. It is of great importance to know the biology behind these antibodies as well as their mechanisms. Anti-oxLDL IgG seems to be proatherogenic in *in vitro* or *in vivo* studies affecting mechanisms such as endothelial apoptosis, inflammation or oxLDL uptake. On the other hand, anti-oxLDL IgM seems to be protective in *in vitro* or *in vivo* studies by for example blocking oxLDL uptake by macrophages.[230]

Investigations sometimes show consistency between human and mouse data. For example, human studies have demonstrated that antibodies against HSP60 are associated to atherosclerosis,[282] and experimental studies on ApoE$^{-/-}$ mice immunized with anti-IgG HSP60 antibodies resulted in acceleration of atherosclerosis.[397] Immunizations with AGE-LDL reduced atherosclerosis in diabetic mice,[398] and AGE-LDL or AGE-proteins are increased in subjects with T2D.[103,375] Moreover, IgG antibodies against ApoA1 are associated to CVD in humans.[399,400] ApoE$^{-/-}$ mice immunized with IgG antibodies against ApoA1 presented increased lesions in aortic roots.[401] Finally, immunization with MDA modified fibronectin, an ECM protein, reduced atherosclerotic lesion formation, and IgG against MDA-fibronectin was inversely associated with CVD events.[99,107] However, in other cases, associations of antibodies with CVD in human studies are not reflected by pro or antiatherogenic roles in mice.[230,402]

In our second study (**paper II**) we investigated whether immune responses against the basement membrane collagen type IV affect atherosclerosis. The result from **paper II** may indicate that the association of MDA-collagen type IV IgG to MI in **paper I** does not reflect a pathogenic role of these antibodies in plaque development. However, it is difficult to compare results between human and mice.

Limitations

First, studies **I**, **III** and **IV** are describing associations of antibodies against aldehyde-modified antigens and cardiovascular disease and do not prove causality of these antibodies. Thus, experimental studies are needed in order to assess and determine a possible protective role of IgM antibodies against MDA-collagen type IV or MGO-p220 or a possible proatherogenic role of IgG against MDA-collagen type IV. Second, no adjustments for multiple testing analyses of the cohorts have been done. Third, in paper **II** antibodies against collagen type IV did not affect atherosclerotic lesion development, indicating that antibodies against collagen type IV do not influence plaque development. However, we can not exclude that

antibodies generated against other epitopes of collagen type IV, native or MDA-modified, can affect atherosclerosis disease. It is also important to keep in mind that studies in mice may not reflect what is happening in humans. For example, antibodies generated by immunizations in mice are different from those present in humans,[373] and the lipoprotein metabolism may differ between species.[403] In addition, the most important locations of clinically manifested atherosclerosis in humans are the coronary arteries, which may lead to atherothrombotic events and consequent myocardial infarction.[44] However, coronary lesions are hard to be fast induced in $ApoE^{-/-}$ or $LDLr^{-/-}$ and usually myocardial infarction is performed either with surgery, with pharmaceutical administration or with specific mouse models.

Concluding remarks

Could analyses of plasma Ig for specific antigens be used clinically as biomarkers to improve prediction of cardiovascular risk? It is an interesting possibility because the measurement of Ig is simple, powerful and relatively cheap. But, which Ig subtype would be the most accurate one? Many studies along the years have analysed antibody levels against multiple and diverse antigens, with disperse results and conclusions. It seems difficult to agree on the clinical value of specific Ig measurements in the prediction of cardiovascular disease, since sometimes the results are controversial, and more studies are needed before a conclusion is reached.

It is also uncertain if the experiments performed on animal models can be extrapolated to human populations. Previous studies have investigated the role of modified ApoB100 peptides and modified ECM proteins in atherosclerosis and CVD, but the projects presented in this thesis are the first studies that analyzed immune responses against MDA-collagen type IV, and against MGO-p220 in a prospective cohort or in subjects with diabetes versus subjects without diabetes as well as a functional role of antibodies against collagen type IV in an atherosclerotic mouse model.

With that said, and to sum up the novelty and significance of this thesis, I have shown that:

→High levels of MDA-collagen type IV are associated with increased risk of myocardial infarction.

→Immune responses against collagen type IV α1α2 peptides do not affect atherosclerosis in mice.

→Individuals with type 2 diabetes without cardiovascular disease at baseline who suffered cardiovascular events during follow-up display low levels of IgM antibodies against MDA-collagen type IV.

→Low levels of MGO p220 IgM are associated with cardiovascular events in subjects without diabetes.

Further studies are need to investigate possible causalities of papers I, III and IV. However, in addition to CV risk factors, antibodies may be considered as potential biomarkers for CVD.

Future perspectives

There is still a lot of work to do in this area and more studies can be done for a further and better understanding of the role of antibodies against MDA-collagen type IV and MGO-p220.

Paper IV was performed in collaboration with Prof. Thomas Rothstein. Based on our findings, we would like to proceed with that collaboration and investigate if antibodies against MGO-p220 play a functional role in atherosclerosis. Our aim is to clone MGO-p220 specific B-1 cells and produce large amounts of IgM against MGO-p220. These antibodies will then be used for *in vitro* or *in vivo* studies, to determine their functional role for example by investigating their role in uptake of glycated LDL by macrophages, or cytokine production by macrophages. To be able to do that, we would have to sort out B cell populations binding to MGO-p220, run single cell PCR to clone heavy and light chain genes into a plasmid, which then could be transfected into HEK293 cells. Monoclonal secreted antibodies could then be collected and used in functional studies. This work is a tedious and long procedure that I had the opportunity to learn during my stay at Prof. Rothstein's lab in Western Michigan University, US; but which I could not apply in the project because of lack of time. Definitely, it would be something fun to work on. It seems promising!

Another interesting study in the future is based on our finding in paper III, where low levels of IgM antibodies against MDA-collagen type IV were associated to high risk of developing CV events within subjects with diabetes. It would be of interest to check for possible functional roles of these antibodies in diabetic mice. If we would be able to isolate and clone B cells expressing IgM against MDA-collagen type IV epitopes to expand these IgM antibodies against MDA-collagen type IV, they could be used for passive immunization into diabetic mice to study the effect on atherosclerosis development.

Systemic Lupus Erythematosus is associated with an increased risk for CVD.[404] Immune complexes are found in the glomeruli of SLE patients and MDA-collagen type IV has been found in kidney glomeruli in a rat model of nephritis.[405] Thus, it would be interesting to analyze antibodies against MDA-collagen type IV in SLE, and possible associations to CVD.

Collagen type IV is present in the basement membrane underlying endothelial cells, and it is possible that antibodies against MDA-collagen type IV may be of importance in plaque erosion. It would be interesting to measure antibodies against MDA-collagen type IV in a cohort consisting of plaque erosions and to compare it with a cohort containing ruptured plaques.

Ultimately, I hope the findings from this thesis will stimulate others to further investigate the possible role of antibodies against native or modified components of the basement membrane in cardiovascular disease and atherosclerosis.

Science for everyone

I know that this book might not be the typical reading material waiting for you on the nightstand after a long day at work, at least not for some of you. Indeed, it might not be a riveting or fun read for some of my family members and friends who are not "that much" into science. For them, and for anyone else who is not willing to read the entire book, but still wants to know what I have done and what has kept me "quite" busy over the last four years of my life, here comes a summary with the best of my intentions. Let us see after reading this section if you think that science can be fun. Ready?

To begin with, I think I should start clarifying two terms that sometimes are mixed up: arteriosclerosis and atherosclerosis. The first one is the result of loss of elasticity in the arteries and it happens with age, whereas in the second one, fatty deposits called plaques are formed in the arteries. The rupture of these plaques could result in a blood clot, which could block the arteries with fatal consequences. If the blocked artery supplies the heart, the consequence is a myocardial infarction (heart attack), but if the blocked artery supplies the brain, the result is a stroke. Some of the risk factors affecting atherosclerosis are well known like smoking, diabetes, hypertension, obesity or LDL. LDL is known as the "bad guy" and HDL as the "good guy".

I am sure everyone has heard about collagen, and probably has watched at least once (if not many times) the typical commercial on TV about how to keep our face without wrinkles or fine lines, and how important it is to regenerate collagens. But the functions of collagen are much more crucial than keeping a pretty face. In fact, collagens are the most abundant proteins in mammals and collagen type IV is the major component of basement membranes. It gives structure to the arteries and interacts with cells, primarily endothelial cells and smooth muscle cells, influencing their functions. These cells are involved in atherosclerosis development and plaque formation, and any type of change in collagen type IV might impede or affect the protein-cell binding resulting in impaired behaviour. During lipid (LDL) accumulation in the plaques, LDL gets oxidized by different factors, and reactive molecules such as malondihaldehyde (MDA) can be released and may, thereby, affect surrounding proteins. These proteins, once modified, may be targets of immune responses and antibodies can be generated. Antibodies (also called immunoglobulins, Ig) are proteins produced by the immune system. They are in

charge of recognizing molecules such as bacteria, viruses, chemicals, inflammatory molecules etc., in our body in order to neutralize them. Antibodies recognize a unique molecule (antigen) in each pathogen. There are five different types of antibodies that differ in structure and function.

In my thesis, I have studied the possible role of IgM and IgG antibodies against MDA-modified collagen type IV in atherosclerosis and cardiovascular disease. In paper I, we asked whether antibodies against MDA-collagen type IV predict risk for development of myocardial infarction. The levels of MDA-collagen type IV IgM and IgG in plasma (the remaining colorless part of the blood after cells and cellular components have been removed) were analyzed in a total of 795 subjects from the Malmö Diet and Cancer study. These subjects were followed for 13 years to see who developed myocardial infarction. It was found that MDA-collagen type IV IgG levels were higher in individuals who developed myocardial infarction during those 13 years than in controls. These immune responses may reflect LDL oxidation in the artery wall, but could also in themselves affect the atherosclerotic disease process.

Based on the previous paper where we demonstrated an association between high levels of antibodies against MDA-modified collagen type IV and the risk of development of myocardial infarction, we asked in paper II whether immune responses against collagen type IV could contribute to vascular injury and promote the development of atherosclerosis. To investigate this, we induced an antibody-response against collagen type IV in a typical mouse model used in atherosclerosis called apolipoprotein E (apo E)-deficient mice. These mice are genetically modified and they develop plaques faster than wild type mice when fed a high fat diet. Our findings demonstrate that presence of antibodies against collagen type IV does not affect atherosclerosis development in ApoE-deficient mice. This may suggest that the association between autoantibodies against collagen type IV and myocardial infarction found in humans does not reflect a pathogenic role of these antibodies.

Type 2 diabetes (T2D) is associated with accelerated atherosclerosis and increased risk for myocardial infarction. The molecular mechanisms behind why subjects with diabetes have a more aggressive atherosclerotic disease process are still not fully understood. Reactive compounds formed from either oxidation of lipids or glucose are increased in plasma from individuals with diabetes. In fact, MDA levels are also elevated in subjects with diabetes, suggesting that MDA can also be important within this disease. Autoimmune responses against modified proteins as well as modified LDL may therefore be of particular importance in diabetes.

In paper III we investigated if antibodies against the MDA-modified collagen type IV are linked to the development of cardiovascular events in T2D. Plasma levels of IgM and IgG against MDA-collagen type IV were measured in another study named SUMMIT, consisting of 1500 subjects. We found that subjects with T2D had lower

levels of IgM autoantibodies against MDA-collagen type IV compared to subjects without T2D. Furthermore, subjects with T2D who suffered from cardiovascular events during follow-up, presented lower levels of IgM against MDA-collagen type IV compared to those who remained event free.

Methylglyoxal (MGO) is another type of reactive molecule that is found in individuals with diabetes because it is derived from glucose, and glucose is elevated in diabetes. MGO can react with ApoB100, the protein part of LDL in humans. In study IV we analyzed whether antibodies against MGO-modified peptide from LDL predict cardiovascular events. Peptides comprising the entire ApoB100 protein (in total 302 peptides) were screened to identify peptides targeted by MGO specific antibodies. It was found that peptide 220 (p220) was the best candidate. IgM and IgG against MGO–p220 in apoB100 were measured in 700 individuals from the Malmö Diet and Cancer Study. It was showed that subjects with low levels of IgM recognizing MGO-p220 had an increased risk to develop cardiovascular events. In addition, we demonstrated that anti-MGO-p220 IgM is produced by B1 cells, which are known to be the source of natural IgM antibodies andconsidered protective in atherosclerosis.

In summary, in this thesis antibodies against MDA modified-collagen type IV as well as antibodies against MGO modified-ApoB100 peptide and their implications in atherosclerosis, cardiovascular disease and cardiovascular complications in diabetes have been investigated. Further studies are needed in order to see if any of these specific antibodies could be used as possible markers in atherosclerosis.

Populärvetenskaplig sammanfattning

Jag vet att denna bok inte är den typiska läsningen som väntar på dig på ditt nattduksbord efter en lång dag på jobbet, i alla fall inte för de flesta av er. Det är sannerligen inte en trevlig eller kul läsning för en del av min familj och vissa av mina vänner som inte är så intresserade av vetenskap. För dem, och för alla andra som inte vill läsa hela boken men fortfarande vill veta vad jag har gjort och vad som hållit mig ”rätt så” upptagen de senaste fyra åren av mitt liv, så har ni här en förhoppningsvis mer tillgänglig sammanfattning av min forskning. Låt oss se om du kanske tycker att vetenskap är roligt efter att du har läst detta avsnitt. Redo?

Åderförkalkning, eller åderförfettning, är en fettbeläggning som formas i artärerna och som kallas plack. När dessa beläggningar brister och frigörs i blodet flyter dem runt som blodproppar som kan blockera artärerna. Om de blockerade artärerna är de som levererar blod till hjärtat är konsekvensen en hjärtattack och om de blockerar artärerna som leder blod till hjärnan är resultatet en stroke. Några av riskfaktorerna för åderförfettning är välkända så som rökning, diabetes, hypertoni, fetma och LDL. LDL är känd som ”det onda” och HDL som ”det goda” kolesterolet.

Jag är säker på att alla har hört om kollagen och har förmodligen sett åtminstone en gång (om inte många gånger) den typiska reklamen på TV om hur man bibehåller ansiktet fri från rynkor och linjer, och hur viktigt det är att återbilda kollagen. Men kollagenets funktion är mycket viktigare än att behålla ett ansikte vackert. Faktum är att kollagen är de vanligaste proteinerna hos däggdjur och kollagen typ IV är huvudkomponenten av basalmembran. Det ger struktur till artärerna och interagerar med celler, främst endotelceller och glatta muskelceller, och påverkar deras funktioner. Dessa celler är involverade i utvecklingen av åderförfettning och bildningen av plack. Någon typ av förändring, modifiering, av kollagen typ IV kan utgöra hinder för eller påverka protein-cell bindningen vilket kan resultera i avvikande beteende. Under lipid (LDL) ackumuleringen i plack oxideras LDL av olika faktorer, och reaktiva molekyler så som malondihaldehyd (MDA) kan släppas ut och påverka proteinerna i omgivningen. Dessa modifierade proteiner kan orsaka ett immunsvar och generera antikroppar. Antikroppar (även kallad immunoglobuliner, Ig) är proteiner producerade av immunsystemet. De är ansvariga för att känna igen molekyler så som bakterier, virus, kemikalier, inflammatoriska molekyler osv i vår kropp för att neutralisera dem. Antikroppar känner igen en unik

molekyl (antigen) i varje patogen. Det finns fem olika typer av antikroppar som skiljer sig åt i struktur och funktion.

I min avhandling har jag studerat den möjliga rollen av IgM och IgG antikroppar mot MDA-modifierat kollagen typ IV i åderförfettning och kardiovaskulära sjukdomar. I artikel I frågade jag om antikroppar mot MDA-kollagen typ IV förutspår risker för utvecklingen av hjärtinfarkt. Plasma (den kvarvarande färglösa delen av blodet efter att celler och cellulära komponenter har tagits bort) nivåer från MDA-kollagen typ IV IgM och IgG har analyserats i totalt 795 individer från Malmö Kost Cancer studien där deltagarna följdes i 13 år för att se vilka som utvecklad hjärtattack. Vi såg att IgG nivåerna för MDA-kollagen typ IV var högre hos individer som fick en hjärtattack jämfört med dem i kontrollgruppen. Detta immunsvar skulle kunna reflektera LDL oxideringen i artärväggen, men själva immunsvaret skulle även kunna vara det som påverkar åderförfettningens sjukdomsprocess.

Baserat på vår tidigare artikel, där vi demonstrerade en koppling mellan höga nivåer av antikroppar mot MDA-modifierade kollagen typ IV och risken för utvecklingen av hjärtinfarkt, frågade vi i artikel II om immunsvar mot kollagen typ IV kunde bidra till vaskulär skada och främja utvecklingen av åderförfettning. För att undersöka detta inducerade vi en antikroppsrespons mot kollagen typ IV i en musmodell som vanligtvis används för åderförfettning. Dessa möss är genetiskt modifierade (saknar apolipoprotein E, ApoE) och utvecklar plack snabbare än vildtypsmöss när de får äta en diet rik på fett. Våra resultat visar att närvaron av antikroppar mot kollagen typ IV inte påverkar utvecklingen av åderförfettning i möss som saknar ApoE. Detta föreslår att kopplingen mellan autoantikroppar mot kollagen typ IV och hjärtinfarkt hos människor inte reflekterar en patologisk roll hos dessa antikroppar.

Typ 2 diabetes (T2D) är kopplat till accelererad åderförfettning och ökad risk för hjärtinfarkt. Den bakomliggande molekylära mekanismen till varför individer med diabetes har en mer aggressiv sjukdomsprocess är fortfarande inte helt förstådd. Reaktiva komponenter formade från oxidering av lipider eller glukos finns i högre nivåer i plasman från individer med diabetes. Faktum är att även MDA nivåerna är högre i individer med diabetes, vilket föreslår att MDA också kan vara viktig inom denna sjukdom. Autoimmuna svar mot modifierade proteiner och modifierad LDL kan därför vara av särskild vikt i diabetes.

I artikel III undersökte vi om antikroppar mot MDA-modifierat kollagen typ IV är länkad till utvecklingen av kardiovaskulär sjukdomi T2D. Plasmanivåer av IgM och IgG mot MDA-kollagen typ IV mättes i en annan studie kallad SUMMIT som inkluderar totalt 1500 deltagare. Vi såg att individer med T2D hade lägre nivåer av IgM antikroppar mot MDA-kollagen typ IV jämfört med personer utan T2D. Deltagarna med T2D som utvecklade kardiovaskulär sjukdom under uppföljningen

hade dessutom lägre nivåer av IgM mot MDA-kollagen typ IV jämfört med dem som förblev fria från kardiovaskulär sjukdom.

Metylglyoxal (MGO) är en annan typ av reaktiv molekyl som finns hos individer med diabetes eftersom det kommer från glukos, och glukos är förhöjd vid diabetes. MGO kan reagera med ApoB100, proteindelen av LDL i människor. I studie IV undersökte vi om antikroppar mot MGO-modifierade peptider från LDL kan förutsäga kardiovaskulär sjukdom. Peptider från det fullständiga ApoB100 proteinet (totalt 302 peptider) kartlades för att identifiera peptider som MGO specifika antikroppar är riktade mot. Vi fann att peptiden 220 (p220) var den bästa kandidaten. IgM och IgG mot MGO-p220 i ApoB100 mättes hos 700 individer från Malmö Kost Cancer Studien. Vi visade att deltagarna med låg nivå av IgM som kände igen MGO-p220 hade en ökad risk att utveckla kardiovaskulär sjukdom. Vi påvisade dessutom att anti-MGO-p220 IgM är producerad av B1 celler, som är kända för att vara källan till naturliga IgM antikroppar och anses vara skyddande vid åderförfettning. Sammanfattningsvis så har jag i denna avhandling studerat antikroppar mot MDA modifierat-kollagen typ IV såväl som antikroppar mot MGO modifierad-ApoB100 peptid och deras innebörd i åderförfettning, kardiovaskulär sjukdom och kardiovaskulära komplikationer i diabetes. Ytterligare studier behövs för att se om någon av dessa specifika antikroppar kan användas som en möjlig markör för åderförfettning.

Ciencia para todos ☺

Seguramente este libro no sea la típica lectura esperando en tu mesilla de noche después de un largo día de trabajo, o por lo menos, no creo que lo sea para muchos de vosotros. De hecho, puede no ser una lectura agradable o divertida para algunos miembros de mi familia o incluso para aquellos amigos alejados del mundo científico. Para ellos, y para todos aquellos que no deseen leer mi tesis al completo pero aún así quieran saber qué me ha mantenido ocupada durante estos cuatro años, aquí tenéis un resumen de mi investigación. Veamos si después de leer este capítulo os puedo convencer de que la ciencia también puede ser divertida. ¿Preparados?

Creo que debería empezar con dos términos que en ocasiones se usan indistintamente pero que no son sinónimos: arteriosclerosis y aterosclerosis. El primero de ellos es la pérdida de elasticidad de las arterias, producido generalmente por la edad; mientras que en el segundo se forman en las arterias depósitos de grasa llamados placas. La ruptura de estas placas puede generar coágulos y puede obstruir el flujo sanguíneo con consecuencias fatales. Si la arteria bloqueada lleva sangre hacia el corazón, podremos sufrir un infarto de miocardio (ataque al corazón), pero, por otro lado, si la arteria bloqueada riega el cerebro, podremos sufrir un ictus. Algunos de los factores de riesgo más conocidos que afectan a la aterosclerosis son fumar, la diabetes, la tension alta, la obesidad o los lípidos, LDL, entre otros. El LDL es conocido como el "malo de la película" mientras que HDL es considerado "el bueno". ¿Quién será "el feo"?

Estoy segura de que todo el mundo ha oído hablar del colágeno, y probablemente haya visto el típico anuncio de televisión sobre lo importante que es regenerar el colágeno para mantener nuestra cara perfecta y sin arrugas. Pero sus funciones son mucho más importantes que las de mantener una cara bonita. De hecho, el colágeno es la proteína más abundante en los mamíferos, y el número IV (colágeno IV), es el componente mayoritario de las membranas basales. Éste confiere estructura a las arterias e interactúa con las células, principalmente endoteliales y de músculo liso, afectando e interfiriendo en sus funciones. Estas células están relacionadas con el desarrollo de la aterosclerosis y la formación de placa, por lo que cualquier tipo de cambio en el colágeno IV podría dificultar o afectar la unión de proteínas a la célula y, como consequencia, afectar el comportamiento de estas células. Durante la acumulacón de lípidos (LDL) en las arterias, el LDL es oxidado por diferentes factores, pudiendo ser liberadas moléculas reactivas como el malondialdehído

(MDA) que podrían modificar las proteínas más cercanas. Estas proteínas, una vez modificadas, podrían ser objeto de respuestas immunes generando anticuerpos. Los anticuerpos (también llamados immunoglobulinas, Ig) son proteínas producidas por el sistema immunológico encargadas de reconocer moléculas extrañas en nuestro organismo y neutralizarlas. Los anticuerpos son capaces de reconocer una parte única (antígeno) de una molécula o patógeno. Existen cinco formas de anticuerpos que difieren tanto en estructura como en función.

En mi tesis, he estudiado la respuesta immunológica (IgM e IgG) frente al colágeno IV una vez ha sido modificado con MDA (MDA-colágeno IV), tanto en la aterosclerosis como en enfermedades cardiovasculares. En el estudio I, investigamos si los anticuerpos que reaccionan frente a MDA-colágeno IV predicen el riesgo de desarrollar un infarto de miocardio. Los niveles de IgM e IgG frente a MDA-colágeno IV han sido analizados en el plasma (la parte transparente de la sangre que queda una vez las células y los componentes celulares han sido eliminados) de 795 personas pertenecientes a un estudio llamado "Malmö Diet and Cancer Study". Estas personas fueron evaluadas durante 13 años para observar quienes sufrían algún infarto. Descubrimos que los niveles de IgG que reaccionan con MDA-colágeno eran más elevados en individuos que habían sufrido algún infarto de miocardio durante estos 13 años que en aquellas personas que no habían sufrido ninguno. Estas respuestas immunológicas podrían reflejar la oxidación del LDL en la pared arterial, que también podrían influenciar en el proceso aterosclerótico.

Basado en los resultados obtenidos en el estudio anterior, donde elevados niveles de anticuerpos estaban asociados con el riesgo de desarrollar infarto de miocardio, en el estudio II nos preguntamos si respuestas immunológicas contra colágeno IV podrían contribuir a producir daño vascular y promover el desarrollo de aterosclerosis. Para investigar esto, indujimos la producción de estos anticuerpos en un tipo de ratón usado normalmente en la investigación de aterosclerosis, el ApoE (ratón deficiente en apolipoproteína E). Este modelo de ratón está genéticamente modificado y es capaz de desarrollar placas mucho más rápido que cualquier otro ratón bajo una dieta alta en calorías. Nuestros resultados demuestran que la presencia de anticuerpos contra MDA-colágeno IV no afecta al desarrollo de la aterosclerosis. Esto sugiere que la asociación entre las respuestas immunológicas frente a MDA-colágeno IV y el infarto de miocardio en humanos no refleja un rol patogénico de estos anticuerpos.

La diabetes tipo 2 (T2D) está asociada con un desarrollo acelerado de aterosclerosis y un elevado riesgo de sufrir infarto de miocardio. Los mecanismos moleculares por los cuales las personas con diabetes presentan un desarrollo de aterosclerosis mucho más agresivo no se conocen completamente. Los compuestos reactivos que son formados durante la oxidación de lípidos o glucosa, pueden observarse en niveles

elevados en el plasma de personas diabéticas. De hecho, los niveles de MDA también son altos en personas con diabetes, sugiriendo que esta molécula puede ser también importante en esta enfermedad. Por consiguiente, las respuestas autoimmunes contra las proteínas modificadas así como el LDL modificado, podrían ser de especial importancia en la diabetes.

En el estudio III, investigamos si los anticuerpos frente a MDA-colágeno IV están relacionados con el desarrollo de problemas cardiovasculates en T2D. IgM e IgG frente a MDA-colágeno IV fueron analizados en el plasma de 1500 individuos pertenecientes a un estudio denominado SUMMIT. Encontramos que las personas con T2D presentaban niveles inferiores de anticuerpos IgM comparados con aquellos individuos que no presentaban T2D. Además, las personas con T2D que sufrieron problemas cardiovasculares durante el tiempo que duró el estudio, tenían los niveles más bajos.

El metilglioxal (MGO) es otro tipo de compuesto reactivo encontrado en grandes cantidades en personas con diabetes, porque se genera a partir de la glucosa, que como ya se sabe, se encuentra en niveles elevados en personas diabéticas. El MGO puede reaccionar con la proteína ApoB100, que forma parte del LDL en humanos. En el estudio IV, analizamos si se podrían predecir problemas cardiovasculares con anticuerpos frente a un péptido de esta proteína una vez modificada con MGO. La totalidad de la proteína ApoB100 (un total de 302 péptidos) fue analizada para identificar qué péptidos eran reconocidos por anticuerpos una vez habían sido MGO modificados. Entre todos, el péptido p220 fue el mejor candidato. Anticuerpos IgM e IgG frente a MGO-p220 fueron analizados en 700 individuos pertenecientes al estudio "Malmö Diet and Cancer Study". En este studio observamos cómo aquellos sujetos con niveles bajos de anticuerpos IgM contra MGO-p220 tienen mayor riesgo de desarrollar problemas cardiovasculares. Además, demostramos que estos anticuerpos son producidos por las células B-1, conocidas como las principales productoras de anticuerpos naturales, y con una función protectora en aterosclerosis.

En resumen, en esta tesis hemos investigado anticuerpos (IgM e IgG) frente a MDA-colágeno IV y MGO-p220 y sus implicaciones en la aterosclerosis, las enfermedades cardiovasculares, así como complicaciones vasculares en diabetes. Estudios adicionales son necesarios antes de poder concluir si alguno de estos anticuerpos pueden ser usados como posibles marcadores de aterosclerosis, siendo possible detectar sus niveles en plasma sanguíneo y asociarlos al riesgo de sufrir enfermedades cardiovasculares.

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