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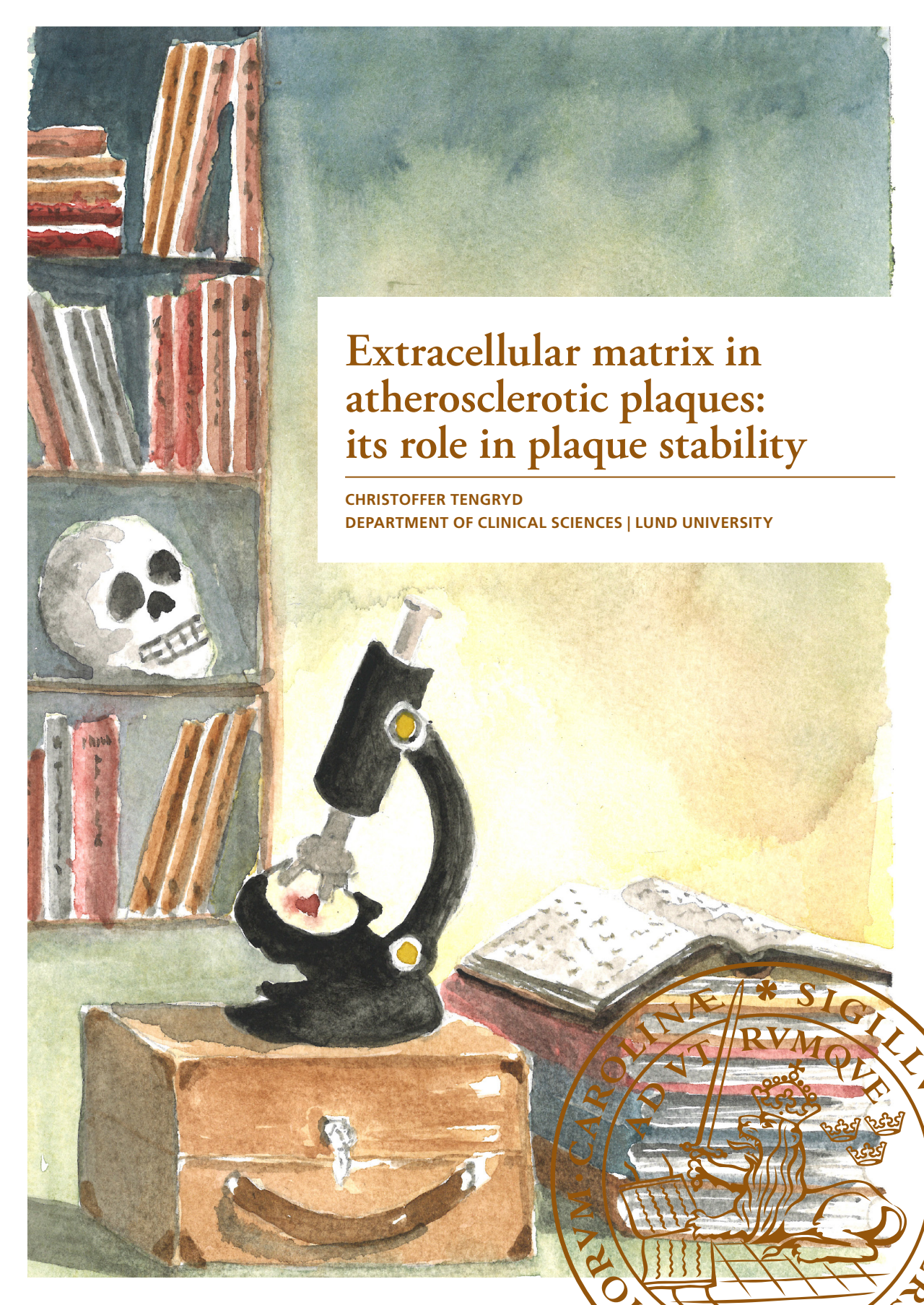
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A watercolor illustration of a laboratory or study. On the left, a bookshelf holds several books with red and brown spines. A human skull is placed on one of the shelves. In the center, a black microscope with yellow accents sits on a brown wooden box. To the right of the microscope, an open book with a detailed anatomical drawing of a human torso lies on a surface. The background is a soft, yellowish-green wash.

Extracellular matrix in atherosclerotic plaques: its role in plaque stability

CHRISTOFFER TENGRYD

DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY



Extracellular matrix in atherosclerotic plaques: its role in plaque stability

Christoffer Tengryd, MD



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DOCTORAL DISSERTATION

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Atherosclerosis is a systemic disease affecting the large arteries caused by the accumulation of lipids in the vessel wall. Cardiovascular disease due to atherosclerosis is today the most common cause of death globally through its end consequences: myocardial infarctions and stroke. The extracellular matrix (ECM) is a network of structural fibrous proteins, proteoglycans and glycoproteins. ECM proteins are involved in all steps of the disease progression from the initial lipoprotein accumulation to the failure of the fibrous cap causing clinical symptoms. It is therefore attractive to focus on these proteins to find novel ways of treating atherosclerosis. The basement membrane is a sheet like structure composed of ECM proteins underlying the endothelial cells and surrounding smooth muscle cells. The two major proteins in the basement membrane are the network forming collagen type IV and laminin. In the clinical context there is a great need of finding the patients that are at high risk of suffering from impending cardiovascular events, thereby qualifying them for a more intensive treatment. The ECM is continuously being degraded by matrix metalloproteinases (MMPs) and synthesized mostly by smooth muscle cells. The balance between these two processes are important for tissue homeostasis and stability in the atherosclerotic plaque. The purpose of this thesis was to explore the role of different parts of the ECM and their association to plaque vulnerability. We measured the the small leucine rich proteoglycans (SLRPs), neoepitopes of degraded ECM proteins and the three isoforms of transforming growth factor-β in human atherosclerotic plaque tissue. Plaque sections, homogenate and blood samples were obtained from the Carotid Plaque Imaging Project (CPIP). In this thesis three different SLRPs, fibromodulin, lumican and mimecan were examined by immunohistochemistry and their association to plaque components, vulnerable plaque features and their association with future cardiovascular events were explored. We also explored the association of serum levels of the degraded basement membrane proteins collagen type IV (C4M), laminin (LG1M) and collagen type I (C1M) and the risk of suffering from future cardiovascular events and death. Finally, we assessed the three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) in 223 plaques, to investigate if they were associated to a specific plaque phenotype and future cardiovascular events. Fibromodulin, lumican and mimecan were all expressed in the atherosclerotic plaque tissue and fibromodulin plaque area was greater in symptomatic and diabetic plaques and correlated to plaque levels of lipids and proinflammatory cytokines. Mimecan plaque area was associated with a vulnerable plaque phenotype, the proinflammatory cytokine MCP-1 and matrix degrading proteinase MMP-9. Larger plaque areas of mimecan were also associated with an increased risk for cardiovascular death. All three isoforms of TGF-β were detected in the human plaque tissue although TGF-β2 was present in much higher levels. TGF-β2 was also found to correlate to features of plaque stability and tissue remodelling. C4M, LG1M and C1M were in varying degrees independently associated with all-cause mortality, cardiovascular death and cardiovascular events. The regulation of ECM synthesis and degradation harbour the potential of novel and beneficial reparatory mechanisms that awaits to be discovered. The findings in this thesis may lead to the discovery of new mechanisms involved in vascular repair.		
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Christoffer Tengryd, MD



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To my family who endured

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Preface

-At last, the skies above are blue.

Etta James

At first sight, a thesis about the extracellular matrix in atherosclerotic plaques may seem boring. However, I hope that I can give you a glance into the fascinating world that have been my part time occupation for the last six years.

Atherosclerosis, the systemic process of formation of plaques in the arteries is the underlying cause of the majority of deaths and disability in the world today. A few of the mechanisms behind atherosclerosis such as the accumulation of lipids in the vessel wall and the aggravating effects of inflammation have been revealed. Despite these advancements and the new treatment options that have emerged in the last decades, the risk of suffering from stroke and myocardial infarctions remains high. The extracellular matrix is a network of secreted proteins surrounding cells in tissues, including in atherosclerotic plaques. The extracellular matrix proteins are not just important for structural stability of the tissue, but they also affect cellular behaviour and function, serve as a reservoir for cytokines and growth factors and they are therefore highly interesting targets when searching for novel mechanisms for making atherosclerotic plaques stable and less prone to cause clinical symptoms.

My hope is that the research conducted in this thesis has contributed to the collective knowledge or perhaps some time in the future will benefit patients suffering from atherosclerosis.

Original Papers

- I. Shami A, **Tengryd C**, Asciutto G, Bengtsson E, Nilsson J, Hultgårdh-Nilsson A, Gonçalves I. Expression of fibromodulin in carotid atherosclerotic plaques is associated with diabetes and cerebrovascular events. *Atherosclerosis*. 2015;241:701-8.
- II **Tengryd C**, Holm Nielsen S, Cavalera M, Bengtsson E , Genovese F, Karsdal M, Dunér P, Orho-Melander M, Nilsson J, Edsfeldt A and Gonçalves I. The proteoglycan mimecan is associated with carotid plaque vulnerability and increased risk of future cardiovascular death. *Atherosclerosis*. 2020;313:88-95.
- III Holm Nielsen S, **Tengryd C**, Edsfeldt A, Brix S, Genovese F, Bengtsson E, Karsdal M, Leeming DJ, Nilsson J, Goncalves I. Markers of Basement Membrane Remodeling Are Associated With Higher Mortality in Patients With Known Atherosclerosis. *J Am Heart Assoc*. 2018;7:e009193
- IV Holm Nielsen S, **Tengryd C**, Edsfeldt A, Brix S, Genovese F, Bengtsson E, Karsdal M, Leeming DJ, Nilsson J, Goncalves I. A biomarker of collagen type I degradation is associated with cardiovascular events and mortality in patients with atherosclerosis. *J Intern Med*. 2019;285:118-23
- V **Tengryd C**, Singh P, Cavalera M, Bengtsson E, Dunér P, Volkov P, Orho-Melander M, Nilsson J, Edsfeldt A, and Gonçalves I. Transforming growth factor- β 2 is associated with atherosclerotic plaque stability and lower risk for cardiovascular events. *Manuscript submitted*

Papers not included in this thesis

- Tomas L, Bengtsson E, Andersson L, Badn W, **Tengryd C**, Persson A, Edsfeldt A, Nilsson PM, Schiopu A, Nilsson J, Gonçalves I, Björkbacka H Low Levels of CD4⁺CD28^{null} T Cells at Baseline Are Associated With First-Time Coronary Events in a Prospective Population-Based Case-Control Cohort. *Arterioscler Thromb Vasc Biol.* 2020;40:426-436.
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- Chen Y, Hultgårdh Nilsson A, Goncalves I, Edsfeldt A, Engström G, Melander O, Orho-Melander M, Rauch U, **Tengryd C**, M Venuraju S, Lahiri A, Liang C, Nilsson J Evidence for a protective role of placental growth factor in cardiovascular disease. Accepted in *Science Transl Med.*

Abbreviations

ECM	Extracellular matrix
CV	Cardiovascular
SLRP	Small leucin rich proteoglycan
TGF- β	Transforming growth factor β
CPIP	Carotid Plaque Imaging Project
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinases
TIMP	Tissue inhibitors of matrix metalloproteinase
IL	Interleukin
DM	Diabetes mellitus
GAG	Glycosaminoglycan
SMC	Smooth muscle cell
EC	Endothelial cell
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PMA	Phorbol 12-myristate 13-acetate
HR	Hazard ratio
CHD	Coronary heart disease
THP-1	Tamm-Horsfall protein 1
LPS	Lipopolysaccharide
MIP-1 β	Macrophage inflammatory protein -1 β
PCI	Percutaneous coronary intervention
eGFR	Estimated glomerular filtration ration
DDR	Discoidin domain receptor
CKD	Chronic kidney disease
ESRD	End stage renal disease
CAD	Coronary artery disease

MI	Myocardial infarction
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
CTGF	connective tissue growth factor
CABG	Coronary artery bypass surgery
LDL	Low density lipoprotein
HDL	High density lipoprotein
TG	Triglycerides
FHS	Framingham heart study
CRP	C-reactive protein

Introduction

"Arteriosclerosis is a chronic disease of the large arteries which is characterized by focal thickenings of the intima, consisting of connective tissue and containing more or less lipids often complicated with calcification and ulceration."

-Nikolai Anichkov, 1934

The word atherosclerosis is derived from the Greek words *ἀθήρα* (*athéra*) and *σκληρώσις* (*sklerosis*), which translates into gruel and hardening. Atherosclerosis is a systemic disease afflicting the arteries with thickenings consisting of accumulated lipids, extracellular matrix (ECM), dead cells, inflammatory cells, debris and calcium. Atherosclerosis is not evenly distributed in the arterial tree as certain arteries are more prone to develop plaques, such as the large and medium sized carotid, coronary, femoral and iliac arteries. Atherosclerosis has been described as consequence of human longevity since ancient times both in ancient Egyptian and hunter gatherer societies¹.

In the last two centuries, the rise of western society associated with unhealthy diet, smoking, sedentary lifestyle and longer lifespan have made atherosclerotic cardiovascular disease (CVD) and some of its end consequences, ischemic heart disease and ischemic stroke, to the most common causes of death globally^{2, 3}. However in the last decades the mortality from cardiovascular disease have decreased^{2, 4} attributed to advancements in both preventive treatments such as cholesterol lowering medication, changes in behavioural risk factors and improvements in acute medical care^{5, 6}. Despite these improvements, CVD remains the most common cause of death worldwide with nearly 80% of CVD death occurring in low and middle income countries^{7, 8}.

In this chapter I will describe the background regarding atherosclerosis formation and its known causes and thereafter more in detail describe the ECM and how it is involved in the atherosclerotic process.

Risk factors for developing atherosclerosis

The German pathologist Adolf Windaus discovered already 110 years ago that the plaque in the aorta contained increased amounts of cholesterol and a few years later the Russian pathologist Nikolai Anichkov conducted experiments where he fed rabbits with a high cholesterol diet and subsequently noticed increased plaque formation^{9, 10}. In the wake of the epidemic growth of coronary heart disease (CHD) and the death of president Roosevelt in hypertensive heart disease and stroke, the Framingham Heart Study (FHS) was established in 1948, being an observational prospective cohort created to evaluate which factors that play a role in the development of CVD¹¹. Thanks to the FHS and several other epidemiological and autopsy studies there are today several well described factors important for developing atherosclerotic lesions, usually called risk factors¹²⁻¹⁵.

These factors can be divided into modifiable risk factors that can be affected by medications or lifestyle changes (circulating lipoproteins, smoking, hypertension and diabetes) and risk factors that are non-modifiable (age, ethnicity, sex and family history of CVD)¹⁶. Intake of alcohol has mostly been associated with reduced risk of CV events and a stable plaque phenotype, albeit heavy alcohol consumption is associated with deleterious effect on CVD¹⁷⁻¹⁹. The association of diabetes with increased occurrence of CVD has been seen in autopsy and hospital-based studies since the first half of the 19th century^{20, 21}. Hypertensions increase the risk for developing both coronary heart disease and stroke²². Inhalation of smoke through cigarettes or cigars is a known risk factor for developing atherosclerosis²³ and smoking cessation reduces mortality risk for non-fatal re-infarction in patients with coronary heart disease²⁴. Women before menopause have a lower risk of coronary heart disease (CHD) compared to men¹⁵. The manifestations of CVD differ in the fact that plaque rupture is a more common cause of coronary thrombosis in men (80%) compared to women (60%)²⁵. Several other diseases with autoimmune aetiologies such as systemic lupus erythematosus and rheumatoid arthritis have also been associated with accelerated atherosclerosis²⁶. Other risk factors for CVD which are not taken into consideration in this thesis are e.g. pollution, socioeconomic factors, level of education, sedentary life style and nutrition patterns.

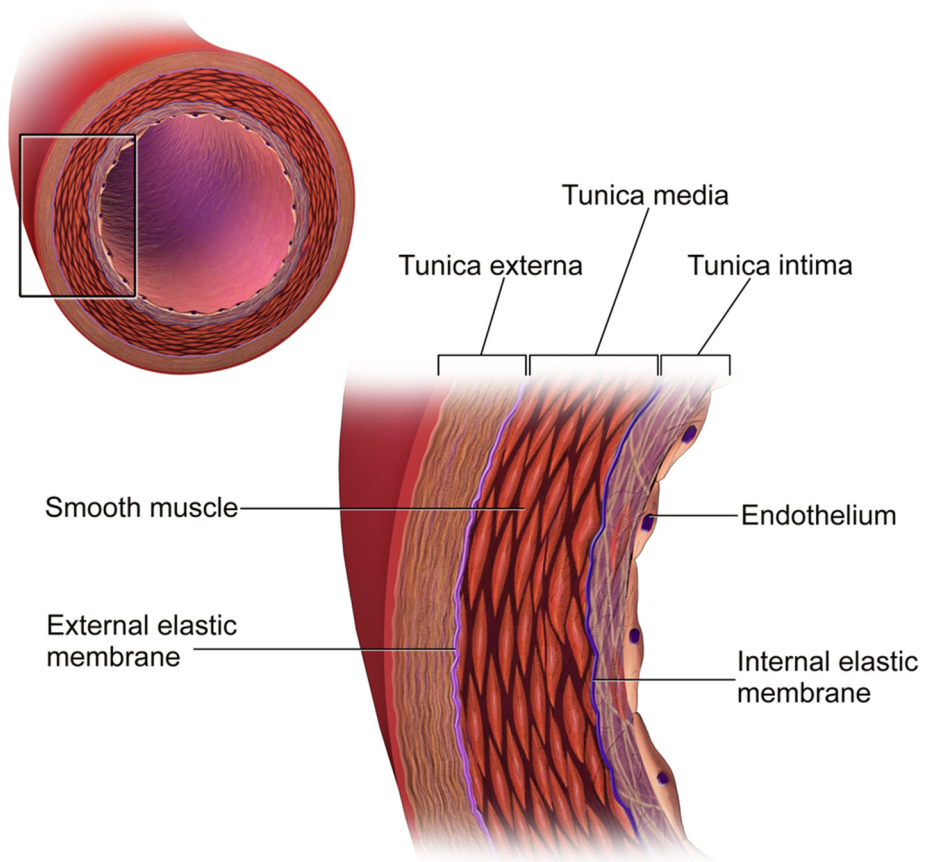


Figure 1

Illustration of an artery unaffected of atherosclerosis visualizing the different layer present. Image courtesy of Blausen27.

The healthy artery

The arteries in the human body have the function of transporting blood rich in oxygen and nutrients to all tissues and organs, whereas the veins transport blood with waste products from the tissues and organs. The blood is pumped from the heart via the aorta to the arteries in a high-pressure system and returns to the heart via the low pressure venous system. The arterial and venous vessels are therefore differently composed and this thesis will focus on atherosclerotic disease, which affects the arteries. The vessel wall of the arteries is organised in different layers separated by layers of elastic laminae. The layer closest to the lumen is called the

intima and consist of a single layer of endothelial cells (ECs) resting upon a basement membrane composed of ECM proteins (Fig 1). Underneath the basement membrane there is a proteoglycan rich subendothelial space is found. The middle layer, which is separated from the intima by the internal elastic laminae, is called the media and consists of several layers of smooth muscle cells (SMC) surrounded by a basement membrane of collagen and elastic fibers, important for tensile strength and adaptation to variations in blood flow. The outermost layer, which is separated from the middle layer by the external elastic laminae, is called adventitia and consists mostly of collagen, fibroblasts and smaller vessels called vasa vasorum supplying nutrients to the vessel wall itself.

Lipoproteins and cholesterol transportation

In the early 19th century the Russian pathologist Nikolaj Anitschkow investigated the role of cholesterol in experimental atherosclerosis by feeding rabbits with a high cholesterol diet inducing plaque formation^{10, 28}. Cholesterol is essential for the human body as a component of cell membranes and a precursor for vitamin D, bile acids and steroid hormones including androgens, estrogens, progestogens, mineralocorticoids and glucocorticoids²⁹. Atherosclerosis is initiated by accumulation of cholesterol in the vessel wall which will be covered more in detail in later chapters. Cholesterol and triglycerides are hydrophobic molecules which are not soluble in water and therefore they need to be transported in the circulation by spheroid molecular complexes called lipoproteins. Lipoproteins were first discovered and isolated in 1950 by Gofman et al³⁰ and they are recognized as key molecules in the formation of atherosclerotic lesions³¹. The lipoproteins consist of a hydrophilic outer layer of phospholipids, free cholesterol and apolipoproteins surrounding a hydrophobic core of phospholipids, cholesteryl esters, triglycerides and fat soluble antioxidants and vitamins.

There are several different lipoproteins: very low density lipoprotein (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), high density lipoproteins (HDL), triglycerides (TG) and chylomicrons with different functions in cholesterol transport. Chylomicrons have a truncated version of the ApoB100 protein named ApoB48 representing the initial 48% of the N-terminal amino acids³². The chylomicrons transport lipids taken up in the intestine and deliver them to tissues where the lipids are hydrolysed by lipoprotein lipase into free fatty acids utilized by the tissues. VLDLs loaded with TG and cholesterol are formed in the liver and deliver fatty acids to peripheral tissues. The remnants of VLDL are called IDL and they are hydrolysed by hepatic lipase forming LDL. LDL is endocytosed by peripheral cells and hepatocytes by the LDL receptor (LDLR). HDL is central for reverse cholesterol transport where excess cholesterol is removed from

peripheral tissue. Plasma levels of cholesterol and LDL are associated with an increased risk for CVD, whereas plasma levels HDL are inversely related to the risk of CVD^{33, 34}. Patients with genetic defects of the cholesterol transport system such as familial hypercholesterolemia accumulate lipids in several tissues and develop advanced atherosclerotic plaques early in life²⁵.

Plaque development

The initiation of atherosclerosis

It is known that early atherosclerotic lesions start to develop already in childhood^{23, 35, 36}. However, it is not until later in life, around the fourth decade of life, that the lesions usually start to cause symptoms. In 1995 Stary *et al* published a definition for classification of atherosclerotic lesion using histological findings³⁷. Lesions are divided into different stages from lesion type I to lesion type VI.

Table 1. Classification of atherosclerotic lesions. Adapted from Stary et al.³⁷

Lesion Type	Histological features	Clinical features	Possible onset	Possible growth mechanism
Type I	Macrophages, lipids and a few foam cells	No clinical symptoms	First decade	Lipid accumulation
Type II (fatty streak)	Mainly intracellular lipid accumulation			
Type III	Intracellular lipids, foam cells, macrophage accumulation and small extracellular lipid pools		Third decade	
Type IV	Intracellular lipids, foam cells, macrophages and a core of extracellular lipids	May develop clinical symptoms		
Type V	Lipid core and fibrotic cap, mainly fibrotic or mainly calcific		Fourth decade	SCMs and collagen
Type VI	Defect endothelium and/or thrombus and/or hemorrhage/hematoma			Thrombosis, intraplaque hematoma

Thickenings of the intima arise in areas with low shear stress or with turbulent blood flow such as in bifurcations of arteries³⁶. In these regions, circulating particles are in prolonged contact with the endothelium which will cause increased deposition of lipoproteins in the intima³⁸. According to *the response-to-retention theory* the initiating factor in the atherosclerotic process is the retention of atherogenic

lipoproteins such as low density lipoproteins (LDL) in the subendothelial space³⁹. LDL particles are retained by attaching to ECM molecules such as proteoglycans. Eventually the LDL particles may become modified by oxidation and are therefore no longer recognized and removed by the classical LDL receptor mediated endocytosis. The oxidised LDL particles are instead taken up by scavenger receptors on either resident macrophages in the arterial wall or blood monocytes that have entered the arterial wall⁴⁰. Blood derived monocytes recruited to the intima engulf the lipoproteins and accumulate the lipids in lipid droplets giving them a foamy appearance. These *foam cells* give rise to *fatty streaks* in the intima constituting the earliest phase of atherosclerosis. The foam cells increase progressively, forming larger lipid pools and subsequently a lipid core. As the plaque evolves, smooth muscle cells (SMC) migrate from the media to the intima and switch phenotype from a quiescent differentiated SMC to a synthetic phenotype producing ECM proteins, which affect plaque composition. A subgroup of SMCs also contain lipid droplets and have a foam cell appearance⁴¹. As the plaque progresses further into more advanced lesion types, intraplaque hemorrhage may occur and the acellular necrotic core, filled with dead cells and debris, will start to develop⁴².

A competing theory of the initiation of atherosclerotic plaque progression is the *response-to-injury* theory in which the denudation, injury or activation of the endothelium is the initiating factor of atherosclerosis⁴³. In the *response-to-injury* theory which was put forward by Ross et al, the main culprit behind the expansion of plaques was the expansion of SMCs in the subendothelial space caused by the injured endothelium⁴⁴. The endothelium regulates several functions in the vessels including vasodilation, vasoconstriction, regulation of SMC and inflammatory cells⁴⁵. A defective endothelium has been proposed to exacerbate atherosclerosis by increasing endothelial permeability, aggregation of platelets, adhesion of leukocytes and increased generation of cytokines^{46, 47}. The damaged endothelium has also been proposed to be a factor leading to thrombosis caused by plaque erosion⁴⁸.

The role of the immune system and inflammation

The immune system is a complex organisation of cells and signalling molecules designed to protect the organism in which it resides. The immune system is generally divided into two large parts, the innate and the adaptive immune systems which both have been implied to have roles in the atherosclerotic process^{49, 50}.

Inflammation is a reaction of the immune system to an injury or external pathogen. The German pathologist Rudolf Virchow described atheromatous vascular lesions already in 1856 and was the first to implicate inflammation in the pathological process of atherosclerosis⁵¹. However, it not until the end of the 20th century it became clear that atherosclerosis is a chronic inflammatory disease⁵².

Macrophages are important for plaque initiation and progression of atherosclerosis and the formation of foam cells is a hallmark of atherosclerosis⁵³. Except for macrophages, a wide range of immune cells have roles in the progression of atherosclerotic lesions⁵⁰. Several different types of T-cells are present in atherosclerotic plaques and recognize and react to antigens such as oxidised LDL, heat-shock protein 60 and chlamydia pneumonia⁵⁴⁻⁵⁶.

Intraplaque hemorrhage

The cells in the plaque get nutrients and oxygen supply from the luminal blood and vessels in the adventitial layer⁵⁷. As the plaque expands and grows there is increased need for oxygen, especially in the core⁵⁸. Together with inflammatory cell infiltration this promotes neovascularization (the formation of new blood vessels). The new vessels formed are often immature and leaky with a compromised endothelial structural integrity which lead to an increased extravasation of erythrocytes and immune cells from the lumen to the plaque^{59, 60}. This intraplaque hemorrhage has been identified as a driving force of plaque expansion by the deposition of erythrocytes contributing to necrotic core enlargement. Intraplaque hemorrhage is also a marker of plaque vulnerability⁶¹

Plaque calcification

Calcifications are common in atherosclerotic plaques and are found in varying sizes from large noduli to smaller microcalcifications⁶². The presence of calcification in the coronary arteries is used as a marker of total atherosclerotic burden measured using coronary computed tomography^{63, 64}. It is associated with other risk factors for atherosclerosis such as kidney failure, ageing, diabetes mellitus, smoking, hypercholesterolemia, osteoporosis, obesity, menopause and lack of physical exercise⁶⁵. The process of arterial calcification was previously viewed as a passive degenerative effect of ageing. However, it has recently been shown that the calcification process in arteries is an active cellular biomineralization process^{65, 66}. The most predominant calcium salt in atherosclerotic lesions is hydroxyapatite^{67, 68} and carotid plaques from symptomatic patients have lower levels of hydroxyapatite compared to plaques from asymptomatic patients⁶⁹. This indicates that plaque calcification might be atheroprotective in some cases, possibly depending of the location of the calcification.

Smooth muscle cells

SMCs in healthy arteries are quiescent cells important for maintaining normal vessel physiology and structure. These medial quiescent SMCs express several contractile

proteins including smooth muscle cell specific α -actin, myosin heavy chain, SM-22 α and calponin^{70, 71}. Proliferation of vascular SMCs was historically believed to be the major cause of plaque growth. Yet, today this notion is more correct in the pathogenesis of the neointimal hyperplasia seen after invasive treatment of atherosclerotic lesions⁷². Vascular SMCs have great plasticity and are affected by signalling from surrounding cells in the intima and adventitia⁷³. The SMCs in the plaque change phenotype from a contractile to a synthetic phenotype when migrating into the intima during plaque progression. The SMCs synthesize ECM proteins that form the fibrous cap, crucial to shield the prothrombotic necrotic core from getting in contact with the blood in the lumen⁷⁴. Apoptosis or cellular death of SMCs is a feature of advanced atherosclerotic plaques⁷⁵. Apoptosis of SMCs have been shown to be associated with increased plaque vulnerability in mice^{76, 77}.

Reports indicate that not all SMCs in the intima originate from the media. A portion of SMCs derived from circulating progenitor SMCs in the circulation have been suggested to be originating from the bone marrow⁷⁸. However this has been questioned by bone marrow transplant studies indicating that the vast majority of SMCs are locally derived⁷⁹.



Figure 2.
Three sections of a human carotid atherosclerotic plaque.

Plaque vulnerability

Early pathology studies indicated that plaque with a large necrotic core, thin fibrous cap and increased plaque inflammation constitute a vulnerable plaque⁸⁰. Autopsy studies have shown that the majority of cardiovascular events are caused by plaque ruptures^{62, 81}. A plaque rupture is thought to be caused by enzymatic degradation of the fibrous cap caused by matrix metalloproteinases and an increased inflammatory activity. Two processes which are commonly identified in the shoulder regions of the plaque⁸². Several morphologic features have been associated with high risk plaques including, a thin fibrous cap and a large lipid core, endothelial denudation and intraplaque hemorrhage. These plaques are commonly termed thin-cap fibroatheromas (TCFAs)^{81, 83}.

Invasive imaging techniques such as near infrared spectroscopy (NIRS) and intravascular ultrasound imaging (IVUS) have been developed to detect these vulnerable TCFA. However only a small percentage of all identified potential vulnerably TCFA become symptomatic during follow up. In a study of 697 patients suffering from acute myocardial infarction (MI), the patients underwent angiography and IVUS after percutaneous coronary intervention (PCI) treatment and then they were subsequently followed up for three years. After 3 years 20.4% of patients had suffered from a new major adverse cardiovascular event (MACE) and the treated culprit lesions were responsible for half of the new events and the other half were due to non-treated lesions, indicating the need for improved methods to identify and to treat “at-risk” lesions⁸⁴. Ultrasonography of the carotid arteries are used in the clinic today (further discussed below). Coronary computed tomography angiography are used to discern coronary plaque features such as outward-remodelling, low attenuation, napkin ring sign and spotty calcifications⁸⁵. Another imagining modality for detecting lesions at high risk of symptoms, is magnetic resonance imaging (MRI) in which plaque characteristics can be detected non-invasively without radiation exposure⁸⁶.

To assess carotid atherosclerotic plaque burden and degree of stenosis Doppler ultrasonography is the most commonly used method in the clinic today (further discussed below).

Plaque erosion

Studies of autopsies of patients who died of sudden coronary deaths indicate that not all plaques show signs of fibrous cap rupture⁸⁷. A substantial portion of culprit plaques are instead rich in SMCs and proteoglycans and have less inflammatory cells. This subtype of culprit plaques are caused by superficial erosion on the luminal surface and Kolodgie et al found that versican, hyaluronan and the hyaluronan receptor cluster of differentiation 44 (CD44) were increased in eroded plaques whereas the proteoglycans decorin and biglycans were decreased⁸⁸.

The carotid plaque

The bifurcation of the carotid artery, where the artery is divided into the internal and the external carotid artery, is a highly susceptible location for atherosclerotic plaque formation. In the clinic, patients with carotid artery stenosis are examined using a Doppler ultrasonography to detect and monitor plaque progression⁸⁹. During an ultrasonography examination, in the early phases of the disease, intima-medial thickness (IMT) can be measured and IMT is associated with future cardiovascular and cerebrovascular events⁹⁰. In the more advanced phases of the disease, other features of plaque morphology can be assessed and have been associated with a high risk for events including echolucency, irregular surface of the plaque and presence

of ulceration^{91,92}. Yet, in the clinic today we commonly assess the degree of stenosis by measuring the peak flow velocity with pulsed wave Doppler⁹³ to identify those patients who are candidates for surgery besides the best medical therapy.

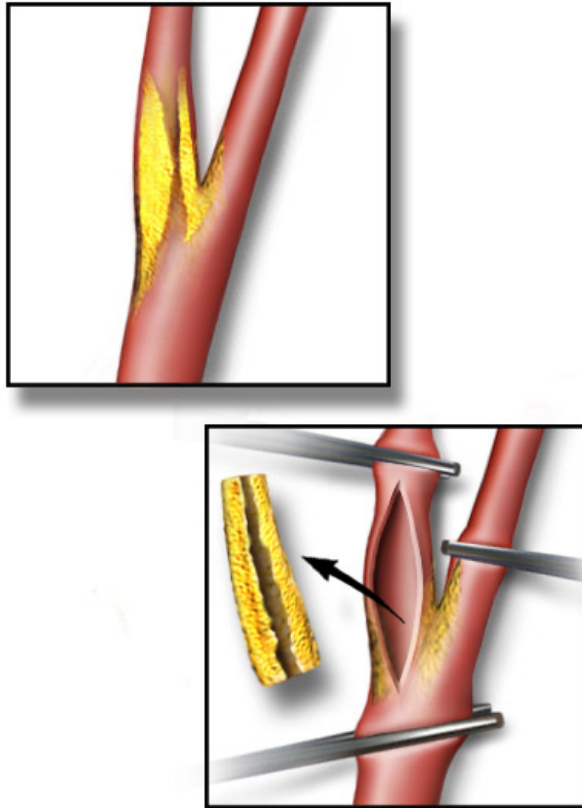


Figure 3. Schematic illustration of the removal of an atherosclerotic carotid artery plaque using endarterectomy. Modified image from Medical gallery of Blausen (2014)²⁷

Endarterectomy

The human atherosclerotic plaques used in this thesis are carotid artery plaques which have been surgically removed. Carotid endarterectomy surgery (Fig 3) was developed in the 1950 and 60s⁹⁴. The first large study of the effect of endarterectomy was the Joint Study of Extracranial Arterial Occlusion, which was published in a series of papers beginning in 1968, in which a lower rate of recurrent transient ischemic attacks (TIAs) and strokes were seen in the surgically treated patients^{95,96}. Since then several large studies have confirmed the beneficial effects of carotid

endarterectomy. The degree of stenosis need in order for an endarterectomy to be beneficial has ranged from 50% up to 90% in different large randomly controlled trials^{97, 98}. However most studies have shown the greatest risk reduction in patients with above $\geq 70\%$ stenosis and only moderate benefit to those with 50-69% stenosis⁹⁹.

Carotid plaques from patients who experienced a stroke or TIA have more unstable plaque features compared to plaques from asymptomatic patients and AF¹⁰⁰. However, it is likely that asymptomatic plaques are a heterogeneous group of plaques which of some may be near rupture and others may be harmless. A recent meta-analysis in asymptomatic patients showed that asymptomatic plaques with clinically detectable high-risk feature had a higher prevalence of ipsilateral ischemic cerebrovascular events, than previously seen¹⁰¹. This shows that there is a great need to find non-invasive markers which can assist the clinicians in choosing the correct treatment for asymptomatic patients.

Treatment of atherosclerosis

To reduce the risk for developing clinical significant atherosclerosis it is of great importance to treat the risk factors such as diabetes, blood pressure and hyperlipidaemia. The benefit of glycaemic control and blood pressure control was shown among others in the UK Prospective Diabetes Study¹⁰² for type 2 diabetes. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) are used widely in the clinic as secondary preventive treatment and in some cases as primary preventive treatment. In the first landmark study, the 4S study, LDL levels were lowered with 35% and both overall mortality and major coronary events were reduced in patients with statin treatment compared to placebo¹⁰³. In a post hoc analysis of four prospective studies, statins lowered LDL levels and raised HDL levels, which was associated with plaque regression in 18 to 24 months¹⁰⁴.

Apart from the cholesterol lowering effect statins may reduce inflammation and have antithrombotic effects¹⁰⁵. It has also been suggested that statins may act by decreasing endothelial dysfunction⁴⁵.

More recently, novel drugs have been attempted against atherosclerosis and its mechanisms. Hoping to target inflammation, the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) focused on patients with previous MI and elevated CRP levels, treating them with a monoclonal antibody against the pro-inflammatory cytokine interleukin-1 β and showed a decrease in the occurrence of recurrent cardiovascular events¹⁰⁶. The CANTOS study was the first large trial of a new era, possibly targeting the inflammatory aspect of atherosclerosis in humans. Another drug tested is methotrexate, an immunosuppressing medication used to treat cancer and decrease inflammation in patients with autoimmune diseases such

as psoriasis, rheumatoid arthritis and Crohn's disease. In contrast to the CANTOS trial, the Cardiovascular Inflammation Reduction Trial (CIRT), where patients with previous MI were treated with low dose methotrexate, did not show any beneficial effects on inflammatory parameters or recurrent vascular events¹⁰⁷.

Besides these drugs, there are invasive treatment strategies, such as endarterectomy, coronary artery bypass surgery (CABG) and PCI that have improved the clinical outcomes for patients. However, these strategies are not targeting the atherosclerotic process, but aim to restore the blood flow in the artery. With endarterectomy, the plaque is excised and removed from the patient; with CABG the coronary plaques often stay untouched in the patient, while mammary artery or vein grafts are used to bypass the stenotic plaques and finally, in percutaneous interventions, the plaques stay in the patients but are dilated and "smashed" by balloons and stents, with subsequent healing and stent re-endothelialisation.

Therefore, the fact is that there still is a huge need for novel therapies targeting the atherosclerotic process directly without the need for invasive treatment alternatives such as endarterectomy, PCI, stenting or CABG.

Even though new specific therapies are needed, the current treatment strategies have had a positive effect on plaque composition. According to a recent longitudinal study, including 1583 endarterectomy plaques obtained between 2002-2011¹⁰⁸, a decrease in the number of plaques with large lipid cores, luminal thrombosis and calcified plaques was identified. This coincided with an increased percentage of patients treated with statins and angiotensin II antagonists, indicating that the increased treatment might have affected the composition of plaques over time. Yet, despite these changes toward a more stable plaque phenotype there was no difference in risk for cardiovascular events during a three year follow up. Even though patients today are treated with the best available options there is still a high level of recurrent events^{6, 109}. This emphasize why the continuous search of new knowledge regarding mechanisms and the therapies targeting them, such as vascular healing/repair or plaque stabilization with ECM proteins are so important in the future.

Biomarkers of atherosclerosis

Atherosclerosis developing in the arteries is often a silent disease up until the moment when an acute event, such as a MI or stroke, occur. There is therefore a great need to find biological markers that can predict the risk for developing these acute events. The term biomarker refers to a quantifiable indicator reflecting a biological and often pathological process. There are different types of biomarkers such as predictive, prognostic and diagnostic biomarkers used in the clinic today. One example in the field of atherosclerosis are the cardiac troponins which have

been the gold standard for defining cardiomyocyte cell death. Another well studied example is the use of C-reactive protein (CRP) for predicting CVD events such as MI or stroke^{110, 111}. CRP is an acute phase protein released into the circulating blood from the liver as a response to increased inflammation (levels of interleukin (IL-6)). However, there are studies that have questioned the predictive capability of CRP¹¹² and it has not become widely used in the clinical setting for predicting CHD.

ECM biomarkers

ECM proteins are an essential part of atherosclerotic plaques and many different proteins are present in and contribute to the structural framework of plaques. In the cap and shoulder regions there is high grade of inflammation, and high turnover, including synthesis and enzymatic degradation of the ECM proteins by e.g. MMPs. This degradation generates fragments of ECM proteins that could potentially serve as diagnostic or prognostic markers of plaque vulnerability and future cardiovascular events¹¹³. These markers such as the ones evaluated here in this thesis may help clinicians in finding patients that are at higher risk of suffering from events.

The extracellular matrix (ECM)

The ECM is the non-cellular component of all tissues and organs and it comprises of over 300 different of proteins with varying functions in healthy and diseased tissues^{114, 115}. The ECM supports the cells in the vessel wall with structural and mechanical properties necessary for proper cellular functions and survival. Besides the purely structural scaffolds for cells the ECM affects cell behaviour and function by regulating gene expression by interacting with specific matrix receptors as well as by binding and storing growth factors. The ECM is involved in embryogenesis, physiological properties and as a reaction to injury to the vessel. A clear evidence of the crucial role of ECM proteins in normal development during embryogenesis is illustrated by several genetic syndromes with lack of functioning proteins¹¹⁶.

Another important function of the ECM involving the collagen, elastic and reticular fibers is tissue integrity and resilience. Proteoglycans are glycosylated proteins with a wide array of functions in normal and pathological circumstances and are further described in the proteoglycan section. Glycosaminoglycans (GAGs) are long linear polysaccharides with repeating disaccharides which are highly negative charged and therefore attract water and work as a lubricant and shock absorber. The GAGs are often attached to the core proteins of proteoglycans with the exception of hyaluronan. Glycoproteins are another type of ECM proteins with multiple functions^{114, 115}.

The ECM is not a passive structural entity. On the contrary it has a multitude of effects on cells¹¹⁷. The ECM proteins can attach to each other forming complex structural protein networks and interact with cells via membrane receptors such as the discoidin domain receptors (DDRs) and integrins^{118, 119}. ECM proteins are degraded by factors such as matrix metalloproteinases (MMPs) and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and synthesized by a several cell types including SMCs, fibroblasts and ECs.

The ECM in the vessel wall

In the healthy artery the ECM have important roles for the tensile strength and viscoelasticity for the vessel wall, as well as serving as the scaffold and milieu for

the different cell types present in the vessel wall¹²⁰. In the intima, the ECs are covered by a layer called glycocalyx consisting of various proteoglycans, glycoproteins and various soluble proteins imperative for proper EC function¹²¹. ECs synthesize the ECM components of the basement membrane to which the EC are attached. In the subendothelial space, proteoglycans retain LDL particles leading to lipid accumulation. The ECs in the intima are separated from the SMCs in the media by a layer of elastin called the internal elastic lamina (IEL)¹²². In the medial layer of the vessel wall SMCs are surrounded by basement membrane containing type IV collagen and proteoglycans¹²³. In the media, collagens types I, III, V are closely associated with elastin fibers. Layer of elastin fibers separate the layers of SMCs and the number of elastin layers depend on the type of artery (muscular or elastic arteries). The outermost vessel layer, the adventitia is a layer rich in collagen fibers, fibroblasts, immune cells and the vasa vasorum which supply the media and outer layers of the vessel walls with nutrients^{57, 124}.

The various ECM proteins and their organised networks affect the formation and vulnerability of atherosclerotic plaques. In the following sections, the ECM proteins most relevant for the work in this thesis and their relation to atherosclerotic plaques will be addressed.

Collagens

Collagens are the most abundant ECM proteins in body¹²⁵ and they are important structural components in both healthy unaffected arteries and in arteries afflicted by atherosclerosis¹²⁶. Currently, 28 different types of collagens have been described and they contribute to mechanical properties, organization and shape of tissues¹²⁷. The common structural feature of collagens is that they contain a triple helical structure consisting of three polypeptide chains called α -chains which differ in size. Collagens interact with cells through several receptor families and affect cellular behaviour such as proliferation, migration and differentiation¹²⁸.

Collagens can be divided into group depending on their conformation and localisation in the matrix^{128, 129}. The classical fibril forming collagens (collagen type I, II, III, V, XI, XXIV and XXVII) are assembled into larger fibers whereas the fibril associated collagen (FACITs) with interrupted triple-helices (collagen type IX, XII, XIV, XVI, XIX, XX, XXI and XXII) contain non-collagenous domains such as thrombospondin and von Willebrand domains. Other groups of collagens are the network forming collagens (collagen type IV, VI VIII and X), the membrane associated collagens with interrupted triple helices (MACITs, collagen type XIII, XVII, XVIII and XXV) and the multiple triple helix domains and interruptions (MULTIPLEXINs, collagen type XV and XVIII).

Collagen fibrillogenesis is a process that starts when collagen mRNA translocates into the rough endoplasmic reticulum where procollagen under several steps of post-translational modifications involving several enzymatic steps¹³⁰. Three procollagen polypeptide chains then assemble and fold into a triple helix¹³¹. The N- and C-propeptides are cleaved off by special proteinases and the lack of C-propeptides triggers self-assembly off the procollagen triple helices into collagen fibrils¹³². The continued growth of the collagen fibrils proceeds by a lateral (side-by-side) and linear (end-to-end) fusion into larger fibers¹³³. The formation of crosslinks are important for strength of the ECM¹³⁴. The enzyme lysyl oxidase activates lysine and hydrolysine residues and forms covalent crosslinks¹³⁵.

Collagens in atherosclerosis

Collagen fibers are important structural proteins in plaque tissue and collagens have been calculated to account for up to 41% of the total protein in fibrous plaques and 61% of the total protein in calcified human aortic plaques from human aortic plaques¹³⁶. The fibrillar collagens type I and III are the most abundant in the vascular matrix, forming a network of fibers together with smaller amounts of collagen type IV, V, VI and XVIII¹³⁷. The network forming collagen type IV is a major part of the basement membrane underneath the ECs and surrounding the SMCs. Collagens in the atherosclerotic plaque is mainly synthesized by SMCs but can also be synthesized by ECs^{138, 139}. The production of collagens type I and III by SMCs are stimulated by the cytokines TGF- β and PDGF but the synthesis of collagen levels are reduced when stimulating with INF- γ ¹⁴⁰.

Collagens interacts with cells by binding through cell surface receptors such as the integrins. Integrins are transmembrane glycoproteins with heterodimeric α and β subunits¹¹⁸. There exist a total of 18 α and 8 β subunits forming 24 different integrin heterotrimers. Integrins receptors are important for cellular cross-talk with the extracellular microenvironment via coupling the internal cellular actin cytoskeleton and extracellular matrix proteins or adhesion molecules on adjacent cells. This coupling allows external forces to affect cellular behaviour, such as changes in shear stress blood flow, which induces an endothelial pro-inflammatory response via the $\alpha\beta3$ integrin receptor¹⁴¹. Integrin signalling affects several cell types in atherosclerotic lesions including, SMCs, macrophages and lymphocytes. Inhibiting the $\alpha5\beta1$ integrin signalling decreased plaque inflammation and atherosclerotic plaque development in apolipoprotein E-deficient mice¹⁴².

The discoidin domain receptors (DDR) are tyrosine kinases that bind to collagen fibers. DDRs are expressed in vessels and are important for signalling between the

ECM and cells. In a study with LDL-knockout mice, the loss of DDR1 caused an attenuation in plaque progression and an increase in ECM deposition¹⁴³.

In the following section collagen type I and III will be described in detail.

Collagen type I

Collagen type I molecules consist of a trimer composed of α (I) and α 2 (I) chains. It is the most abundant collagen in tissues and forms large fibrils important for the strength and structural stability of the plaque. Its expression in atherosclerotic plaques is focal and especially prevalent in the fibrous cap and vascularized regions¹⁴⁴. It is expressed together with collagen type III in plaques and comprises two thirds of the total collagen in human aortic plaques¹⁴⁵. In paper IV, we measured the MMP-generated type I collagen neoepitope marker (C1M). Previously, in the PERF study of postmenopausal women in Denmark, women with high serum levels of C1M was found to be associated with a higher risk of CV mortality, cancer and CV diseases¹⁴⁶. In data from the same study women with C1M levels in the highest quartile was shown to be an independent risk factor for acute MI during a 15 year follow up time¹⁴⁷.

Collagen type III

Collagen type III is formed by a homotrimer of the α 1(III) polypeptide chains. It coexists on the same fibrils as type I collagen and is crucial for collagen fibrillogenesis during cardiovascular development^{148, 149}. Collagen type III a fibrillar forming collagen and mutations in the COL3A1 gene leads to type IV Ehler-Danlos syndrome with fragile blood vessels and skin¹⁵⁰. In atherosclerotic plaques, it is expressed in similar locations as collagen type I¹⁵¹. In a recent bioinformatics study the gene COL3A1 was downregulated in ruptured plaques compared to stable plaques¹⁵².

The basement membrane

The basement membrane is a thin sheet like extracellular matrix. It is situated at the basal aspect of the endothelium in vessels, in the epithelium in skin and it is also found surrounding SMCs^{153, 154}. The composition of the basement membrane is tissue specific, heterogenous and dynamic during pathological processes¹⁵⁵. The basement membranes in different tissues are highly specific and are formed by continuous synthesis and degradation of basement membrane proteins¹⁵³

The basement membrane is self-assembled on cell surfaces and contains a multitude of interacting proteins including laminins, type IV and XVIII collagen, perlecan, nidogens and von Willebrand factor. The two main structural proteins are laminin and collagen type IV which form independent fibrillar structural networks. During early development when the basement membranes are formed, a network of laminins are deposited, which is sufficient for maintaining the structure of the basement membrane. However, during later phases of development there is a crucial need for the formation of the type IV collagen network as it has been shown in studies with mice knockout models for type IV collagen¹⁵⁶.

The nidogens are small proteins consisting of the globular domains which help to mediate the binding of the laminin and type IV collagen network in the basement membrane¹⁵⁷. Even in the absence of nidogens, formation or maintenance of the basement membrane is not affected (in nidogens knockout mice)¹⁵⁸. Perlecan is a large multidomain heparan sulfate proteoglycan (HSPG) that bridges the laminin and type IV collagen networks. Perlecan is the major proteoglycan in the basement membrane and it is important for the basement membrane formation, lipoprotein binding, storage of growth factors and cell adhesion¹⁵⁹⁻¹⁶².

Type XVIII collagen is another basement membrane HSPG and it is found in three different isoforms with varying multi-domains interacting with other matrix proteins¹⁶³. Knocking out type XVIII collagen in a mouse model for atherosclerosis caused enhanced lipid accumulation, angiogenesis and vascular permeability¹⁶⁴. A third HSPG is agrin which has been found to be present in atherosclerotic lesions in mice and humans¹⁶⁵. Several other proteins are part of the complex protein network of the basement membrane including the glycoprotein osteonectin (SPARC) which binds and sequesters growth factors like PDGF and VEGF¹⁶⁶.

Laminins

Laminins are a family of large heterotrimeric glycoproteins which were discovered in 1979¹⁶⁷. Laminins are multidomain proteins unique to basement membranes and consist of three laminin subunits, the α - β - and γ -chains which get together in a long coiled-coil domain¹⁶⁸. There are five different α -chains, four β -chains and

three γ -chains forming 16 specific heterotrimers in a cross or crucifix shaped configuration¹⁵³ (Fig 4). Laminins binds to several ECM macromolecules including integrins and dystroglycans. The laminins self-assemble into networks by attachment to other laminins and are also linked to type IV collagen via the glycoproteins nidogens-1 and nidogens-2. The α -chains are mainly responsible for cell surface adhesion whereas the β - and γ -chains are important for nidogens binding, polymerisation and modulating receptor binding.

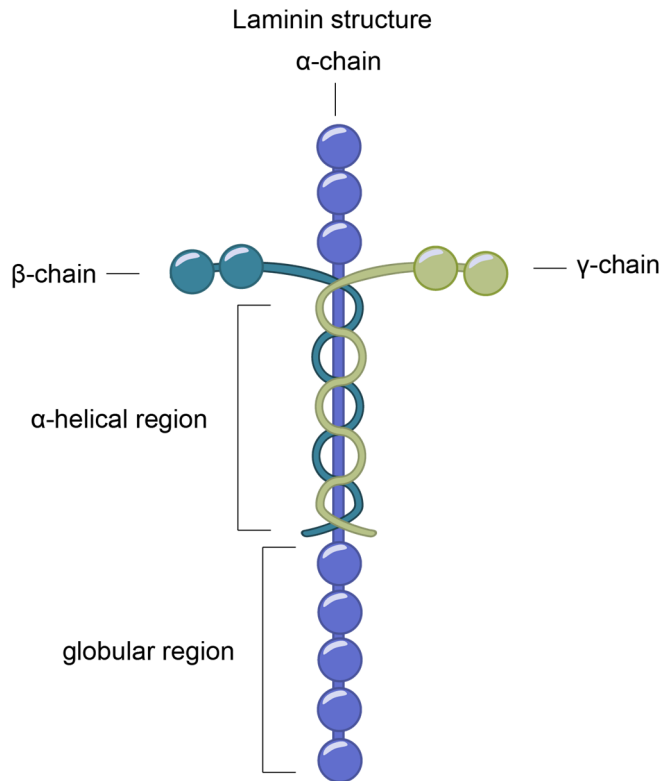


Figure 4. Schematic structure of laminin, with the α - β - and γ -chains forming a crucifix shaped protein.

In a study of different laminin isoforms in mouse and human arteries by Rauch et al¹⁶⁹ laminin α 2, α 4, α 5, β 1, β 2, γ 1 and γ 3 chains were present in varying amounts in both human and mouse atherosclerotic lesions.

The laminin γ 1-chain is found in most basement membrane and is widely expressed in different tissues and have its highest expressions in the following tissues: placenta, breast, heart, smooth muscle, skeletal muscle, lung, pancreas, kidney and urinary bladder^{170, 171}. It was found to be generally present in structures of neovascularization and at the luminal plaque border in human and mouse

atherosclerotic plaques¹⁶⁹. A recent study which compared stable plaques to ruptured plaques from freshly harvested carotid plaques using proteomics found that the majority of proteins that differed were basement membrane proteins including laminin $\alpha 5$, $\beta 2$ and $\gamma 1$ chains, which were all reduced in the ruptured plaques¹⁷².

In paper III, we measured the MMP-9 generated neo-epitope fragment of the laminin $\gamma 1$ -chain (LG1M). In patients with chronic kidney disease (CKD) serum levels of LG1M were associated with development of end stage renal disease (ESRD) during 1 year follow up.

Type IV Collagen

Type IV collagen is the main collagen type in basement membranes and it belongs to the network-forming collagen and is essential for the structural strength of basement membranes.

Collagen type IV is made up of six different α -chains ($\alpha 1$ to $\alpha 6$) that are encoded by the genes COL4A1 to COL4A6. Each α -chain consists of a collagenous 7S domain in the N-terminal, a central triple helical collagen domain and a C-terminal global non-collagen domain (NC1)¹⁷³. The α -chains assemble into triple helical heterotrimeric protomers (Fig 5), by binding of the NC1, into only three different isoforms the $\alpha 1\alpha 1\alpha 2$ (IV), $\alpha 3\alpha 4\alpha 5$ (IV), and $\alpha 5\alpha 5\alpha 6$ (IV)¹⁷⁴. Subsequently the triple helical protomers form complex lattice-shaped networks in the ECM by forming dimers via binding by the NC1 and tetramers by binding the 7S domains. The $\alpha 1\alpha 1\alpha 2$ (IV) is the predominant isoform in most tissues. In paper III, we measured the MMP-generated neo-epitope fragment of type IV collagen $\alpha 1$.

In atherosclerotic lesions type IV collagen is detected in the basement membrane region and surrounding elongated SMCs¹⁵¹. *In vitro* experiments show that SMCs plated on type IV collagen express elevated contractile markers compared to SMCs plated on type I collagen¹⁷⁵. Autoantibodies against collagen type IV did not affect atherosclerosis development in ApoE knockout mice¹⁷⁶

Alleles in the locus 13q34, where the genes for the $\alpha 1$ and $\alpha 2$ chains for type IV collagen, COL4A1 and COL4A2 are situated, have been found to be associated with an increased risk for coronary artery disease (CAD) in GWAS studies¹⁷⁷ *se. In vitro* experiments with primary SMCs and ECs from patients with mutations in the single nucleotide polymorphism (SNP) rs4773144 in the COL4A1/ COL4A2 locus show increased apoptosis in the patients homozygotic for the mutation¹⁷⁸. In the same study, patients homozygotic for the SNP rs4773144 had less type IV collagen in their atherosclerotic plaques and reduced fibrous cap thickness. In another group of patients with angiographically documented coronary disease there was an association with a higher rate of MI in patients homozygotic for the SNP. Furthermore, rare genetic mutations in the COL4A1 or COL4A2 have been

associated with increased risk of haemorrhagic stroke and small vessel disease^{179, 180}.

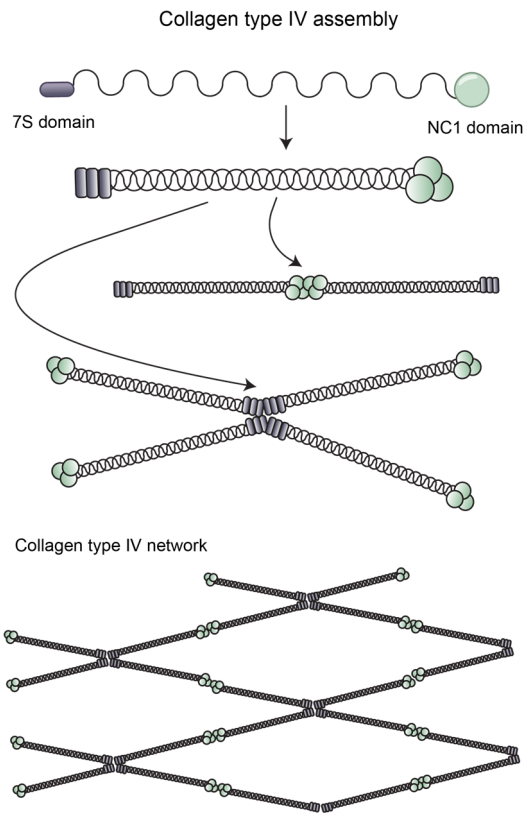


Figure 5. Illustration of the steps of type IV collagen formation. From α -chains into triple helical heterotrimeric protomers which form the type IV collagen network by forming dimers and tetramers.

Elastin

Elastin is an ECM protein is sometimes referred to as “the rubber band of the body”. Elastin has multiple functions in several tissues such as providing elasticity to joints and tendons in the body¹⁸¹. Elastin is also important for the elasticity the arteries, maintaining constant adaptation to different haemodynamic changes during the cardiac cycle. The elastin is found throughout the whole arterial system and is arranged into layers termed laminae which are formed by SMCs and partly by ECs during embryogenesis and postnatal development¹⁸². The elastin in the vessel wall is degraded by elastases and can be synthesised by macrophages in an disorganized way¹⁸³

Glycoproteins

Glycoproteins are proteins with covalently attached carbohydrate chains (glycans). The glycoproteins can be divided based on the type of linkage to the protein backbone: either via N-glycan, O-glycan or via an glycosphospholipid (GPI) anchor¹⁸⁴. Several of the glycoproteins are membrane bound and coat the outer layer of cells and carry several important functions with implications in atherosclerosis such as the receptor for p-selectin which is the p-selectin, glycoprotein ligand-1 (PSGL-1) important for leukocyte attachment to the endothelium^{185, 186}. Serum levels of several other types of glycoproteins including the lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1)^{187, 188} and galectin-3¹⁸⁹ have been associated with an increased risk for future cardiovascular events.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are long, linear and heterogenous polysaccharides with a repeating disaccharide structure of uronic acid and amino sugar. There are several different types of GAGs chains including chondroitin sulfate/dermatan sulfate, heparane sulfate and keratan sulfate GAG chains. The GAG chains are often attached to proteoglycan core proteins and GAGs bind to the ApoB100 protein of LDL and are involved in the initiation and progression of atherosclerotic plaques^{190, 191}. In the progression from normal intima to an atherosclerotic plaque in human aorta the total amount of GAGs decreases, with the exception for dermatan sulfate which instead increases¹⁹². Hyaluronan is a large non-sulfated GAG that is expressed during atherosclerotic plaque progression¹⁹³

Proteoglycans

By definition proteoglycans are proteins consisting of a core protein with one or several covalently attached sulfated carbohydrate chains (GAGs) Proteoglycans are proteins that developed early in the evolution of life and they are essential for several functions in multicellular organisms¹⁹⁴. Proteoglycans can be divided into different groups based upon their cellular or subcellular location and their different structural domains. Currently there are 43 genes encoding for proteoglycans¹⁹⁵. However due to alternative splicing the number of proteins are much higher¹⁹⁵. In the atherosclerotic plaque, proteoglycans affect both cellular functions and structural plaque features. Several proteoglycans have been shown to be involved in the atherosclerotic process and are suggested to have a crucial role in plaque erosions.

One example is the chondroitin sulfate proteoglycan versican which is synthesized by smooth muscle cells and binds lipoproteins and contributes to the formation of atherosclerotic plaques¹⁹⁶.

Small leucine rich proteoglycans (SLRPs)

Small leucine-rich proteoglycans (SLRPs) represent a group of proteoglycans with a small protein core (36-42 kDa), in comparison to larger proteoglycans such as aggrecan and versican¹⁹⁵. SLRPs also have unique structural organization made up of tandem leucine-rich repeats (LRRs) creating a characteristic arched solenoid shape¹⁹⁷. The SLRPs are divided into five classes (I-V) and contain different covalently attached side GAG sidechains. The most common GAG side chains are chondroitin, dermatan, keratan, tyrosine or polyglucosamin sulfate chains. Several SLRPs are involved in the process of collagen fibrillogenesis and are important to attain the correct size distribution of collagen fibers¹⁹⁸. SLRPs are located on the surface of collagen and bind to collagen with their central domain.

The three SLRPs studied in this thesis (Fig 6) are described briefly below.

Fibromodulin

Fibromodulin is a class II SLRP with covalently attached keratan sulfate GAG chains to the protein core. Fibromodulin is important for several processes in various tissues¹⁹⁹ and was discovered in 1989 in Lund^{200, 201}. Fibromodulin binds to collagen type I and is involved in fibrillogenesis and crucial for correct collagen fibrillogenesis in tendons during embryonal development^{202, 203}.

In a study by Shami *et al*, fibromodulin deficiency was found to cause an abnormal collagen structure and fibromodulin deficiency reduced total plaque burden as well as aortic root and carotid plaque size in mice²⁰⁴. In the same study macrophage like cells RAW265.7 were cultured on fibromodulin-deficient ECM which reduced the accumulation of lipids in the macrophages.

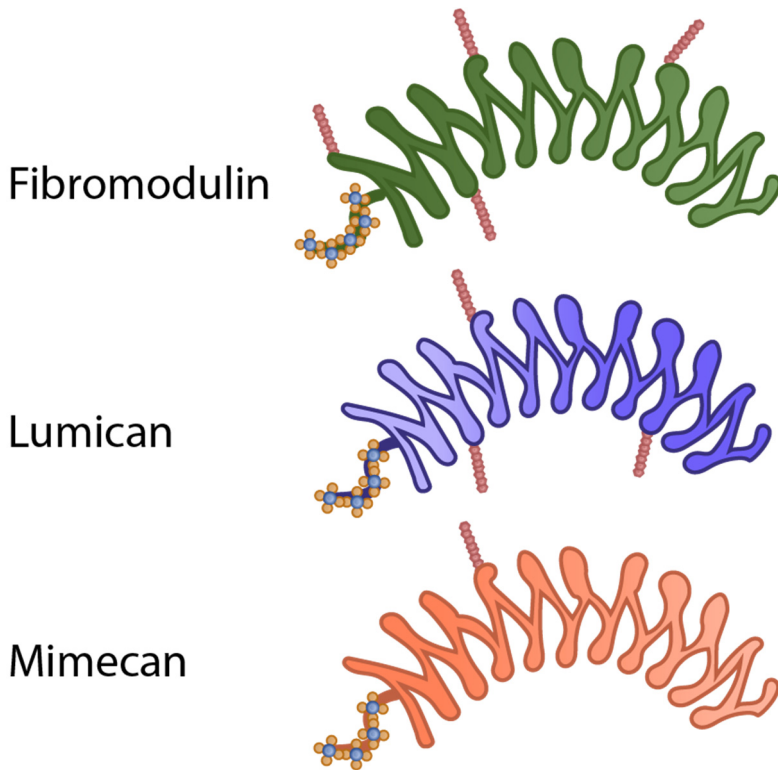


Figure 6 . Schematic structure of the three SLRPs studied in paper I and II. Sulfated tyrosine residues in the N-terminal (left) and various attached keratan sulfate GAG chains.

Lumican

Lumican is a class II SLRP with keratan sulfate GAG chains covalently attached to the core protein. Lumican is known to regulate collagen fibrillogenesis. Lumican shares a 48% sequence homology with fibromodulin. Lumican is overexpressed in wound healing in the cornea. Lumican is expressed in the thickened intima of coronary arteries²⁰⁵. Talusan et al investigated levels proteoglycans in the intima of atherosclerosis-prone and atherosclerosis-resistant arteries and found that there was an upregulation of lumican in the atherosclerosis-prone carotid artery²⁰⁶. The lumican mRNA levels were elevated in a human aorta from patients with CAD²⁰⁷. Lumican measured in the serum of hypertensive patients were increased in patients with carotid artery plaque compared to patients without carotid plaques²⁰⁸.

Mimecan

Mimecan a class III SLRP with keratan sulfate GAG chains²⁰⁹. Mimecan affects several biological processes including the regulation of collagen fibrillogenesis²¹⁰. Mimecan binds to vascular endothelial growth factor receptor 2 (VEGFR2) and was shown to affect angiogenesis in an ischaemic mouse model²¹¹. Mimecan has also shown to be expressed in the intima and below the endothelium in atherosclerotic plaques in rabbits²¹². Shanahan *et al* detected mimecan mRNA in human coronary arteries and found it to be expressed in low levels in advanced plaques and downregulated in intimal SMCs²¹³. Mimecan is upregulated in rat carotid arteries after balloon injury suggesting that it is also involved in vascular remodelling²¹³

Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc containing endopeptidases that can degrade a wide array of ECM proteins²¹⁴. The members of the MMP family are commonly classified by the cleavage substrates and structural domains into collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), matrilysins (MMP7 and MMP-26), membrane-type (MT)-MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP23, MMP-24 and MMP-25), furin-containing MMPs (MMP-11, MMP-21 and MMP-28) and other MMPs (MMP-12, MMP-19, MMP-20, MMP-22 and MMP-27).

The MMPs are expressed in atherosclerotic lesions and degrade collagen fibers which are important for maintaining the structural stability of the plaques²¹⁵. MMPs can also regulate the functions of other proteins by cleavage of cell surface receptors, release of apoptosis ligands such as the FAS ligand or inactivation of chemokines^{216, 217}. MMPs are involved in several biological processes in the atherosclerotic plaques, including angiogenesis, apoptosis, cell migration and tissue repair.

The collagenase MMP-1 was the first MMP to be discovered in 1962²¹⁸ and is expressed in human atherosclerotic lesions²¹⁹. MMP-3 and MMP-9 knockout mice form smaller brachiocephalic plaques, indicating that MMP-3 and -9 may be important for plaque progression²²⁰. MMP-9 levels in serum have also been associated with intima-media thickness, total plaque area and plaque instability in patients examined with carotid ultrasonography²²¹. MMP-7 cleaves and inactivates the apoptosis ligand FAS ligand²¹⁷.

Cytokines

Several cell types present in the atherosclerotic plaque can produce cytokines including SMCs, ECs, T-cells and macrophages²²². Cytokines are secreted proteins which have a wide array of functions important cell communication and regulation and subsequently in plaque development and progression²²³. Several cytokines can increase the synthesis of type I and III collagen such as PDGF and TGF- β , whereas other cytokines such as the pro-inflammatory INF- γ reduce the synthesis of collagen type I and III in SMCs¹⁴⁰.

IL-10 is an anti-inflammatory cytokine expressed in atherosclerotic plaques which can decrease inflammation by affecting macrophages and T-cells²²⁴. Platelet derived growth factor (PDGF) is a mitogen and functions as a chemokine for SMCs²²⁵. Vascular endothelial growth factor (VEGF) is an inducer of angiogenesis and maintenance of endothelial function²²⁶. RANTES (CCL5) is a chemokine associated with unstable plaques²²⁷.

MCP-1 is a potent monocyte chemoattractant secreted by several cell types including ECs and SMCs. MCP-1 mRNA was extracted in macrophage-derived foam cells from rabbit plaques and MCP-1 was also expressed in macrophage rich areas in human and rabbit atherosclerotic plaques²²⁸. Soluble CD40 Ligand (sCD40L) is a pro-inflammatory and immunoregulatory molecule that is expressed on several cell types in atherosclerotic plaques and CD40/CD40L signalling affects several key processes in atherosclerosis^{229, 230}.

Macrophage inflammatory protein -1 β (MIP-1 β or CCL4) is a T-cell derived cytokine expressed in carotid artery plaques and high plasma levels of MIP-1 β predict future TIA²³¹. Serum levels of MIP-1 β was also able to predict major adverse cardiac event (MACE) in patients with coronary plaques²³².

Inhibition of the pro-inflammatory cytokine TNF- α can reduce atherosclerosis in ApoE knockout mice²³³. IL-1- β is a pro-inflammatory cytokine affecting several cells in atherosclerotic plaques such as SMCs, monocyte/macrophages and ECs²³⁴. IL-1- β induces the expression of adhesion markers VCAM-1 and ICAM-1 on human vascular SMCs²³⁵. IL-6 is a pro-inflammatory cytokine that can predict future CV death²³⁶. Injection of IL-6 in ApoE knockout mice increase plaque size²³⁷. On the contrary, in another study in mice in which both IL-6 and ApoE were knocked out there was increase in plaque size²³⁸.

Extracellular matrix degradation and remodelling

The state and structural form of the ECM affects the behavior of cells. In tissues EMC proteins are constantly being synthesized and degraded in a homeostatic manner. In inflamed tissues, such as atherosclerotic plaques, MMPs and other degrading enzymes are released by either tissue resident cells or cells attracted to the vessel wall from the bloodstream. This can lead to an excess of degradation and consequently plaque rupture. On the other hand, exacerbated ECM synthesis may lead to restenosis as seen in the neointimal formation after invasive coronary treatment with PCI.

The term remodelling refers to the process during plaque growth in which the vessel adapts in response to the plaque growth. Outward remodelling is the adaptive expansion of the elastic lamina and the media to accommodate the plaque growth. Inward remodelling is the opposite response in which the lumen is narrowed due to decrease in the radius of the vessel often seen in smaller arterioles and resistance arteries^{239, 240}. The degradation of ECM proteins leads to the formation of bioactive fragments often termed matrikines or matricryptines²⁴¹.

Transforming growth factor β (TGF- β)

Transforming growth factor β (TGF- β) belongs to a family of secreted dimeric cytokines and growth factors that was initially discovered in cells infected with cancer causing viruses. The name transforming growth factor was given due to the cytokines ability to transform normal fibroblast *in vitro*²⁴².

The TGF- β super family consist of cytokines and growth factors encoded by 33 different genes in humans and mice. The proteins in this family have a wide variety of functions in embryonic development, differentiation of cells and tissues, homeostasis of tissues in both the healthy conditions and several disease states²⁴³. There are three different TGF- β isoforms called TGF- β 1, TGF- β 2 and TGF- β 3. They have common signaling pathways and are structurally related, yet they are encoded by different genes. Knockout mice of the individual isoforms have shown varying embryonal defects²⁴⁴⁻²⁴⁶.

TGF- β ligands form dimers (Fig 7) and signal through the type I and type II membrane receptors²⁴⁷ which phosphorylates SMAD2 and SMAD3, which in turn translocate to the nucleus and activate target genes such as connective tissue growth factor (CTGF) and collagen type I²⁴⁸

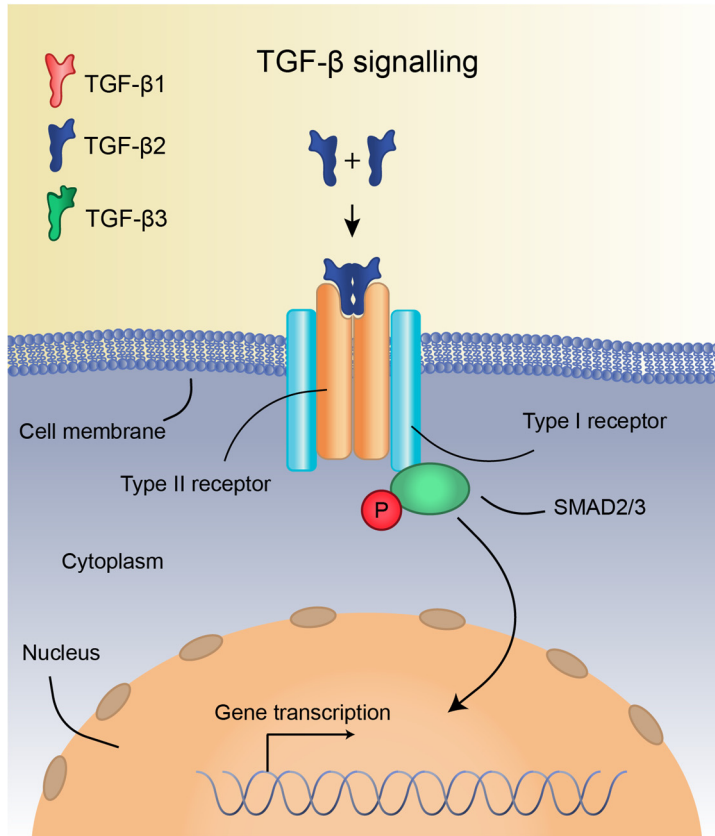


Figure 7. Simplistic overview of the TGF- β signalling pathway for the TGF- β ligands TGF- β 1, TGF- β 2 and TGF- β 3.

The TGF- β proteins are secreted in a biological inactive form attached to binding proteins. There are four different latent TGF- β proteins LTPB1-4 which bind to the LAP complexes and are important for binding to ECM proteins in tissues²⁴⁹. The inactive LAP complexes are cleaved by proteases such as MMP-2 or MMP-9²⁵⁰. TGF- β 1 is activated by plasmin²⁵¹. The LDL variant lipoprotein(a) inhibit the transition of plasminogen to plasmin. The inhibition of plasmin formation decrease the amount of activated TGF- β , which could potentially be a mechanism for lipoprotein(a) to cause increased atherosclerosis²⁵².

TGF- β in atherosclerosis

The overwhelming amount of TGF- β research conducted have been about TGF- β 1²⁵³. TGF- β are generally considered atheroprotective since TGF- β 1 stimulate ECM matrix synthesis. However certain function may atherogenic effects such as the inhibition of SMC proliferation and specifically stimulating proteoglycans in SMCs which may led to increased lipoprotein retention in plaques^{254, 255}.

When neutralizing TGF- β 1, TGF- β 2 and TGF- β 3 simultaneously in mice, they develop atherosclerotic plaques with an unstable plaque phenotype with less collagen and more inflammation²⁵⁶. Also specifically knocking out TGF- β -signalling in T-cells accelerate plaque formation in mice²⁵⁵.

In one study the three TGF- β isoforms were measured in serum of patients with chronic kidney disease (CKD) it was found that TGF- β 1 and TGF- β 2 were lower in patients with carotid plaques compared to patients without carotid plaques and all the isoforms were inversely correlated with carotid IMT²⁵⁷. Furthermore, TGF- β 2 and TGF- β 3 but not TGF- β 1 were independent predictors of subclinical atherosclerosis²⁵⁷.

Methods

Carotid Plaque Imaging Project

The data in this thesis is mainly derived from the CPIP (Carotid Plaque Imaging Project) biobank which was started November 2005 at Skåne University Hospital by Isabel Goncalves. Patients with carotid stenosis are included in the study and biobank when they become eligible for a carotid endarterectomy. At the time of writing this thesis more than 1200 patients, blood, living cells and plaques are included in the biobank. The general aim of the CPIP is to identify useful markers of vulnerable atherosclerotic plaques to characterize the mechanism leading to destabilization of plaques, as well as to identify patients with vulnerable plaques with a high risk for CV events such as MI or stroke.

All patients are examined with a preoperative ultrasound of the carotid arteries to evaluate the degree of stenosis and are assessed by a neurologist. Patients were considered symptomatic if they suffered from amaurosis fugax (AF), TIA or ischemic stroke within one month prior to surgery. The indications for carotid endarterectomy were 1) ipsilateral symptoms and stenosis > 70% or 2) asymptomatic patient with > 80% stenosis, assessed by Doppler ultrasound.

Carotid plaques are snap-frozen in liquid nitrogen in the operation theatre directly after surgical removal. From the most stenotic part of the plaque a 1 mm thick section is taken for histology and embedded in optimal cutting medium (OCT, Sakura Finetek Europe BV, Japan) and cryosectioned into 8 μ m sections. An adjacent section of 1 mm was kept for RNA sequencing. The rest of the plaque is homogenized in a standardised way described earlier⁶⁹. Briefly, the plaques are weighed and cut into smaller pieces while still frozen. They are subsequently homogenize with a motorized blender at 1600 rpm with a Teflon pestle. The plaques were homogenized in a 5 mL buffer containing, Tris-HCl, sucrose, tris(2-carboxyethyl) phosphine HCl (TCEP.HCL), NaF, Na-orthovanadate, Na-glycerophosphate, Na-pyrophosphate, benzamidine, phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Complete, EDTA-free).

Clinical data

The clinical data is collected during patient enrollment into the study using standardised forms and requisition of information from patient's journals. Information regarding medical treatments and cardiovascular risk factors are collected at the time of inclusion, including history of diabetes, smoking, dyslipidaemia, smoking status, renal function and familial history of CVD diseases. Blood samples were obtained the day before surgery by the research nurse/doctor including the patients and analysed in the lab.

These studies were approved by the local ethics committee and all patients gave written informed consent to participate in the study. All the studies conformed to the principles of the Declaration of Helsinki.

Histology

Histology, the examination of biological tissues with a microscope, was first employed by the Italian Marcello Malpighi in the 17th century to examine tissue and organs in different animals²⁵⁸. The development of the microscope, and the embedding techniques and stainings used in sample preparation were essential in many medical discoveries in the fields of pathology, bacteriology and atherosclerosis during the last two centuries.

In the studies in this thesis, sections were fixed with Histochoice™®, a patented fixative designed to replace formaldehyde and other fixatives. Immunohistochemistry was employed in paper I, II and V of this thesis and its entails the use of antibodies specific for certain proteins (antigens). Immunohistochemistry was developed in the 1960 and 70s and made it possible localise multiple antigens in tissue sections²⁵⁹. Primary antibodies are directed to the specific antigens and the secondary antibodies with detection systems such as ABC (avidin/biotin) method or Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) bind to the primary antibodies and finally an enzyme substrate such as 3,3'-diaminobenzidine (DAB) is added and the enzymatic process induce a colour change indicating where the primary antibody has bound.

Plaque sections from carotid endarterectomy samples were stained for several proteins using primary antibodies against including the SLRP; fibromodulin, lumican and mimecan. In paper I, plaques from 153 patients were stained for fibromodulin and lumican. Fibromodulin was stained with a primary antibody kindly provided by late prof D.Heinegård, Lund University Sweden. Lumican was stained with a rabbit monoclonal antibody (ab168348; Abcam, Cambridge, UK). A

biotinylated goat anti-rabbit (Vector BA-1000, Vector Laboratories Inc, Burlingame CA, USA) and rabbit anti mouse IgG (ab98668, Abcam, Cambridge) were used as secondary antibodies.

In paper II 223 human carotid plaques were obtained from 218 patients who underwent carotid endarterectomy. Mimecan was stained with a rabbit polyclonal antibody (PA5-48255, Invitrogen, Waltham, MA) and a MACH3 rabbit probe and horseradish peroxidase-polymer (RP531H, Biocare Medical, Pacheco, CA) was used as a secondary antibody. A rabbit IgG polyclonal isotype control antibody was used as a control for each plaque (ab27478, Abcam, Cambridge, UK).

In paper V, mouse monoclonal antibodies were used to stain for TGF- β 1 (Abcam, Cambridge, UK, ab27969, 0.06 μ g/ml) and TGF- β 2 (Abcam, Cambridge, UK, ab36495, 0.25 μ g/ml) overnight at 4°C on paraffin-embedded frozen sections of carotid atherosclerotic plaques. A mouse monoclonal isotype control antibody was used as a control (Abcam, Cambridge, UK, ab81032, at corresponding concentration). To detect staining a MACH3 probe and horseradish peroxidase (HRP) polymer (Biocare Medical, Pacheco, CA, USA) was used.

In paper I, II and V several plaque components were examined using immunohistochemistry. Primary antibodies against the red blood cell marker Glycophorin A (M0819, Dako Sweden, Stockholm, Sweden) was used to detect intraplaque hemorrhage. Glycophorin A is a sialoglycoprotein exclusively expressed in the cell membrane of red blood cells.

Macrophages were detected using a mouse anti-human antibody for CD68 clone KP1 (DakoCytomation, Glostrup, Denmark). CD68 is a widely used marker of smooth muscle cells. To detect SMCs a mouse anti-human smooth muscle actin clone 1A4 (DakoCytomation) was used. Followed by biotinylated rabbit anti-mouse Ig(DakoCytomation) as secondary antibody, as previously described²⁶⁰. The markers used for detecting smooth muscle cells and macrophage are commonly used in the literature. However recent data have emerged showing that these markers are not entirely specific and that a certain overlap exists.

To stain for neutral triglycerides and lipids in sections the lysochrome (a dye soluble in fat) Oil Red O was used. Sections were submerged 0.4% Oil Red O in (60%) isopropanol for 20 min. Calcified areas were measured by quantifying the areas of calcium deposits, as well as the empty areas with surrounding calcium generated when the slides were prepared. Sections were counterstained with the hematoxylin-eosin staining Mayer's Hematoxylin (Histolab, Gothenburg, Sweden) staining to visualise general tissue structures. The hematoxylin stain acidic structures such as nuclei, ribosomes and rough endoplasmic reticulum in blue/purple. The eosin stains basic structures such as the cytoplasm and ECM in pink.

To stain for the extracellular matrix and collagen in paper I the Masson's trichrome technique Fuchsin-Ponceau, (Chroma-Gesellschaft, GmbH, Germany) Phosphomolybdic Acid and Fast Green (Sigma Aldrich Chemie GmbH, Germany) aniline blue (BDH, Dorset, England) was used to assess plaque collagen content. In a smaller subset of plaques type I and III collagen primary antibodies (ab6308 and ab6310 respectively; Abcam, Cambridge, UK) were used. In paper II the Russell-Movat Pentachrome staining was used to stain for ECM proteins. were collagen fibers were stained yellow. In paper I, Elastin staining was performed with Accustain Elastic Kit (Sigma-Aldrich).

Sections were scanned using Aperio ScanScope digital slide scanner (Aperio Technologies, Inc, Vista, CA) and positive immunoreactivity with the different antibodies and stainings were quantified blindly using Biopix iQ 2.3.1 (Biopix Ab, Gothenburg, Sweden).

Biochemical assays for ECM components

The ECM components glycosaminoglycans (GAG), collagen and elastin were analysed in plaque homogenate with colorimetric assays as described previously⁶⁹. Briefly, an aliquot of homogenate was centrifuged to remove heavy calcium/apatite salts. Plaque homogenate was treated with guanidine hydrochloride and collagen was dye precipitated and quantified with Sircol collagen assay (Bicolor, Carrickfergus, UK). The assay measured acid-soluble and pepsin-soluble type I, II, III, IV and V collagens. The Sircol Dye contains picric acid which the dye used in the collagen stain Sirius red. It is an anionic dye with sulfonic side chains which binds to the continuous repeating tri-peptide amino acid sequence (with glycine every third amino acid) found in fibrillary collagen.

Fastin elastin assay (Bicolor, Carrickfergus, UK) was used to quantify elastin. The assay measures soluble tropoelastins, insoluble cross-linked elastins that have been solubilized and lathyrogenic elastins. The dye used was 5,10,15,20-tetraphenyl-21H,23H-porphine tetra-sulfonate (TPPS) which binds to non-polar and basic amino acids found in elastin²⁶¹.

To analyse total sulfated GAGs, homogenates were treated with Pronase supplemented with Ca^{2+} to release serine glycosaminoglycans. Subsequently Blyscan proteoglycan and glycosaminoglycan assay (Bicolor, Carrickfergus, UK) was used. The GAG hyaluronic acid (hyaluronan) is not detected by this assay, due to the lack of sulfate groups.

MMPs

In paper I, II, III and V. MMPs were measured with two different methods. The MMP-1, -2, -3, -9 and -10 were measured using the Mesoscale ultra-sensitive kits (Mesoscale, Gaithersburg, MD, USA) which employ wells precoated with capture antibodies for specific MMPs. Sample is added to the wells in this sandwich immunoassay with electrodes in each well. Detection antibodies labelled with an electrochemiluminescent compound which emit lights after voltage is applied to the well and read by a plate reader.

In paper I the levels of MMP-7 and MMP-12 were measured in plaque homogenates and in paper III the levels of MMP-1, -3, -7, -10 and 12 were measured in the circulation using Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD96996 reagents kit. (Olink Bioscience, Uppsala, Sweden). Data are presented as arbitrary units. The measurement were done at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala.

PEA is an immunoassay analysing several biomarkers simultaneously. For each marker, a matched pair of antibodies (proximity probes) linked to unique oligonucleotides bind to the target protein. When the probes are get close they hybridize each other. Subsequently a DNA polymerase extend the hybridising oligo, bound to one of the proximity probes. This created a DNA amplicon which can be quantified with qPCR²⁶².

Tissue inhibitors of MMPs (TIMPs 1, 2 and 3) in plaque homogenate supernatants were analysed using MILLIPLEX® MAP Human TIMP Magnetic Bead Panel (Milliplex, MA, USA) using the Luminex® xMAP® bead-based multiplex assay platform. It is an immunoassay using analyte-specific capture antibodies which are bound to beads. The beads are incubated with samples, washed and incubated with a secondary biotinylated antibody. Added Streptavidin-phycoerythrin bind to the biotin of the secondary antibodies. Luminex instrument are able to measure the individual beads and the signal from the enzymatic reaction streptavidin-conjugated R-Phycoerythrin.

Cytokines

The cytokines IL-10, monocytes inflammatory protein- β (MIP-1 β), s-CD40L, PDGF-AB/BB, Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), TNF- α and vascular endothelial growth factor (VEGF) were measured in plaque homogenate supernatants (Human Cytokine/Chemokine Immunoassay, Millipore Corporation, MA, USA) and analysed with Luminex 100 IS 2.3 (Austin,

TX, USA) as previously described²⁶⁰. The procedure was performed according to the manufacturer's instructions and all cytokines were normalized to plaque wet weight.

In paper II MCP-1, IL-6 and MIP-1 β were measured in plaque homogenate supernatants using Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD96x96 reagents kit (Olink Bioscience, Uppsala, Sweden).(see MMP section for details). Data are presented as arbitrary units.

In paper I, active Caspase 3 (cleaving at the Asp175/Ser176 site) was measured in carotid plaque homogenate using Human Caspase-3 ELISA (Invitrogen, Life Technologies, Carlsbad, CA).

Plaque TGF- β 1, - β 2 and - β 3 analysis

TGF- β 1, - β 2 and - β 3 were assessed in 25 μ L of supernatant from plaque homogenate after centrifugation for 5 min at 8000 RPM in 4°C, using the Milliplex Map TGF- β Magnetic Bead 3 Plex Kit - Immunology Multiplex Assay from MerckMillipore (TGFBMAG-64K-03, Billerica, MA, USA) according to the manufacturer's instructions and measured using Luminex 100 IS 2.3 (Austin, TX, USA). Levels of TGF- β 1, - β 2 and - β 3 were normalized to plaque wet weight.

In vitro THP-1 stimulation

In paper II and V, human blood monocytes Tamm-Horsfall protein 1 (THP-1 THP, 88081201, Sigma-Aldrich, St Louis, USA) cells belonging to a human leukaemia cell line were cultured in RPMI-1640 medium (11875093, Thermo Fisher Scientific, Waltham, USA) supplemented with 10 % fetal bovine serum (FBS, 10270106, Thermo Fisher Scientific, Waltham, USA) and 50 U/mL penicillin-streptomycin. The THP-1 cells were stimulated with phorbol 12-myristate 13-acetate (PMA, cat #78139 Sigma- Aldrich, Saint Louis, USA) for 24 hours, which differentiated the cells into a macrophage-like state adherent "M0"cell²⁶³.

In paper II the cells were subsequently seed into wells and treated with TGF- β 2 (T2815, Sigma-Aldrich, Saint Louis, USA) and MCP-1 (RP-8648, Thermo Fisher Scientific, Waltham, USA) before measuring mimecan levels using immunofluorescence microscopy using a rabbit polyclonal antibody (PA5-48255, 175 Invitrogen, Waltham, MA). Mimecan mRNA levels were measured using quantative real time polymerase chain reaction (qPCR) in cell supernatants.

In paper V the “M0” macrophages were treated with TGF- β 2 for 48 hours before they were treated with lipopolysaccharide (LPS L2630, Sigma-Aldrich, Saint Louis, USA) for 15 hours to polarise toward a pro-inflammatory “M1” macrophage phenotype. Subsequently the mRNA levels of IL-6, MCP-1 and MMP-9 were measured using qPCR. In both papers, each reaction was performed in duplicate and results were normalized by geometric mean of two housekeeping genes 18S ribosomal RNA (18S) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

MMP-generated neo-epitope fragments

The MMP-generated neo-epitope fragments measured in paper III and IV were developed at Nordic Biosciences (Herlev, Denmark)²⁶⁴⁻²⁶⁶. In paper III the MMP-9 of the laminin γ 1-chain (LG1M) and the MMP-2, -MMP-9 and MMP-12 generated neo-epitope fragment of type IV collagen α 1 were measured in serum from 787 patients from the CPIP biobank. In paper IV, the MMP-2, MMP-9 and MMP-13 generated neo-epitope fragment of collagen type I (C1M) was measured in serum from 787 patients from the CPIP biobank.

Briefly the peptide fragments generated by *in vitro* cleavage of the different ECM proteins, were analysed with mass spectrometry and the first six amino acids sequence of the fragments were then injected (with adjuvant) into mice to induce an antibody response. Hybridoma cells from the immunized mice were generated by fusion of mouse spleen cells and myeloma fusion cells. The hybridoma cell generated antibodies were then labelled with an HRP-polymer and technically validated in the assay for C4M, LG1M and C1M.

In paper III and IV the three ECM markers were measured in serum from 787 patients in the CPIP biobank. Briefly, a 96-well ELISA plate coated with streptavidin was coated with the synthetic peptide. The plate was washed in washing buffer between the different steps. Thereafter the standard peptide or sample were added, followed by peroxidase conjugated antihuman monoclonal antibodies. The incubation reaction was stopped by the adding a stop solution (1% H₂SO₄) and the plate was analysed using an ELISA reader. Samples below the lower limit of quantification were assigned the value of LLOQ, while samples above upper limit of quantification were assigned the value of ULOQ.

RNA sequencing

In paper V, the expression of the three TGF- β genes were evaluated from global transcriptome RNAseq data collected from 72 plaques. RNA was prepared from

standard total RNA extraction with Trizol cleared of Ribosomal RNA using RiboZero™ Magnetic Kit from (Epicentre). Strand specific RNAseq libraries were prepared with ScriptSeq™ v2 RNA-Seq Library v2 Preparation Kit (Epicentre).

Paired-end sequencing libraries for 72 RNA samples were generated and sequenced using high-output kit version 2, HiSeq2000 platform, Illumina, USA. Adapter sequences and bases with Phred score less than 20 were removed from the sequencing reads using TrimGalore! (Version 0.3.7, Babraham Bioinformatics, Cambridge, UK). Next, quality controls were performed using FastQC (Version 0.11.2, Babraham Bioinformatics, Cambridge, UK) and summarized using mutliqc (Version 0.9).

Follow up

The patients in the biobank were followed up by research nurses after one and two years following their endarterectomy by telephone calls to the patients and retrieving information in the medical journals. CV events were identified through data retrieved from the Swedish National patients register (99% of all discharge codes registered)²⁶⁷ using hospital discharge codes. Patients were located through their personal identification number and the following ICD-10 codes were used to identify CV events: G45.3, G45.9, G46, I63.1-5, I63.8-9, I64.5, I21-22, I24.8-9, I25.1-2, I25.5-6 and I25.8.). The CV events variable was composite end-points which included M), transient ischemic attacks (TIA), amaurosis fugax (AF) and vascular interventions not planned at the time of the operation such as carotid endarterectomy (CEA), carotid artery stenting (CAS), coronary artery bypass grafting (CABG) or percutaneous coronary artery intervention (PCI) and all deaths with an underlying cardiovascular cause of death.

Data regarding the cause of death was extracted from the Swedish cause of death register²⁶⁸. The underlying cause of death for each patient was registered as ICD code. The underlying cause indicate the disease that contributed the most to the death of the patient. In the whole dataset the following ICD-codes indicate that the patient died from CV cause of death: I10, I11.0, I11.9, I13.0, I13.2, I20.9, I25.9, I46.9, I48, I48.9, I49.0, I50.1, I50.9, I60.9, I61.9, I64, I69.3-4, I70.0, I70.2, I71.0-6, I71.8-9, I70.9, I72, I73.9, I74, I74.9, I99.9 and E78.5.

For patients suffering of multiple events only the first one was taken into account in the survival analysis. During the time between the publication of paper I to the publication of paper II (from 2015 to 2020) the follow up data was updated with more patients, longer follow up time and therefore also more events. Furthermore, in the follow up analysis in paper I all events that occurred within 24 hours were

considered procedure related whereas this time frame was extended to 72 hours when the follow up analysis for paper II was performed.

Statistical methods

In papers I-V, continuous variables were non-normally distributed and are thus presented as median with interquartile range (IQR), while categorical variables are expressed as percentages. For continuous variables Mann-Whitney U test and Spearman's rank correlation were used and for categorical variables Chi-square test was performed. In paper II and V, for the *in vitro* experiments in THP-1 cells one-way ANOVA with Holm Sidak's adjustment for multiple comparisons was used for statistical comparison.

Kaplan-Meier survival analyses were performed with grouping in either above or below median in paper II, III and IV, tertiles in paper I and quartiles in paper V. The differences in the different division were based upon the distribution of the measurements and the separation of the Kaplan-Meier curves. The significance between groups were assessed by the Log-rank test.

Cox proportional hazard regression analysis (hazard ratios (HR) with 95% CI) was used to analyse future risk of CV events, CV Death and all-cause death. The Cox multivariate regression models were adjusted for varying covariates in the different papers. The covariates adjusted for in the multivariate models were either based upon conventional risk factors from the Framingham heart study or the variables which were significantly correlated to the values to the marker examined.

A p-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 24.0 (IBM Corp., Amonk, NY, USA). In paper V, p-values were corrected for multiple comparisons using Bonferroni-Sidak correction in GraphPad Prism v7.03.

RNA sequencing analysis

In paper V for the RNA sequencing analysis, Surrogate Variable Analysis using svaseq²⁶⁹ identified 4 significant surrogate variables. Next, fold changes between symptomatic and asymptomatic patients were estimated using DESeq2²⁷⁰ with the surrogate variables as covariates and genes were considered differentially expressed if Wald test p-value was < 0.05.

PCA and OPLS-DA

In paper V, in order to examine if the three TGF- β was associated to asymptomatic plaques and to examine which factors were the most relevant for separating symptomatic plaques from asymptomatic plaques, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were carried out in SIMCA-P software package (version 14.1, Umetrics, Umeå, Sweden) using log transformed, mean-centered and scaled to unified variance input variables.

Overall contribution of each variable to group discrimination was ranked by VIP values (variable influence on projection). Variables with VIP value of >1 were considered relevant to group discrimination. Ellipse based on Hotelling's T^2 represents the 95% confidence interval of modelled variation in score plots.

Discriminatory power of selected variables ($VIP > 1$) to cluster into two groups was further examined by K-means unsupervised clustering in Qlucore omics explorer v 3.5. Hierarchical clustering of variables was performed using Euclidean distance and average linkage method. Dataset was log transformed, mean centered and normalized to unit variance for analysis in Qlucore omics explorer v 3.5 and presented as a heatmap.

Aims

- What is the role of the proteoglycans fibromodulin, lumican and mimecan in human atherosclerotic carotid plaques and are they associated to plaque vulnerability and future CV events?
- Does the cleavage neoepitopes of basement membrane proteins collagen IV (C4M) and laminin γ -1 chains (LG1M) have the ability to predict CV events, CV death and all-cause death?
- Can a collagen type I neoepitope (C1M) levels, measured in serum from patient with atherosclerotic plaques, predict future CV events, CV death and all-cause death?
- Which TGF- β isoform is the most prevalent in human carotid atherosclerotic plaques and are these isoforms associated to plaque vulnerability and future CV events?

Results

Paper I

In paper I, the two SLRPs fibromodulin and lumican were assessed in atherosclerotic carotid plaques using immunohistochemistry. Plaque areas stained positive for both proteoglycans were identified in the fibrous cap, core and the shoulder regions. Fibromodulin and lumican were often, but not always, found in the same regions. Plaque areas of both proteoglycans correlated positively with lipid plaque area, measured by Oil Red O, and the plaque area stained positive for intraplaque hemorrhage, measured by the red blood cell marker Glycophorin A. Both fibromodulin and lumican were detected in lipid rich areas and to a lesser extent in macrophage rich plaque areas. Fibromodulin, but not lumican, was inversely correlated with the area stained positive for alpha-actin (SMC marker), whereas lumican correlated with the area stained positive for CD68 (macrophage marker) and elastin. Neither proteoglycans correlated with plaque calcification nor the collagen stained plaque area (masons trichrome). However, fibromodulin correlated with collagen type I plaque area.

Fibromodulin significantly correlated with plaque tissue homogenate levels of the pro-inflammatory cytokines MIP-1 β and sCD40L. Furthermore, the growth factor VEGF correlated positively with fibromodulin. There was an inverse correlation for fibromodulin to the anti-inflammatory cytokine IL-10. Lumican correlated positively with the chemotactic cytokine RANTES (CCL5). Fibromodulin and Lumican were both correlated with the proteolytic enzymes MMP-1 and MMP-9 and the active form of the apoptosis enzyme caspase-3. Fibromodulin also correlated inversely with MMP-12 and lumican correlated positively with MMP7. Both fibromodulin and lumican correlated with TIMP1.

Fibromodulin plaque area was greater in plaques that recently had caused a stroke, TIA or amaurosis fugax. Fibromodulin plaque area was also greater in plaques from patients with diabetes. There was no difference of the lumican plaque area in plaques from symptomatic compared asymptomatic patients or plaques from patients with diabetes compared to patients without diabetes.

However, when comparing symptomatic and asymptomatic plaques from patients with diabetes, fibromodulin plaque area was significantly larger in the symptomatic

group. Indicating that the larger fibromodulin plaque areas in symptomatic plaques were likely due to the larger fibromodulin plaque areas in plaques from patients with diabetes. Lumican plaque area was instead significantly larger in symptomatic plaques from patients with diabetes compared to asymptomatic plaques from patients without diabetes.

Finally, a larger fibromodulin positive plaque area was associated with the occurrence of post-operative cerebrovascular events during follow-up (mean follow up time 37.1 months). The higher risk for future cerebrovascular events remained significant after adjusting for clinical risk factors (age, gender, diabetes, hypertension, CHD, smoking and statins) in a Cox Proportional Hazard model. Lumican expression was not associated with an increased risk for cerebrovascular events and neither fibromodulin nor lumican plaque areas were associated with an increased risk for AMI, cardiac or vascular interventions or CV death.

Paper II

In paper II another proteoglycan was studied. Mimecan was stained in human carotid atherosclerotic plaques and was located in areas of collagen fibers, close to the core region and in close approximation to calcified plaque areas. It was inversely correlated to the area stained for smooth muscle cells (SMC α -actin) and positively correlated to lipids (Oil Red O) and macrophages (CD68) and intraplaque hemorrhage (glycophorin A).

Furthermore, mimecan plaque area correlated with plaque tissue homogenate levels of MMP-9, one of the main extracellular matrix degrading enzymes known to be associated with human plaque vulnerability, and the proinflammatory cytokine MCP-1. In vitro studies showed that stimulation of PMA differentiated THP-1 cells ("M0"macrophages) with MCP-1 increased the mRNA expression and the protein expression of mimecan. These findings indicate that mimecan might be upregulated by the inflammatory plaque milieu.

When comparing the patient characteristics between patients with high or low (above or below median) plaque areas of mimecan, it was noted that patients with higher mimecan levels were older, more commonly suffered from diabetes and had higher HbA1c levels.

Patients with greater plaque area stained for mimecan also had a higher risk of suffering from future CV events and CV death. In a Cox regression model the risk for CV death remained significant after adjusting for age, sex, diabetes, body mass index (BMI), HDL, TG and estimated glomerular filtration rate (eGFR).

Paper III

In paper III two neoepitope fragments of basement membrane proteins collagen type IV (C4M) and laminin (LG1M) were measured in the blood of 787 patients undergoing carotid endarterectomy due to advanced atherosclerotic disease.

When dividing the patients in high and low (above and below median) levels of C4M, it was noted that the patients with above median levels of C4M had higher levels of cholesterol, LDL, CRP and lower levels of HDL and reduced estimated glomerular filtration ration (eGFR) The group of patients with above median levels of LG1M had higher prevalence of current smoking, higher CRP levels and lower HDL levels. C4M levels correlated inversely with eGFR. Current smokers had higher levels of LG1M. C4M correlated with MMP-1, MMP-7, MMP-10 and MMP-12. LG1M correlate with MMP-7, MMP-10 and MMP-12, indicating that there is an association between MMP-induced cleavage and the levels of the circulating neoepitopes.

Patients who were operated due to a symptomatic plaque had higher levels of C4M compared to patients who were operated due to an asymptomatic plaque. When investigating the potential predictive role of the two basement membrane remodelling markers, C4M predicted future all-cause mortality in a multivariate Cox regression model together with diabetes and eGFR. However, C4M was not associated with an increased risk for CV events or CV death during the follow up time of 6 years.

LG1M was not higher in serum from symptomatic patients compared to asymptomatic patients, even if a trend was noted. In regard to the risk for future CV events, LG1M was associated with a higher risk to suffer from future CV events but the association did not remain significant when adjusting for potential confounders. Patients with above median levels of LG1M had a higher risk for CV death, corroborated in a univariate Cox regression using continuous values, but not with above and below median. However, it did not remain significant in the multivariate model.

Finally, patients with high levels of LG1M had a higher risk for future all-cause mortality in univariate Cox regression and in a multivariate Cox regression analysis LG1M was associated with a higher risk for all-cause death together with diabetes and eGFR in the multivariate analysis.

Paper IV

In the fourth paper (Paper IV) we focused on another neoepitope: MMP-generated neo-epitope marker of type I collagen (C1M). It was measured in serum from 787 patients who underwent a carotid endarterectomy. The patients were divided into two groups using the median as cut-off. The patients with above median values of C1M were significantly older, had higher CRP levels, and higher total cholesterol, LDL and triglyceride levels but lower HDL and eGFR (worse kidney function). In the patients with high C1M there were also more female, hypertensive and patients who were operated due to symptomatic plaques rather than asymptomatic plaques.

Follow up data regarding CV events, CV mortality and all-cause mortality was available for 473 patients (mean follow-up time 6.8 years). A total of 64 patients died and out of those 39 deaths (60.9%) were due to CV causes. During the follow up period 101 (21.4%) patients suffered from cardiovascular events. The levels of C1M were significantly higher in patients with a symptomatic plaque compared to patients without symptomatic plaques.

According to the Kaplan Meier analysis, patients with higher C1M levels had a higher risk of suffering from CV events, CV mortality and all-cause mortality. Using a Cox proportional hazard regression model, adjusting for potential confounders defined by the Framingham Heart Study (age, gender, diabetes, current smoking, total cholesterol and hypertension), C1M was shown to be a predictor of CV mortality and all-cause mortality together with age and diabetes. In summary, the neo-epitope marker of type I collagen C1M is associated with clinical features of high CV risk and predict future CV events, CV mortality and all-cause mortality in patients with advanced atherosclerosis

Paper V

In the final paper (Paper V) we measured the three proteins TGF- β 1, TGF- β 2 and TGF- β 3 in carotid plaque tissue homogenates from patients who underwent carotid endarterectomy. All three TGF- β isoforms were present in the plaque tissue and TGF- β 2 was more abundant compared to TGF- β 1 and TGF- β 3. In fact, TGF- β 2 was 20 times more abundant than TGF- β 3 and seven times more abundant than TGF- β 1.

In order to identify if any of the three isoforms were associated with an asymptomatic plaque phenotype, a PCA was performed. The PCA included several variables including TGF- β isoforms, inflammatory cytokines, ECM proteins, histological markers, MMPs, TIMPs and lipoproteins. The PCA analysis showed a good separation between symptomatic and asymptomatic plaques. To identify the

most relevant variable for separation of symptomatic and asymptomatic a supervised OPLS-DA was performed. Interestingly, this analysis showed that TGF- β 2 was the strongest biological component for segregating asymptomatic plaques and symptomatic plaques.

To examine the potential association of TGF- β and plaque vulnerability, histological plaque components were measured and showed that TGF- β 2 was the only isoform to correlate with histological plaque components. TGF- β 2 correlated positively with the plaque area stained positive for SMCs (α -actin) and inversely with the plaque area stained positive for intraplaque hemorrhage (Glycophorin A), indicating that TGF- β 2 is associated with histological features of a stable plaque phenotype. Neither TGF- β 1 nor TGF- β 3 correlated with any histological features. TGF- β 2 levels were also higher in plaques from asymptomatic patients compared to plaques from symptomatic patients which was not observed for TGF- β 1 or TGF- β 3. RNA sequencing in a subgroup of 37 plaques showed that the mRNA levels of TGF- β 2 were higher in asymptomatic plaques compared to symptomatic plaques which is in line with the finding at protein level.

All the three isoforms correlated with plaque collagen levels. TGF- β 2 and TGF- β 3 also correlated to plaque levels of GAGs and elastin. However, of the three measured TGF- β isoforms, TGF- β 2 was the isoform with the strongest correlations to the ECM proteins. TGF- β 1 did not correlate with any MMPs or TIMPs whereas TGF- β 2 correlated inversely with MMP-9 and TIMP2 and positively with TIMP-1. TGF- β 3 correlated with MMP-2. In the group of symptomatic plaques, the levels of all three TGF- β isoforms correlated with the time passed between the occurrence of symptoms and the carotid endarterectomy. This indicated that the TGF- β isoforms increase after the occurrence of a plaque rupture and might be part of the repair processes following the rupture.

TGF- β 1 is known to reduce the inflammatory responses, yet we found that TGF- β 2 seemed to be the most relevant isoform in human carotid atherosclerotic plaques. Therefore, we investigated if TGF- β 2 may have anti-inflammatory properties. Interestingly, TGF- β 2 correlated inversely with the pro-inflammatory cytokine MCP-1 and there was a trend towards an inverse correlation to IL-6. In THP-1 cells that were stimulated with PMA, the pre-treatment of TGF- β 2 decreased the mRNA levels of MCP-1, IL-6 and MMP-9.

K-means clustering using 11 components that contributed to the separation between symptomatic and asymptomatic plaques, identified two main clusters. Cluster 1 had high TGF- β 2 levels, elastin and more SMCs whereas cluster 2 had high MMP-levels, inflammatory cytokines, lipids and greater plaque areas of intraplaque hemorrhage. Cluster 1 also had a higher percentage of asymptomatic plaques compared to cluster 2.

During the follow-up period of 67 months, patients with plaque TGF- β 2 levels in the top quartile had a significantly lower risk to suffer from CV events compared to the patients in the three lower quartiles. In the highest quartile only 22% of the included patients suffered from CV events compared 45% of the patients in the three lower quartiles. In a Cox regression analysis, the higher quartile showed a hazard ratio (HR) of 0.4 compared to the three lower quartiles. The lower risk to suffer from a future CV event remained significant in a multivariate Cox regression even after adjusting for cardiovascular risk factors (age, sex, hypertension, LDL, HDL and current smoking) with and HR of 0.45.

Discussion

The role of SLRPs in plaque vulnerability

In this thesis we studied three SLRPs. In paper I, we studied fibromodulin and lumican and in paper II we studied mimecan. The finding in paper I that fibromodulin was increased in symptomatic plaques and that the fibromodulin staining correlated to lipid staining, indicate that fibromodulin might be associated with plaque vulnerability. This notion was corroborated by previous findings by our group that ApoE/fibromodulin double knockout mice had decreased total plaque burden and smaller plaques in the aortic roots²⁰⁴. Furthermore, in the same study, low shear stress lesions, formed by a stress-modifying cast placed around the mice carotid arteries displayed decreased lipid content and less macrophages in the ApoE/fibromodulin knockout mice compared to ApoE knockout mice.

The findings that fibromodulin and lumican stainings correlated to lipid staining and that the stainings of both of these SLRPs was found in the core region suggested that fibromodulin and lumican might have roles in the binding of lipids as seen with other SLRPs²⁷¹. Rather than direct binding to lipids which has not been described for keratan sulfate SLRPs, the presence of fibromodulin on collagen fibers might be relevant for lipid uptake by cells such as macrophages. This idea fits nicely with the findings in the previously mentioned study on fibromodulin, where there was a decreased lipid uptake by macrophages that were cultured on fibromodulin deficient collagen²⁰⁴. The finding that the lumican staining also correlated to macrophage staining is in agreement with the notion that lumican binds to macrophages as observed *in vitro* in a previous study²⁷².

The correlation of fibromodulin and lumican to intraplaque hemorrhage is interesting since it is a known feature of vulnerable plaques⁶⁰. Furthermore, fibromodulin staining correlated with VEGF levels. VEGF induces angiogenesis which leads to the formation of immature neovessels that are dysfunctional and cause intraplaque hemorrhage²²⁶.

Fibromodulin and lumican stainings correlated with MMP-1 and MMP-9 which have been shown to be expressed in higher levels in shoulder regions of atherosclerotic plaques^{273, 274}. In our study, lumican staining correlated to MMP-7, an MMP which have previously been shown to be expressed in macrophages in

carotid artery plaques²⁷⁵: Surprisingly, fibromodulin was inversely correlated to MMP-12 which is expressed in macrophages in atherosclerotic plaques and have been associated with plaque destabilization²⁷⁵⁻²⁷⁷. However, in a study of human cartilage proteolysis, MMP-12 was the preferred MMP for cleavage of fibromodulin, which could explain the inverse correlation found in our study²⁷⁸. Fibromodulin also correlated to MIP-1 β and sCD40L and lumican correlated to RANTES, which are all considered proatherogenic cytokines^{230, 231, 279}. The findings that fibromodulin and lumican stainings were detected in the shoulder regions of the plaques and that they were correlated to several MMPs and pro-inflammatory cytokines, further strengthens the notion that these SLRPs are associated with plaque vulnerability.

There was no difference in lumican staining between plaques from symptomatic patients and plaques from asymptomatic patients, indicating that lumican might be less relevant to plaque vulnerability in advanced atherosclerotic plaques. In early stages of plaques development however, lumican was the only SLRP found to be upregulated in the carotid artery²⁰⁶. Yet, since all the plaques in our study were advanced atherosclerotic plaques, we could not study the potential role of lumican in early atherosclerotic disease in our experimental design.

Fibromodulin and lumican stainings were also detected in some regions of the fibrous cap regions and in regions with dense ECM. The staining in the fibrous cap makes sense since the cap contains collagen type I, to which both fibromodulin and lumican bind with their core proteins during the process of collagen fibril formation¹⁹⁸. In line with this, fibromodulin staining correlated with collagen type I staining. The lack of correlation of fibromodulin and lumican to the Masson staining might be due to the fact that the Masson staining stains all types of collagen, including collagen type III, which is present in carotid artery plaques²⁸⁰, but does not bind to fibromodulin or lumican.

In ApoE/fibromodulin knockout mice, the collagen fibers of the fibrous plaques generated by a stress-modifying carotid cast were thicker and more heterogeneous showing that fibromodulin deficiency alters the collagen fibers in mouse lesions. It is not known if this is true in human plaques.

We found that the plaque area stained for fibromodulin correlated with the areas stained for lumican and that the molecules were found in similar regions. Fibromodulin and lumican bind to the same site of type I collagen which could explain the similarities in the staining patterns. Furthermore, fibromodulin and lumican have overlapping functions in collagen fibrillogenesis²⁰³.

Fibromodulin stained areas were larger in plaques from patients with diabetes. This might be due to an impaired repair response, as described previously in plaques from patients with diabetes²⁸¹, where a decrease of MMP-2 was seen. However, no

correlation between MMP-2 and fibromodulin was seen in our study. On the other hand, another study looked at fibromodulin cleavage *in vitro* and found that only MMP-13 but not MMP-2, MMP-8 or MMP-9 was able to cleave it *in vitro*. Finally, an alternative theory could be that fibromodulin could be upregulated in a response to plaque rupture and that upregulation was enhanced in plaques from patients with diabetes.

In paper II, mimecan staining was associated with several features of vulnerable plaques as it correlated with lipids, intraplaque hemorrhage and macrophages. The correlation of mimecan staining and staining for lipids could indicate a role for GAGs chains attached to mimecan in lipid binding. However the fact that there was no difference in plaque size or composition between in the ApoE/mimecan knockout mice and ApoE knockout mice indicates that the lack of mimecan does not necessarily affect lipid uptake. Unfortunately, *in vitro* studies on a mimecan deficient matrix to evaluate macrophage uptake of lipids are lacking.

An inverse correlation of mimecan staining to SMC staining could be explained by the fact that mimecan can be downregulated in intimal SMCs²¹³. Mimecan staining correlated with glycophorin A, a marker of intraplaque hemorrhage. Mimecan negatively regulates the ischemia-induced angiogenesis by affecting endothelial cell tube formation, proliferation and migration²¹¹. This regulation might generate defective neovessels and increased intraplaque hemorrhage, a feature of vulnerable plaques. In our study mimecan staining was observed in areas of collagen staining with Movat Pentachrome. Mice in which mimecan have been knocked out develop larger collagen fibril diameters in several tissues including skin²¹⁰, indicating that mimecan is important during collagen fibrillogenesis and pointing to a potential way for mimecan to affect plaque vulnerability. Mimecan is also known to strengthen the collagen during the wound healing process in the myocardium after MI.²⁸² In the same study an overexpression of mimecan with adenovirus improved the collagen quality, with more mature, tightly packed collagen fibrils observed in the mice where mimecan was overexpressed. However, the effect on collagen quality after mimecan overexpression in human atherosclerosis has not been studied previously.

Mimecan staining correlated with the pro-inflammatory cytokines MCP-1 which is a chemokine for macrophage recruitment in atherosclerosis^{283, 284} and plaque destabilisation²⁸⁵. In our study mimecan gene expression was upregulated in macrophages after stimulation with MCP-1, indicating that mimecan is upregulated by an inflammatory milieu such as in the atherosclerotic plaque. Mimecan is expressed in circulating and resident macrophages²⁸⁶, which could be related to the correlation seen between mimecan staining and macrophage staining. Patients with above median staining of mimecan had a higher frequency of diabetes and higher HbA1c and a trend for higher BMI. Mimecan has been identified as a regulator of

glucose and energy homeostasis,²⁸⁷ which could explain the higher prevalence of diabetes and increased levels of HbA1c levels found in the group of patients with high mimecan staining.

Mimecan staining also correlated with the pro-atherogenic MMP-9 and mimecan levels above median were associated with increased risk for CV death and CV events. Indeed several SLRPs can become cleaved by MMPs and mimecan is no exception. A MMP-9 and -12 generated degradation marker MMCN-151, developed by Nordic Bioscience has been considered to reflect mimecan remodelling in ApoE knockout mice²⁸⁸.

MMP generated ECM neo-epitopes as markers for plaque vulnerability

The extracellular matrix is constantly remodelled by the degradation by proteases such as MMPs and ADAMTs. At the same time the synthesis of ECM proteins is needed to keep the ECM in equilibrium. The basement membrane is an ECM network surrounding SMCs and ECs and the two major protein networks are the type IV collagen and laminin, forming intertwined networks.

In paper III, two MMP-generated neopeptide basement membrane markers MMP-2, -9 and -12 generated type IV collagen $\alpha 1$ chain (C4M) and the MMP-9 neo-generated laminin $\gamma 1$ -chain (LG1M) were measured in serum from 787 patients. The patients were followed up for 6 years and the main outcomes studied were CV events, CV death and all-cause mortality.

When dividing the patients into groups with high or low (above and below median) levels of C4M, the group with above median C4M levels were more often female, had higher cholesterol, higher LDL, lower HDL, higher CRP levels, lower eGFR (decreased kidney function) and fewer statin treated patients compared to the group with low levels of C4M. These are all factors that traditionally have been associated with a higher risk of CVD, except sex. For women, the risk for CVD increases after menopause²⁸⁹ and the mean age was over 70 in our cohort. Chronic kidney disease and patients with reduced eGFR level have an increased risk for stroke^{290, 291}. The group with high C4M levels also had significantly more patients that were operated because of a symptomatic carotid artery plaque than due to a plaque not associated with symptoms. Since symptomatic plaques more commonly have features of plaque vulnerability it could be expected that the high C4M group would be at higher risk for future CV events and CV death.

Yet, C4M did not reach significance when predicting the risk for CV events and CV death. However, when looking at all-cause mortality, patients with high C4M had

an increased risk of all-cause mortality according to the log rank test. In the Cox regression model C4M predicted all-cause mortality together with diabetes and eGFR.

In the group with high LG1M levels there were more current smokers, they had a higher degree of plaque stenosis, higher CRP and lower HDL levels compared to the group of patients with low LG1M levels. There was also a trend ($p=0.050$) for more patients operated due to symptomatic plaques than asymptomatic plaques in the high LG1M group. The higher levels of LG1M in serum of patients who were operated due to symptomatic carotid plaques might reflect an increased degradation of the laminin γ -1 chain occurring in high-risk plaques and fragments leaking to the circulation.

In patients with high LG1M levels there was no increased risk for CV events during follow up compared to the group of patients with low LG1M levels, However when instead using continuous values in the Cox regression instead, an increased risk was observed in the univariate analysis Yet, this did not remain significant in the multivariate analysis. The patients in the group with high LG1M levels had a higher risk for CV death in the log rank test compared to the group of patients with low LG1M values. However this did not remain significant in the univariate or multivariate Cox regression. When using continuous values in the Cox regression LG1M predicted CV death in the univariate analysis, however it did not remain significant in the multivariate analysis. In the group of patients with high LG1M values there was an increased risk for all-cause mortality in the log rank test and in the univariate Cox regression compared to the group of patients with low LG1M levels. In the multivariate Cox regression LG1M predicted all-cause mortality together with diabetes and eGFR.

In vitro the C4M neo-epitope was generated by MMP-2, MMP-9 and MMP-12²⁶⁶. According to our analysis, C4M correlated with four MMPs, MMP-1, MMP-7, MMP-10 and MMP-12, of which MMP-12 had the highest r-value and lowest p-value ($r=0.191$, $p=<0.001$). *In vitro* the LG1M neo-epitope was generated by MMP-9²⁶⁵. In our study LG1M correlated with levels of MMP-7, 10 and 12. This indicates that the neo-epitopes C4M and LG1M might be generated by other MMPs *in vivo*. The correlation to the different markers could also reflect a general higher level of MMP activity in the blood of the patient. Several MMPs are associated with a higher risk for CV risk, including MMP-12²⁹²

Several studies have suggested that the classical vulnerable plaque phenotype might have changed²⁹³ (due to improved life-style and preventive strategies) and that percentage of events caused by plaque erosion have increased. Since the basement membrane is important for endothelial cell survival and dysfunctional endothelial cells are implicated in erosions, the degradation of the basement membrane could be relevant to predict the risk for erosion. However, in this cohort C4M and LG1M

were not able to predict CV event or CV death but instead predicted all-cause mortality supporting an even broader importance of the basement membrane possibly in other organs beyond the arterial wall.

These markers of basement membrane degradation are likely not to be exclusive to the plaques. Other organs likely to contribute to the amount of C4M and LG1M are the kidneys. LG1M was increased in serum and urine from patients with chronic kidney disease (CKD) and associated with progression into end-stage renal disease (ESRD) and mortality²⁶⁵.

Collagen type I is important for the stability and integrity of the fibrous cap. In paper IV we measured a type I collagen fragment generated by matrix metalloproteinases (MMP) -2, -9 and -13 (C1M). Several MMPs are known to degrade collagen type I^{74, 126}. The degraded type I collagen fragments from atherosclerotic plaques likely end up in the circulation and are measured with the specific neo-epitope antibody for C1M.

The group with high levels of C1M were older, more often female and hypertensive patients compared to the patients with low levels of C1M. Their plaques were more often associated with symptoms and they had higher cholesterol, higher LDL, lower HDL levels, lower eGFR and higher CRP levels compared to the group with low levels of C1M. Like for C4M and LG1M this group represents a potential high risk group with several risk factors for developing future CV events. Indeed, the C1M marker predicted CV events independently in a multivariate Cox regression. For CV death and all-cause mortality C1M predicted them both together with age and diabetes. CRP was elevated in all the groups with high levels of the three neo-epitope makers C4M, LG1M and C1M, which could reflect an increased systemic inflammation associated with an increased formation of the neo-epitopes.

For patients with high levels of C1M there was increased risk for all-cause mortality, indicating that C1M levels might also reflect other diseases than atherosclerosis. In fact, in a study of postmenopausal women who developed cancer, C1M levels were associated with survival during a 3 year follow up²⁹⁴. However, the low number of patients who died of cancer in our study, did not allow for subgroup analysis and our design was not primarily aimed for that analysis either.

We did not find any difference in the serum levels of the neo-epitopes C4M and LG1M or C1M when comparing diabetic patients to non-diabetic patients. Furthermore, levels of ECM proteins like collagen have been shown to be lower in plaques from patients with diabetes²⁸¹. Still, diabetes was one of the covariates predicting CV death and all-cause death confirming the well-known high risk for CVD in diabetic patients²⁹⁵.

TGF- β and plaque repair

The three TGF- β isoforms have unique genes expressed on different chromosomes. Studies of the knockout mice for each isoform rendered different congenital deformities. TGF- β 1 knockout mice die after a few weeks with inflammation in several tissues, with infiltration of monocytes and lymphocytes²⁴⁵. The TGF- β 2 knockout mice suffer from perinatal death and severe malformations in heart, lung and spinalcord²⁴⁴. The TGF- β 3 knockout mice die within hours and suffer from cleft palate and undeveloped lungs²⁹⁶. These genetic functions in the different isoforms are non-overlapping indicating the individual role of the TGF- β isoforms during embryogenesis. The three isoforms share common signalling pathways and, in many cases, exert similar functions including the stimulation of ECM synthesis.

In atherosclerosis TGF- β 1 is the mostly studied isoform. Bobik et al. studied TGF- β 1 and TGF- β 3 isoforms in atherosclerosis and found them expressed in atherosclerotic aorta, but TGF- β 2 was not examined. TGF- β 1 and TGF- β 2 were found to be expressed in coronary arteries and saphenous veins using immunohistochemistry²⁹⁷. However, until now no quantification of all three TGF- β isoforms have been performed in atherosclerotic lesions. In paper V, all the three isoforms were detected simultaneously and the TGF- β 2 isoform was the most abundant, TGF- β 1 being seven times lower and TGF- β 3 20 times lower than TGF- β 2.

TGF- β isoforms are involved in wound healing and tissue repair response²⁹⁸. Therefore, we examined if any of the three TGF- β isoforms were associated with an asymptomatic plaque phenotype by using a PCA on a variety of biological factors and saw a clear separation of asymptomatic and symptomatic plaques. In an OPLS-DA it became clear that TGF- β 2 was the strongest factor that differentiated asymptomatic plaques from symptomatic plaques. K-clustering of the data from the OPLS-DA was performed and identified two clusters. This could give clues to which proteins or factors are particularly associated with TGF- β 2.

In our study we saw that the TGF- β 2 isoform was higher in asymptomatic plaques both in protein level and at the mRNA levels. No differences were found for the other isoforms, TGF- β 1 and - β 3. The increased TGF- β 2 in asymptomatic plaques could indicate that the elevated TGF- β 2 constitutes a protective factor in plaques.

One study showed that low levels of TGF- β 2 increased SMC proliferation and higher levels of TGF- β 2 stimulated collagen production²⁹⁹. Both of these scenarios fit with our findings since we found that TGF- β 2 correlated with smooth muscle cells and biochemically measured collagen. TGF- β 2 can inhibit tube formation of ECs which is a relevant step in angiogenesis. This could support a role for TGF- β 2 decreasing plaque angiogenesis, in line with our inverse correlation between TGF- β 2 and intraplaque hemorrhage.

MMP-9 is a proinflammatory protease found in human atherosclerotic plaques. Our data showed that TGF- β 2 was inversely correlated to the levels of MMP-9, suggesting that TGF- β 2 could be involved in decreasing MMP-9 plaque levels. TGF- β 3 correlated with MMP-2, which has been suggested to be a plaque stabilizing MMP by facilitating SMCs migration in plaques.^{300, 301}

TGF- β 2 was also inversely correlated to the pro-inflammatory chemokine MCP-1 and there was a trend for an inverse correlation to IL-6. These correlations indicated that TGF- β 2 might have anti-inflammatory abilities. So, to test this hypothesis we used THP-1 cells and treated them with PMA to induce adherent “M0” macrophages. The cells were then pre-treated with TGF- β 2 for 48 hours before stimulated with LPS to induce an inflammatory response. Interestingly, we found that the pre-treatment with TGF- β 2 reduced mRNA levels of IL-6, MCP-1 and MMP-9 upon LPS stimulation. These results show that TGF- β 2 can reduce an inflammatory response from macrophages in vitro. Together, these observations might have an exciting clinical application in order to dampen inflammation and increase plaque stability in atherosclerosis.

Conclusions

Paper I

- The two SLRPs fibromodulin and lumican were detected in the fibrous cap, the core and the shoulder regions in human carotid atherosclerotic plaques. They correlated with histological features of plaque vulnerability, pro-inflammatory cytokines, MMPs and apoptosis. Fibromodulin was higher in plaques that caused preoperative symptoms as well as in plaques from patients with diabetes. Fibromodulin was also associated with a higher risk to suffer from postoperative cerebrovascular events.

Paper II

- The SLRP mimecan was found to be present in carotid atherosclerotic plaques close to collagen, core regions and areas of calcification. Mimecan staining correlated to histological vulnerable plaque feature including MMP-9. Mimecan was correlated to the pro-inflammatory cytokine MCP-1 and MCP-1 treatment of macrophages increased mimecan levels *in vitro*. Patients with above median mimecan staining in their plaque had higher risk to suffer from postoperative CV death.

Paper III

- In patients with advanced atherosclerosis, the MMP-generated neo-epitope fragments of the basement membrane proteins type IV collagen (C4M) and laminin γ -1 chain (LG1M) were associated with an increased risk of all-cause mortality in a Cox regression model together with diabetes and eGFR

Paper IV

- In patients with advanced atherosclerosis, The MMP-generated neo-epitope fragment of type I collagen (C1M) was associated with an increased risk for CV events, CV death and all-cause mortality together with diabetes and age

Paper V

- TGF- β 1, TGF- β 2 and TGF- β 3 were detected in carotid atherosclerotic plaques. TGF- β 2 was the most abundant of the three TGF- β isoforms and TGF- β 2 protein levels were higher in plaques from asymptomatic patients. TGF- β 2 correlated inversely with MCP-1, MMP-9 and was associated with

a stable histological plaque phenotype. TGF- β 2 reduced the gene expression of the pro-inflammatory cytokine MCP-1 and the matrix degrading proteinase MMP-9 in macrophages *in vitro*. Patients with high plaque levels of TGF- β 2 were associated with a lower risk of cardiovascular events.

Concluding remarks and future perspectives

Further exploration of the ECM

The ECM is a large and constantly expanding set of proteins. In 2011 Naba *et al.* used a bioinformatic technique combined with proteomics to define a core “matrisome” consisting of 278 ECM genes and additionally 778 genes coding for “matrisome associated proteins”^{115, 302}. The genes included in the core matrisome contain 43 collagen genes, 35 proteoglycan genes and 200 glycoprotein genes. These genes also include some that do not have a known function in the ECM but have the structural domains characteristics and represent potential novel ECM proteins to be explored. In the matrisome associated proteins, 352 secreted factors (TGF- β , cytokines etc), 250 ECM regulators (MMPs, crosslinking enzymes etc) and 176 ECM affiliated proteins (galectins, mucins) were included.

A limiting factor in the exploration of the ECM is the narrowing of the searchlight towards single proteins or culprit genes. Many findings might reflect association to complex ongoing processes in which the protein examined is only a part. In the case of the double knockout mice for ApoE and mimecan, no difference in the plaque size or composition was seen which could have been due to the compensation of other proteoglycans from the same class working in cooperation to cover up for the loss of mimecan since birth^{303, 304}. The ability to look at whole sets of protein levels simultaneously such as when using proteomics, transcriptomics, lipidomics or metabolomics might reflect the larger picture of what is going on during the atherogenic process³⁰⁵⁻³⁰⁷. The next step is to integrate all of this available data using advanced bioinformatics to develop algorithms which can decipher novel pathways or unravel new potential drug targets.

In this thesis the three SLRPs fibromodulin, lumican and mimecan were examined in atherosclerotic plaques. However, there are multiple interesting proteins that remain unexplored in atherosclerosis patients. Asporin is one interesting SLRP that bind to collagen type I and calcium³⁰⁸, which could potentially affect plaque calcification and is under study by our lab.

When the genome is translated into mRNA the splicing of introns and exons might generate splice variants of proteins. The TGF- β ligands and its receptors have several splice variants, including a splice variant for TGF- β 2^{309, 310}. However, these different splice variants have not been evaluated in atherosclerosis.

Could ECM proteins help to detect the vulnerably plaque?

In this thesis we have shown that proteoglycans correlate with histological plaque features and that several of these proteoglycans can predict future CV events. To be able to test if the levels of these proteoglycans (or other ECM proteins) can determine plaque vulnerability or future CV events they could potentially be measured using non-invasive methods such as for example MRI or ultrasound.

Using specific gadolinium based contrast agents, the amount of specific proteoglycans could potentially be measured as has been the case for elastin-specific contrast agent in detecting plaque disruption in rabbits³¹¹ or using the gadolinium based contrast agent gadoflourine M, which is specific for ECM³¹². Nevertheless, the low amount of several proteoglycans detected in plaques may not be sufficient using the resolutions achieved in clinical MRI machines and MR/CT-PET today. The recently developed collagen type I specific probe CM-101 might be useful in measuring the amount of collagen and thereby potentially detecting stable plaques³¹³. Disadvantages of using imaging with specific probes is that so far, the methods are only semi-quantitative and the low availability of these sophisticated equipments.

Another option for utilizing the ECM proteins to distinguish the patients with a high risk for CV events, would be to measure the ECM proteins in the serum as a biomarker¹¹³. In Paper III and paper IV of this thesis specific degradation neoepitopes were measured in blood from patients operated with endarterectomy, with this vision of detecting novel marker to improve risk stratification.

These markers are unfortunately not plaque-specific and the protein detected in the blood might originate from other non-healthy tissues organs such as kidney, lungs or liver which might also leak proteins to the circulation. Another possible option to measure markers could be in the urine from patients. The neo-epitope marker LG1M is excreted in the urine²⁶⁵ and these alternatives can also be attractive in the future.

Populärvetenskaplig sammanfattning

Åderförkalkning, även kallat ateroskleros, är en sjukdom som drabbar de stora arteriella blodkärlen i kroppen. Ateroskleros orsakar förträngningar i kärlen som kallas för plack. Aterosklerotiska plack utvecklas i det tysta och märks oftast inte förrän de orsakar en akut hjärtinfarkt eller stroke,

De aterosklerotiska placken uppstår när fetter från blodet lagras in i det innersta lagret av kärlväggen. Med tiden kommer de inlagrade fetterna att härskna vilket leder till att deras ytstruktur förändras. Makrofagerna, en typ av celler som ingår i kroppens immunförsvar, försöker äta upp fetterna men kan inte begränsa intaget av de härskna fetterna. Detta leder till att makrofagerna fylls av fetter och bildar så kallade skumceller. Skumcellerna kommer på sikt att dö vilket leder till att det skapas en inflammatorisk miljö som attraherar fler inflammatoriska celler. Då fler celler attraheras till kärlväggen och fler celler dör kommer det aterosklerotiska placket att växa till och det bildas en kärna av döda celler, den nekrotiska kärnan.

Som ett svar på den inflammatoriska reaktionen kommer en annan typ av celler, glatta muskelceller, att börja vandra in från kärlväggens mellersta lager in till placket. Muskelcellerna producerar proteiner och skapar en bindvävshinna, den fibrösa kappan, som anses stabilisera placket. Den fibrösa kappan består till stor del av glatta muskelceller och en typ av bindvävsfiber som kallas kollagen. Den fibrösa kappan skyddar blodet som cirkulerar i kärlet från att komma i kontakt med plackvävnaden. I vissa fall blir dock den fibrösa kappan så tunn, när den bryts ner av inflammatoriska enzymer, att den brister och den underliggande plackvävnaden kommer då i kontakt med det cirkulerande blodet. Detta leder till att blodet leverar sig mycket snabbt och bildar en propp som minskar eller upphäver blodflödet i kärlet. Det minskade blodflödet leder i sin tur till syrebrist i den vävnad som skall förses av syrerikt blod och ger då upphov till hjärtinfarkt och stroke, beroende på vilket kärl som drabbats.

Aterosklerotiska plack har förekommit hos människan sedan lång tid tillbaka i historien och man har funnit plack i kärlen från Egyptiska mumier. Ateroskleros är således ingen ny sjukdom. Däremot kan förekomsten av plack och dess komplikationer ha ökat i samband med att medelöverlevnaden har blivit längre och vår livsstil har förändrats.

Det finns flera faktorer som ökar risken för att man skall bilda aterosklerotiska plack, så kallade riskfaktorer. Faktorer som har visats spela stor roll är diabetes, rökning, högt blodtryck och höga blodfetter. De senaste årtiondena har behandlingen av hjärt- och kärlsjukdomar orsakade av ateroskleros förbättrats och nya akuta och förebyggande behandlingar har tillkommit. Men trots alla framsteg så är hjärt- och kärlsjukdomar fortsatt den vanligaste dödsorsaken i Sverige och världen. Således finns det ett starkt behov av att öka kunskapen kring ateroskleros för att upptäcka nya behandlingsvägar och markörer för att finna personer med hög risk att drabbas.

De aterosklerotiska plack som ligger till grund för forskningen i denna avhandling kommer från patienter som inkluderats i forskningsstudien Carotid Plaque Imaging Project (CPIP). Studien startades 2005 av professor Isabel Goncalves vid Skånes Universitetssjukhus i Malmö och är idag en av världens största aterosklerotiska biobanker.

Patienter med aterosklerotiska förträngningar på halspulsådern kommer till sjukhuset för en operation där man opererar ut det aterosklerotiska placket. Patienter som ska opereras tillfrågas om de vill delta i studien innan operationen och lämnar i samband med detta blodprov som analyseras. När placket avlägsnats under operationen fryses det ner direkt i flytande kväve och förvaras därefter i biobanken tillsammans med blodet. Syftet med CPIP-studien är att utveckla metoder att upptäcka och hitta de aterosklerotiska plack med hög risk att orsaka hjärtinfarkt eller stroke, så kallade vulnerabla plack. Genom obduktionsstudier på patienter som avlidit till följd av hjärtinfarkt eller stroke har man hittat flera kännetecken för de vulnerabla placken. De kännetecken som brukar anges är en stor nekrotisk kärna med mycket fetter, en tunn fibrös kappa, blödningar inuti placket, mycket inflammatoriska celler och få glatta muskelceller.

I denna avhandling fokuserade vi på bindvävens roll i det aterosklerotiska placket. Bindväv (även kallat extracellulär matrix) är ett nätverk av ett stort antal bindvävsproteiner med viktiga funktioner i kroppens vävnader. Dessa bindvävsproteiner kan bilda långa fiber som gör vävnader stabila (liksom balkar och armeringsjärn i byggnader) samt påverka cellers funktion. Bindvävsproteiner utgör stommen i vävnader och skapar specifika miljöer för de cellerna som vistas där. Beroende på vilka bindvävsproteiner som finns i vävnaden får vävnaden således olika egenskaper.

Det mest förekommande bindvävsproteinet i kroppen är kollagen. I dagsläget har man upptäckt 28 olika typer av kollagen. Kollagenfibrer är viktiga för kärlväggens struktur och hållfasthet. Elastiska fibrer är en annan typ av fibrer som ofta också kallas för ”kroppens gummiband” och är viktiga för kärlväggens elasticitet. Proteoglykaner är proteiner som har en eller flera fastbundna sockerkedjor. Proteoglykaner reglerar flera processer i kroppen t.ex. bildningen av kollagenfiber

och inlagringen av blodfetter i kärlväggen. I det första delarbetet undersökte vi de två proteoglykanerna fibromodulin och lumican i 153 aterosklerotiska plack med hjälp av immunohistokemiska färgningar. Fibromodulin och lumican förekom båda rikligt i de aterosklerotiska placken. Fibromodulin förekom i större områden i plack som orsakat symptom och i plack från patienter med diabetes. Färgningarna för fibromodulin och lumican korrelerade med färgningarna för fetter och blödning, två viktiga kännetecken för vulnerabla plack. Fibromodulin färgningen korrelerade även med låga nivåer av färgning för glatta muskelceller. Dessutom korrelerade färgningarna för fibromodulin och för lumican med en ökning av inflammatoriska proteiner. Under uppföljningen fann vi också att de patienter med mer fibromodulin i placken hade en högre risk att drabbas av stroke. Fynden talar för att de två studerade proteoglykanerna är associerade med en farligare typ av plack med mer fetter, inflammation och blödningar.

I det andra delarbetet färgade vi aterosklerotiska plack för proteoglykanen mimecan. Mimecan-färgningen korrelerade med färgningarna för fetter, blödning samt makrofager (samtliga är kännetecken för vulnerabla plack). Vi såg även att mimecan-färgningen korrelerade med låga nivåer av färgning för glatta muskelceller. Mimecan-färgningen korrelerade även med nivåer av det inflammatoriska proteinet MCP-1 och det bindvävsnedbrytande enzymet MMP-9. När vi stimulerade odlade makrofager med MCP-1 kunde vi även se att detta ledde till en ökning av mimecan uttryck. Slutligen såg vi att de patienter med mer mimecan i placken hade en ökad risk att dö till följd av kardiovaskulära komplikationer. Detta talar för att syntesen av mimecan stimuleras av en inflammatorisk miljö och att ökade nivåer av mimecan är kopplat till patienter med högre risk att drabbas av kardiovaskulära komplikationer.

I det tredje delarbetet mätte vi två markörer som reflekterar nedbrytning av de två bindvävsproteiner, kollagen typ IV och laminin, i blodet från 787 patienter. Laminin och kollagen typ IV finns i en tunn bindvävshinna (basalmembran) som finns runt omkring glatta muskelceller och under endotelceller i kärlväggen. Hos patienter med höga nivåer av markörerna för nedbrytning av kollagen typ IV och laminin sågs högre nivåer av den inflammatoriska markören CRP. De två markörerna var också korrelerade med flera bindvävsnedbrytande enzymer, så kallade matrix metalloproteinaser (MMPs). Under uppföljningen såg vi att patienter med höga nivåer av markörerna för nedbrytning av kollagen typ IV och laminin hade en högre risk för framtida död av alla orsaker men ingen ökad risk att drabbas framtida kardiovaskulär död eller kardiovaskulära komplikationer (t.ex. stroke eller hjärtinfarkt). Sammantaget är dessa markörer troligen inte specifika för den aterosklerotiska sjukdomen men kan fortfarande indikera samsjuklighet och en högre risk för död.

I det fjärde delarbetet mätte vi en markör som reflekterade nedbrytning av kollagen typ I, i blodet från 787 patienter. Kollagen typ I är tillsammans med kollagen typ III den vanligaste kollagentypen i aterosklerotiska plack. Patienter med höga nivåer av markören för kollagen typ I nedbrytning hade högre nivåer av CRP, kolesterol och en sämre njurfunktion. Patienter med höga nivåer av markören för kollagen typ I nedbrytning hade även en ökad risk att drabbas av kardiovaskulär död, kardiovaskulära komplikationer samt död av alla orsaker. Resultaten visar att cirkulerande nivåer av markören för nedbrytning av kollagen I kan fungera som en markör för att identifiera patienter med högre risk för både kardiovaskulära komplikationer och död.

Bindvävsproteiner tillverkas av de celler som finns i placken och olika proteiner kan stimulera cellerna att producera bindväv. Ett exempel på sådana proteiner är TGF- β som vi har studerat i det femte delarbetet. I det femte arbetet analyserade vi tre olika TGF- β proteiner: TGF- β 1, TGF- β 2 och TGF- β 3 i 223 plack. Samtliga TGF- β proteiner detekterades i plackvävnaden men TGF- β 2 påvisades i markant högre nivåer. TGF- β 2 nivåer var tydligast sammankopplade med aterosklerotiska plack som inte orsakat symptom (asymtomatiska plack). TGF- β 2, men inte TGF- β 1 och TGF- β 3, var högre i asymtomatiska plack i jämförelse med symptomatiska plack. Nivåerna av TGF- β 2 var associerade med mer glatta muskelceller, mindre plackblödning, lägre nivåer av det inflammatoriska proteinet MCP-1 samt lägre nivåer av det bindvävsnedbrytande enzymet MMP-9. Genom att förbehandla makrofager med TGF- β 2 kunde vi se att deras nivåer av MCP-1 och MMP-9 minskade när de exponerades för inflammatoriskt stimuli. Dessutom kunde vi påvisa att patienter med höga nivåer av TGF- β 2 hade en lägre risk att drabbas av framtida kardiovaskulära händelser. Tillsammans talar således våra fynd för att TGF- β 2 kan vara en viktig faktor för att bromsa den inflammatoriska processen i placket och att TGF- β 2 kan bidra till ett stabilare plack med lägre risk för att brista och orsaka komplikationer.

Sammanfattningsvis visar vi i denna avhandling att proteiner som bygger upp bindväven, markörer för nedbrytning av bindväven och proteiner som stimulerar produktion av bindväv kan ha både skyddande och skadliga effekter i den aterosklerotiska processen. Fynden i studierna talar för att en balans i omsättning, nybildning och nedbrytning av bindväven i de aterosklerotiska placken är viktigt för plackens stabilitet. Slutligen indikerar våra fynd att de olika komponenterna kan fungera som markörer för att hitta individer med hög risk för död men också som möjliga framtida behandlingsmål för att förhindra kardiovaskulära komplikationer.

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About the author



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