Immi Kormi

TRANSLATIONAL PERSPECTIVES ON MATRIX METALLOPROTEINASE 8 AND OTHER INFLAMMATORY BIOMARKERS IN CARDIO-VASCULAR DISEASES
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OTHER INFLAMMATORY BIOMARKERS
IN CARDIOVASCULAR DISEASES

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Cardiovascular diseases (CVD), and especially atherosclerotic vascular diseases (ASVD), are the largest cause of morbidity and premature death worldwide. Coronary heart disease (CHD) and cerebrovascular disease (stroke) are common and severe manifestations of ASVD.

Atherosclerosis is a chronic inflammatory disease and lipoprotein metabolism disorder. If the regulation of inflammatory process is disturbed, the systemic release of pro-inflammatory mediators, including matrix metalloproteinases (MMPs), may lead to a low-grade systemic inflammation, which is a risk factor for CVDs. MMPs are enzymes that are responsible for the degradation of the extracellular matrix (ECM) during growth and tissue renewal but also in many pathological conditions. These ECM degrading proteases and their regulators play an important role in atherogenesis and subsequent plaque rupture, leading to acute cardiovascular manifestations. The pivotal role of MMPs in atherosclerosis has raised interest in the development of drug therapies targeting these proteases. Doxycycline has inhibitory effects on some MMPs in addition to its antimicrobial properties.

The main objective of this thesis project was to investigate the potential of these inflammatory mediators as biomarkers, risk factors, and therapeutic targets in CVD. The special focus was on MMP-8 and its main regulator, tissue inhibitor of matrix metalloproteinase (TIMP)-1.

The results of this study show that a high serum MMP-8 concentration indicates an acute cardiac condition and predicts a future CVD event. In addition to MMP-8, MMP-7 is a potential biomarker for incident CVD. The balance between these MMPs and their tissue inhibitor may indicate vulnerability to plaque rupture. Measurement of serum MMP-8 concentration is reliable, anti-invasive and inexpensive and can be done in hospital settings. We also show that regular-dose doxycycline decreases the systemic inflammatory burden in patients with earlier myocardial infarction and is a promising anti-inflammatory therapy in the prevention of CVDs with relatively minor side effects.

In conclusion, MMP-8 and TIMP-1 can be considered inflammatory risk markers of CVD events and death, and they can be utilized both for diagnostic and screening purposes. The inhibition of MMP-8 by doxycycline may reduce the systemic inflammatory burden in patients with myocardial infarction.

Keywords: atherosclerosis, biomarkers, cardiovascular disease risk factors, low-grade inflammation, MMP-8, TIMP-1
Kormi, Immi, Translationaalisia näkökulmia matriksin metalloproteinaasi 8:aan ja muihin tulehdussellisiin biomarkereihin sydän- ja verisuonisairauksissa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Oulun yliopistollinen sairaala; Helsingin yliopisto, Lääketieteellinen tiedekunta; Helsingin yliopistollinen keskussairaala

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Tiivistelmä

Sydän- ja verisuonisairaudet, erityisesti aterioskleroottiset valtimosairaudet, ovat maailman yleisin sairastuvuuden ja ennenaikaisen kuoleman syy. Sepelvaltimitautti ja aivohaveri ovat aterioskleroottisen valtimosairauden yleisiä ja vakavia ilmenemismuotoja.

Aterioskleroosi on krooninen tulehdus sairaus ja lipoproteinsaarevainhundann häiriö. Jos tulehdustapahtuma häiriintyy, elimistöön vapautuvat tulehdusvälineet, kuten matriksin metalloproteinaasit (MMP), voivat aiheuttaa elimistön matalaaasteisen tulehduksen, joka on sydän- ja verisuonisairauksien riskitekijä. MMP:t ovat entsyymejä, jotka pilkkovat solunvälis- 
anetettä kasvun ja kudosten uusiutumisen mutta myös monien tautitilojen yhteydessä. Nämä solu-
välisäinetta hajottavat proteaasit ja niiden säätelijät ovat tärkeässä roolissa aterioskleroottisen 
plakin muodostumisessa ja repeämisessä, joka johtaa äkillisiin sydänkohtauksiin. Matriksin 
metalloproteinaasien keskeinen rooli aterioskleroosissa on herättänyt kiinnostuksen uhan 
kohdistuvaan tulehdusvälittämiseen. Doksisykiinillä on joidenkin MMP-entsyymien toiminta 
estä kykenevä antimikrobialisten ominaisuuksien lisäksi.

Tämän väitöskirjan tutkimuksen päätavoitteena oli tutkia näiden tulehdusvälittäjäaineiden 
kuuluvuutta ja käyttöä sydän- ja verisuonisairauksissa. Erityisen kiinnostuksen kohde oli MMP-8 ja sen pääsääntäjä ja kudosestäjä, tissue 
inhibitor of matrix metalloproteinase (TIMP)-1.

Tämän tutkimuksen tulokset osoittavat, että seerumin korkea MMP 8 pitoisuus viittaa akuut- 
iihin sydäntautiin ja ennakoi tulevaa sydäntautitapahtumaa. MMP-8:n lisäksi MMP-7 on lupaava 
sydäntautitapahtuman biomarkkeri. Niden matriksin metalloproteinaasien ja niiden kudossääntöjä 
TIMP-1:n välinen tasapaino voi liittyä aterioskleroottiin plakin haurauteen. Seerumin MMP-8:n 
mittaus on luotettavaa, kajoamantona ja edullista, ja mahdollista toteuttaa myös sairaalaolosuhtei- 
teissa. Näyttää olevan muutos, että doksisykiinillä vähentää elimistön tulehdustaakkaa sydäntäirtikin 
sairastaneilla potilailla ja että se on sydäntautien ehkäisyssä lupaava anti-inflammatorinen lää-
ke, jolla on suhteellisen vähän sivuvaikutuksia. 

Johtopäätöksenä on, että MMP-8:aa ja TIMP-1:tä voidaan pitää lupaavina sydän- ja verisuoni- 
tauttien sekä koelaman biomarkeereina sekä diagnostiikka- että seuontakäytössä. Lisäksi tutki-
mustulokset osoittavat, että MMP-8:n esto doksisykiinillä voi vähentää elimistön tulehdustaak- 
kaa sydänkohtauksen sairastaneilla potilailla.

Asiasanat: aterioskleroosi, biomarkkerit, matala-asteinen tulehdus, MMP-8, sydän- ja verisuonisairauksien riskitekijät, TIMP-1
Acknowledgements

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Oulu, March 2017

Immi Kormi
Abbreviations

AAA abdominal aortic aneurysm
apo apolipoprotein
ACE angiotensin converting enzyme
ACS acute coronary syndrome
AMI acute myocardial infarction
ASVD atherosclerotic vascular disease
BM basement membrane
BMI body mass index
BSA bovine serum albumin
CAD coronary artery disease
CHD coronary heart disease
CI confidence interval
CMIA chemiluminescent microparticle immunoassay
CRP C-reactive protein
CV coefficient of variation
CVD cardiovascular diseases
DALY disability-adjusted life year
DTPA diethylene-triamine-penta-acetic acid
EC endothelial cell
ECG electrocardiogram
ECM extracellular matrix
ELISA enzyme-linked immunoabsorbent assay
γ-GT gamma-glutamyl transferase
GWAS genome-wide association study
HDL high-density lipoprotein
HOCl hypochlorous acid
HR hazard ratio
hsCRP high-sensitivity C-reactive protein
HUCH Helsinki University Central Hospital
IDI integrated discrimination improvement
IDL intermediate-density lipoprotein
IFMA immunofluorometric assay
IHD ischemic heart disease
IL interleukin
IQR inter-quartile range
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LPA</td>
<td>lipoprotein(α)</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MeSH</td>
<td>medical subject heading</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NE</td>
<td>neutrophil elastase, PMN elastase</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NRI</td>
<td>net reclassification improvement</td>
</tr>
<tr>
<td>NS</td>
<td>not statistically significant</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>non-ST elevation myocardial infarction</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDD</td>
<td>subantimicrobial-dose doxycycline</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST elevation myocardial infarction</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>UAP</td>
<td>unstable angina pectoris</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


*The authors contributed equally to the study.

Publication IV has been included in the doctoral thesis of Hatem Alfakry.
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1 Introduction

Cardiovascular diseases (CVD) is the predominant disease group in the adult population and the most common cause of death in Finland. During the last decades, many preventable and treatable risk factors for CVD have been recognized. Because of successful awareness campaigns and advanced therapies, the prognosis of CVD patients has improved measurably. Still, nearly half of the premature deaths of working-age people are caused by cardiovascular diseases. Many of these fatal CVD outcomes occur without prior symptoms.

High age, male sex, high blood pressure, smoking, dyslipidaemias and diabetes are well known risk factors for CVD, among many others. At tissue level, it is known that the vulnerability of atherosclerotic plaques is the key risk factor. A rupture of an atherosclerotic plaque and thrombus formation is the main cause of acute myocardial infarction and stroke. In atherogenesis, inflammation plays a pivotal role, affecting all stages of the development and progression of CVD. The inflammatory process contributes to lipid accumulation and subsequent macrophage foam cell formation, as well as remodelling and rupture of atherosclerotic plaques. Plaques are more prone to rupture when they are characterized by thin, highly inflamed, and collagen-poor fibrous caps and contain elevated levels of proteases, including metalloproteinases (MMPs).

MMPs, earlier also known as matrixins (Nagase & Woessner 1999), are genetically different but structurally related group of enzymes that can collectively process all extracellular matrix (ECM) protein components. They are also able to affect cellular processes and modify immune responses by regulating a wide range of non-matrix bioactive substrates, including chemokines, cytokines, serpins, and cell signalling mediators. (Sorsa et al. 2006). The controlled action of MMPs is part of physiological tissue turnover and development, but disturbed proteinase balance is linked to destructive inflammatory diseases and conditions, such as rheumatoid arthritis (Sorsa et al. 1992) and premature birth (Becher et al. 2010). Furthermore, the expression of MMPs is upregulated by the other inflammatory mediators involved in atherosclerosis (Bäck 2010). In atherosclerotic plaques, MMPs localize in the vulnerable shoulder regions (Galis et al. 1994). The proteolytic effect of MMPs weakens the protective cap covering the plaque, which increases the risk of rupture and subsequent coronary outcome.

Because of their strong proteolytic potential, the expression and function of MMPs is closely regulated. The main regulators of MMPs are tissue inhibitors of metalloproteinases, TIMPs. It has been suggested that the balance of MMPs and
their inhibitors is crucial in the development and progression of atherosclerotic diseases. Expression of TIMPs is increased in patients with an acute coronary syndrome (ACS) (Inokubo et al. 2001), and TIMPs are an independent predictor of cardiovascular events in patients with prevalent CVD (Lubos et al. 2006).

The role of these tissue-degrading proteinases as risk factors for CVD was of particular interest of this study. The special focus is on MMP-8. The measurement of inflammatory biomarkers in the blood has been recommended as a non-invasive and cost-effective tool for diagnosis and monitoring of cardiovascular diseases. (Zucker et al. 1999). Despite of the strong evidence showing the important role of MMP-8 in atherogenesis, there are still few studies investigating its significance in the assessment of cardiovascular risk and use as a therapeutic target. The study was expected to provide novel knowledge about the role and regulation of MMP-8 in CVD.
2 Review of the literature

2.1 Atherosclerosis and cardiovascular diseases

Cardiovascular diseases (CVD) refers to a group of conditions that involve pathologies of the heart and circulatory system. Four major groups of CVDs are arteriosclerosis, vasculitides, tumours, and congenital and acquired structural anomalies such as vascular aneurysms. The disease category is wide, and pathophysiology varies depending on the disease in question. The main cause of CVDs is arteriosclerosis, also known as atherosclerotic vascular disease (ASVD), which is a slow, progressive loss of arterial elasticity due the thickening of the arterial walls. (Kumar et al. 2005)

The predominant manifestation of arteriosclerosis is atherosclerosis. It is a slow, chronic disease characterized by inflammation and accumulation of lipid plaques in the intima layer of medium- and large-sized arteries. The other forms of arteriosclerosis are arteriolosclerosis, which is a hypertension-related disease of the small arteries, and Mönckeberg arteriosclerosis, where calcium deposits are found in the tunica media of medium- and large-sized arteries. The collection of the plaque thickens and reduces the elasticity of the artery walls. The symptoms and complications of atherosclerosis occur in the late, advanced phases of the disease. At the final stages, accumulation of plaque may narrow the arterial lumen and cause impaired blood flow. In coronary arteries, the plaque stenosis is usually more than 50% of the lumen diameter, until reduced blood flow causes chest pain symptoms during exertion (Grech 2003).

A severe complication of atherosclerosis is infarction, a sudden rupture of a soft plaque in coronary or cerebrovascular artery, causing subsequent thrombus formation and ischemia of surrounding tissues. In lower limbs, atherosclerosis can manifest as claudication, which is an ischemia due to poor blood flow. Many of the complications, even infarctions, may be ‘clinically silent’, i.e. occur without sensible symptoms.
2.1.2 Overview and epidemiology of different CVD manifestations

Cardiovascular diseases cause deaths and severe injuries more than any other disease group both in Finland and in Europe. It is estimated that the main cause of CVDs, atherosclerosis, causes annually more than 17 million deaths worldwide (WHO 2011, Dahlöf 2010) and 47% of all deaths in Europe (http://ehnheart.org/cvd-statistics.html). The number of CVD deaths is increasing, mainly due to heart diseases and stroke. (WHO 2011, Mathers & Loncar 2006) In addition to death, CVDs can manifest as coronary artery diseases (CADs), such as ischemic heart disease (IHD) and myocardial infarction (MI), arrhythmias, heart failure and stroke.

In Finland, CVD mortality has declined remarkably in the last 40 years. Nevertheless, because of the high mortality rates of the 1970s, especially the CAD mortality rate is still quite high in Finland compared to most other Western countries (Salomaa et al. 2016). Opposite to the global trend, it is estimated that the favourable development of CVD mortality rates is likely to continue in Finland (Salomaa et al. 2013).

CAD

Coronary artery disease (CAD), which is also known as coronary heart disease (CHD) and ischemic heart disease (IHD), is a major group of atherosclerotic diseases and includes stable and unstable angina, silent ischemia, myocardial infarction (MI), and sudden cardiac death. In CAD, atherosclerotic plaques grow within the walls of coronary arteries. CAD is the most common cause of premature death in the world. Each year, approximately 3.8 million men and 3.4 million women die of CAD, and in 2020, it is estimated that it will be the cause a total of 11.1 million deaths globally (Mathers & Loncar 2006). In addition to its mortality burden, CAD is a leading cause of disability-adjusted life years (DALYs) and can therefore result in high expenditures (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators 2016). For example, in 2003 CAD-related costs totalled approximately EUR 45 billion in the EU (Leal et al. 2006).

MI

Myocardial infarction, or acute myocardial infarction (AMI), commonly known as a heart attack, is a severe manifestation of CAD. The cause of MI often
involves the complete blockage of a coronary artery caused by the rupture of an atherosclerotic plaque. Myocardial infarctions are generally classified into ST elevation MI (STEMI) and non-ST elevation MI (NSTEMI).

Worldwide, approximately 8.6 million MIs occurred in 2013 (Global Burden of Disease Study 2013 Collaborators 2015). MI causes more than a third of deaths in developed nations annually (Yeh et al. 2010), and it affects more than seven million individuals worldwide each year. The economic expenses are high: for example, in 2010 more than 1.1 million US hospitalizations were caused by myocardial infarction, with direct costs of at least USD 450 billion (Weintraub et al. 2011).

**Fig. 1.** ECG of a 79-year-old man who was brought to the emergency department because of prolonged shortness of breath. The ST segment elevations and deep Q waves in leads V2–V6 show myocardial infarction.

**Stroke**

Stroke is a cerebral accident caused by insufficient blood flow to the brains. The two main types of stroke are ischemic, due to central nervous system infarction, and haemorrhagic, due to intracerebral haemorrhage. Stroke is the second most common cause of death and a major cause of disability worldwide. The incidence of stroke has remained stable, and as a result of advanced therapies, the mortality has declined over the past decades (Hankey 2016). Still, because of the ageing population, the number of incident strokes, prevalent stroke survivors, disability-
adjusted life years (DALYs) lost due to stroke, and stroke-related deaths will increase greatly during the next years, especially in developing countries. In 2010, there were approximately 16.9 million incident strokes, which caused 5.9 million deaths worldwide. In the same year, it was estimated that 102 million DALYs were lost, making stroke the third leading cause of DALYs lost worldwide. (Feigin et al. 2014)

![Computed tomography shows an ischaemic stroke in the territory of the left middle cerebral artery of a 60-year-old female. Patient had several risk factors for stroke including high blood pressure, cigarette smoking and diabetes.](image)

Fig. 2. Computed tomography shows an ischaemic stroke in the territory of the left middle cerebral artery of a 60-year-old female. Patient had several risk factors for stroke including high blood pressure, cigarette smoking and diabetes.
2.2 Pathogenesis of atherosclerosis

Atherosclerosis is a lipoprotein-driven disease that leads to plaque formation at focal areas in the arteries through intimal inflammation, necrosis, fibrosis, and calcification. (Usman et al. 2015) Atherogenesis, or the build-up of atheromatous plaque, is a slow process developing over several years through a complex series of cellular events occurring within the arterial wall. In the last decades, it has been shown that atherosclerosis is both a chronic inflammatory disease and a lipid metabolism disorder (Ross 1999, Lusis 2000, Libby 2002, Libby & Aikawa 2002). Inflammation is a complex protective immunovascular response to damaging stimuli such as microbes and damaged cells. (Ferrero-Miliani et al. 2007) The function of inflammation is to eliminate the initial cause of cell injury, to clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. If regulation of the inflammatory process is disturbed, the systemic release of bacteria, bacterial products and pro-inflammatory mediators, including matrix metalloproteinases (MMPs), C-reactive protein (CRP), tumour necrosis factor (TNF-α), and myeloperoxidase (MPO) among others, may lead to low-grade systemic inflammation. Inflammatory mediators and cells play a crucial role in all stages of atherogenesis, including the increased vulnerability of an atherosclerotic plaque to rupture. (Ross 1999, Lockhart et al. 2012)

2.2.1 Arterial wall structure

The vascular wall of muscular arteries is composed of three layers: intima (lined by endothelium), media and adventitia. The inner layer, i.e. the intima, consists of a monolayer of endothelial cells (ECs) facing the lumen and thin connective tissue with few smooth-muscle cells (SMC) supporting it. The intima is mainly composed of extracellular matrix (ECM) components, namely collagens, predominantly of type I and III, and proteoglycans, elastin and matrix glycoproteins. The integrity of the endothelial layer is crucial in the maintenance of vascular functions since damage to it may cause adherence of circulating white blood cells (WBC) or initiation of thrombus formation. The intima is separated from the media by an internal elastic lamina. (Standring 2016, Kumar et al. 2005, Lusis 2000)

The fibro-muscular middle layer, i.e. the tunica media, consists mostly of SMCs and elastic fibres and is the thickest layer of the arterial wall. It is lined
from the adventitia by an external elastic lamina, containing type IV collagen. The surrounding outermost layer, the tunica adventitia, consists of a loose mesh of connective tissue and some elastic fibres which join the adventitia to the adjacent tissues. The adventitia is rich in lymphatic veins, vasa vasorum and nerve fibres.

Fig. 3. Progression of atherosclerosis. (a) LDL-cholesterol enters the intima and undergoes modification to oxLDL. OxLDL stimulates endothelial cells to express adhesion molecules and chemokines that attract circulating macrophages. The monocytes transmigrate the intima and differentiate into macrophages. (b) Macrophages uptake oxLDL and transform into foam cells. Foam cells secrete cytokines and growth factors and recruit circulating leukocytes. (c) The growing plaque promotes neovascularization. The migration of smooth muscle cells stabilizes the growing fibrous plaque. (d) The foam cells undergo apoptosis and release their debris and lipids, which leads to the formation of necrotic core. Modified from (Steinl & Kaufmann 2015). Reprinted with the permission of MDPI Publishing Services.

2.2.2 Initiation of atherosclerotic lesion

Continuous high levels of circulating lipoprotein particles, LDL, IDL and VLDL, initiate their accumulation to arterial walls. The invasion of these particles is possible only if the endothelium is injured, for example, because of hypertensive
physical stress, hyperglycaemia, cigarette smoke constitutes, or pathogen irritation. (Libby 2002, Ross 1993, Ross 1999, Avogaro et al. 2011). Endothelial dysfunction leads to the expression of adhesion molecules. That attracts circulating leukocytes, especially T-lymphocytes and monocytes, which adhere to ECs and subsequently migrate to the intima and mature to macrophages. The differentiated macrophages phagocytize oxidized LDL and convert into lipid-laden foam cells. The foam cells secrete various cytokines and growth factors and induce further recruitment of circulating leukocytes, activation of T-lymphocytes in the intima, and migration of smooth muscle cells from the media into the intima (Glass & Witztum 2001, Libby 2002, Ross, 1999, Hansson & Hermansson 2011). After the migration into the intima, smooth muscle cells change from the contractile to proliferative phenotype and undergo proliferation. In the intima, they can secrete extracellular matrix proteins, particularly type I and III collagens and proteoglycans (Newby 2006). An initial atherosclerotic lesion is called a fatty streak. Fatty streaks are asymptomatic and present already in the blood vessels of children and adolescents, but do not necessarily lead to atherosclerosis. (Stary 1994)

2.2.3 Lesion progression and development

If the blood LDL levels are continuously high, the imbalance between the influx and efflux of cholesterol and the continuing accumulation of LDL, macrophage-foam cells, T-lymphocytes, and SMCs lead to the development and gradual growth of atherosclerotic plaque. Especially the macrophage foam cells conduct the further progression and destabilization of the plaque: secretion of cytokines and chemokines, generation of reactive oxygen species (ROS), presentation of immune activation markers to lymphocytes or macrophage scavenger receptor, and production of matrix-degrading proteases, including MMPs. The accumulation of macrophage foam cells may lead to their apoptosis or necrosis, which causes release of inflammatory debris into the plaque. The foam cell remnants form the necrotic core of the atherosclerotic lesion. The migrated SMCs secrete extracellular components such as collagens and proteoglycans, and form a subendothelial fibrous cap to cover the lesion.
2.2.4 Plaque rupture

The atheromatous plaques are classified on the basis of their vulnerability status as low-risk or stable plaques and high-risk or unstable plaques. A consensus paper on the definition of vulnerable plaques describes the characteristics of unstable plaques: a thin fibrous cap with a large lipid core, active inflammation, endothelial denudation with superficial platelet aggregation, a fissured plaque, or stenosis of more than 90% luminal narrowing. Additional criteria include intraplaque haemorrhage, superficial calcified nodule, and positive observable remodelling. (Naghavi et al. 2003)

If the inflammatory circumstances in the atherosclerotic lesion continue, the fibrous cap of the atherosclerotic plaque weakens especially at the fragile shoulder areas of the lesion. The cap can resist the function of several proteases, but activated macrophages, SMCs and endothelial cells of atherosclerotic lesions express matrix metalloproteinases (MMPs) that are able to degrade its collagen composition and inhibit collagen synthesis.

Rupture of the collagen cap exposes and releases the core of the lesion to arterial lumen and causes subsequent coagulation and thrombus formation. Vulnerability of atheromatous plaques is the reason for approximately 60% of symptomatic CADs and about 75% of acute coronary events. (Usman et al. 2015) Most of the remaining MIs result from endothelial erosion (Virmani et al. 2006). Both the pathogenesis and the risk factors for endothelial erosion differ from those of atherosclerotic plaque rupture, and endothelial erosion is more common among females (Virmani et al. 2000, Arbustini et al. 1999, Burke et al. 2001). Overproduction of MMPs may be an underlying cause of endothelial erosion. (White et al. 2016)

2.3 Risk factors for atherosclerosis

The pathophysiology of atherosclerosis is complex and multifactorial and varies in different manifestations. There are several well-known risk factors which can contribute to the initiation and progression of atherosclerosis. These risk factors can be divided into non-modifiable, modifiable and environmental factors. The non-modifiable risk factors include family history of atherosclerotic diseases, increasing age and male gender. The modifiable risk factors with a strong hereditary background comprise dyslipidaemias including high serum LDL, VLDL or lipoprotein(a) (Lp(a)) concentrations, low serum HDL concentration,
metabolic syndrome, diabetes, obesity, depression, and hypertension, which is one of the main risk factors for stroke. The environmental risk factors include high-fat and high-sugar diets, smoking, some infectious diseases, and lack of exercise, among many others. Most of the risk factors have both genetic and acquired components. (Lusis 2000) Socioeconomic status monitored by years of education and, especially in Finland, geographic area are considered considerable confounders of CVDs. (Laatikainen 2000, Havulinna et al. 2008).

Population-based risk stratification scoring systems based on these traditional risk factors are widely considered as useful clinical tools in risk assessment and primary prevention of CVDs, both globally and in Finland (Lloyd-Jones et al. 2004, Ridker et al. 2007, Vartiainen 2007). Nevertheless, some studies have shown that scoring systems are unreliable in the prediction of CVD risk or incident CHD events (DeFilippis et al. 2015, Yeboah et al. 2012). CVD events may occur without any known traditional risk factors, symptoms or signs (Khot et al. 2003, Fazzini et al. 1993). On the other hand, some individuals who never develop CVD may have one or more risk factors (Greenland et al. 2003). This has provided the rationale for the research of novel biomarkers and risk factors to improve both the understanding of the pathophysiology of CVDs and the development of clinical practices. In addition, biomarkers and other objective measurements are more reliable than questionnaires that are based on subjective interpretations.

Fig. 4. The basic protein structure of MMPs. (a) This structure is the most common among MMPs and seen in MMPs -1, -3, -8, -10, -12, -13, -18, -19, -20, -22, and -27. Other MMPs may lack this domain or have additional domains. (b) The ‘minimal-domain’ MMPs -7 and -26 have only the pre-, pro- and catalytic domains. The following abbreviations are used in the figure: ‘Pre’ refers to the pre-domain or N-terminal signal
peptide that directs MMPs into the endoplasmic reticulum; ‘Pro’ to the pro-domain with zinc-interacting thiol (SH) group; ‘S---S’ means the disulphide bond; and ‘Zn2+’, refers to catalytic zinc. Modified from Egeblad et al. 2002, Parks et al. 2004 and Sternlicht & Werb 2001.

2.4 Matrix metalloproteinases

2.4.1 Classification of MMPs

The group of MMPs is genetically distinct but structurally and functionally related, and consists of a total of 24 vertebrate MMPs, 23 of which have been found in humans (Visse & Nagase 2003). According to their domain organization, substrate specificity, and sequence similarity, MMPs can be classified into nine different subgroups: (1) collagenases (MMPs -1, -8 and -13), (2) gelatinases (-2 and -9), (3) stromelysins (-3 and -10), (4) stromelysin-like MMPs (-11 and -12), (5) matrilysins (-7 and -26), (6) transmembrane MMPs (-14, -15, -16 and -24), (7) glycosyl-phosphatidyl-inositol (GPI)-type MMPs (-17 and -25), (8) MMP-19-like MMPs (-19 and -28), and (9) other MMPs (-18, -20 and -23). (Klein & Bischoff 2011, Nissinen & Kähäri 2014) (Table 1 and Figure 4). There are also alternate classification systems of MMPs; for example, classification according to their domain structure (Sternlicht & Werb 2001). For example, MMP-8 is sometimes included in the interstitial collagenase group with MMPs -13 and -14, and MMPs -7 and -11 in the stromelysin group in addition to MMPs -3 and -10. (Newby 2012)

2.4.2 Regulation of MMPs

The role and function of MMPs have been investigated for decades, but their mechanisms of actions have not been completely characterized. (Noël et al. 2008, Page-McMcCaw et al. 2007). Because of their strong proteolytic potential, the MMP levels and activity is maintained at low levels in healthy tissues, and their regulation is rigidly coordinated. The regulation takes place at least at four different levels (Figure 4):

1. Gene expression

MMP gene expression is mainly regulated at the transcriptional level, and it is regulated by several stimulatory and suppressive factors such as hormones,
growth factors and pro-inflammatory cytokines. There is also post-transcriptional regulation, which is based on mRNA stabilization.

2. Compartmentalization

Compartmentalization enables regulating accumulation of MMPs in the different sites in cytoplasm. The concentration of MMPs close to the potential substrates regulates their activity efficiently, for example, by enabling a rapid release of MMPs from exocytic vesicles to extracellular space.

3. Zymogen activation

All MMPs are produced as inactive prepro-enzymes. Most of them are secreted into the ECM, and their N-terminal signal peptide (‘pre-domain’) is cleaved off. The activation of this latent pro-form, also called zymogen, requires further removal of hydrophobic pro-domain. The pro-domain includes a ‘cysteine switch’, a conserved cysteine residue (Van Wart & Birkedal-Hansen 1990). As long as the cysteine residue interacts with the catalytic site zinc ion, it stays in a catalytically inactive state. The exposure of the catalytic site can happen by several mechanisms, the most important of which is the direct cleavage by other proteinases, such as PMN elastase, reactive oxygen species (ROS) produced by MPO, and microbial proteinases. MMPs can also activate each other to form proteolytic activation cascades (Sorsa et al. 2006). The cleavages enable a water molecule, which is essential for MMPs catalytic functions, to bind to the Zn²⁺ (Nagase & Woessner 1999).

4. Enzyme inactivation

General protease inhibitors, such as α-macroglobulin and tissue factor pathway inhibitor-2, can endogenously inhibit MMP activity in tissues. Still, the most important physiological inhibitors are the specific tissue inhibitors of MMPs (TIMPs). In vertebrate tissues, altogether four TIMPs (TIMPs -1, -2, -3 and -4) have been identified. Their expression is tightly regulated and they affect many physiological and pathological conditions alongside MMPs. TIMPs have biological functions independent of their MMP inhibition.
<table>
<thead>
<tr>
<th>Enzyme subgroup</th>
<th>MMP</th>
<th>Molecular weight latent/active (kDa)</th>
<th>Alternative names</th>
<th>Main substrates</th>
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<td>unknown</td>
<td>-</td>
<td>Gelatine and casein in chickens</td>
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</tbody>
</table>

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MMPs are regulated at least at four levels: 1. Gene expression at transcriptional level but also post-transcriptionally based on the mRNA stability; 2. compartmentalization; 3. zymogen activation; and 4. enzyme inactivation.

2.5 Inflammatory biomarkers of CVD

The term ‘biomarker’, or ‘biological marker’, was introduced in 1989 as a Medical Subject Heading (MeSH) term: ‘measurable and quantifiable biological parameters (e.g. specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiologic studies, etc.’ Later, biomarker was defined by the National Institute of Health (NIH) as a characteristic that is objectively measured.
as an indicator of normal biologic processes, pathogenic processes or pharmacologic response to a therapeutic intervention (Biomarkers Definition Working Group 2001). In CVDs, molecular biomarkers are commonly utilized for three purposes: (1) screening biomarkers to identify vulnerable patients, (2) as diagnostic biomarkers to identify ischemia or injury, and (3) as prognostic biomarkers to recognize patients who can benefit from certain treatments.

**MMPs as CVD biomarkers**

In CVD events, initiation of collagen breakdown in plaques requires MMP family members. Compared to other proteinases, MMPs have probably attracted most as they directly degrade ECM components and are efficient at neutral pH. (Newby et al. 2009) Because of that, the measurement of MMPs in the blood has been suggested as a non-invasive diagnostic and monitoring tool in CVDs. (Zucker et al. 1999). MMPs contribute to several steps of atherogenesis in concert with serine proteinases and cysteinyl cathepsins. Of the known MMPs, at least 11 have been explored in the context of atherothrombosis (Bäck et al. 2010). MMPs are present in: (1) migration of circulating WBCs into the arterial intima, which leads to the degradation of subendothelial basement membrane proteins and interstitial collagens (Gong et al. 2008, Laxton et al. 2009, Matias-Roman et al. 2005, Newby 2008, Wagsater et al. 2009); (2) migration of smooth muscle cells from the media into the intima and within the intima (Newby 2006); (3) processing of ECM proteins that are involved in atherogenesis (Laxton et al. 2009, Newby 2006, Nissinen & Kähäri 2014); and (4) weakening atherosclerotic plaque structural stability and promoting plaque rupture (Kuzuya et al. 2006, Johnson et al. 2005). As MMPs can promote tissue turnover and wound healing, there is still the uncertainty of whether MMPs are involved in the initiation of tissue damage in CVD progression or in the repair mechanism, or in both.

**MMP-8**

MMP-8, also known as collagenase-2, or neutrophil collagenase, was first isolated and characterized by McCartney & Tschesche in 1983. It is a major member of the collagenase subgroup of MMPs and most efficient in degrading type I collagen (Hasty et al. 1987) but also capable of processing other ECM proteins, such as type II, III, VII and X collagens, gelatin, aggrecan, and tenascin. (Fosang et al. 1994, Van Lint & Libert 2006)
As its former name, neutrophil collagenase, implies, it was thought that MMP-8 is expressed mainly by neutrophils since it was cloned from RNA extracted from peripheral blood leukocytes of a patient with chronic granulocytic leukaemia (Hasty et al. 1990). Later, Herman et al. (2001) demonstrated that MMP-8 is also expressed by endothelial cells (ECs), smooth muscle cells (SMCs) and macrophages within human atherosclerotic lesions. The PMN-type MMP-8 secreted by neutrophils is more glycosylated and its molecule weight is larger compared to non-PMN-type MMP-8s (Hasty et al. 1986, Ding et al. 1997, Balbin et al. 1998). Herman et al. also demonstrated that MMP-8 synthesis and release by EC, SMC and macrophages require a prolonged exposure to inflammatory cytokines, but neutrophils store MMP-8 zymogen in intracellular granules and release the collagenase nearly instantly on stimulation.

MMP-8 has been found in various cells, e.g. in rheumatoid synovial fibroblasts (Hanemaaijer et al. 1997), chondrocytes (Chubinskaya et al. 1999), plasma cells (Wahlgren et al. 2001), oral SCC cells (Moilanen et al. 2002), and breast cancer cells (Agarwal et al. 2003). The MMP-8 gene is located in the MMP gene cluster in the long arm of chromosome 11q22.3. (Pendas et al. 1996). Its expression is inducible and upregulated by various inflammatory cytokines, such as interleukin-1β, tumour necrosis factor-α, and CD40 ligand (Herman et al. 2001).

Like other MMPs, MMP-8 is produced in the cells as a prepro-enzyme. When it is secreted to ECM, the pre-domain is cleaved off. MMP-8 can be rapidly released from secretory granules of neutrophils and is not that dependent on transcriptional regulation. The zymogen activation takes place in ECM, and the pro domain may be removed by various factors, including ROS (Saari et al. 1990), tumour-associated trypsinogen-2 (Moilanen et al. 2003), bacteria derived proteases (Sorsa et al. 1992), and other MMPs, including MMP-3 (Knäuper et al. 1993), MMP-7 (Dozier et al. 2006), MMP-10 (Knäuper et al. 1993), and MMP-14 (Holopainen et al. 2003). This indicates that MMP-8 activation is under strong regulation and mostly limited to the sites of inflammation instead of the systemic circulation.

MMP-8 can cleave all three interstitial collagens (collagen types I–III), and it is the most efficient proteinase in the degradation of type I collagen (Welgus et al. 1981). MMP-8 contributes to the progression of many human diseases (Figure 6) (Dejonckheere et al. 2011, Sulkala et al. 2007) but may also have a protective role in skin and oral cancers (Balbin et al. 2003, Korpi et al. 2008) and anti-inflammatory character in pulmonary inflammation (Owen et al. 2004, Gueders et
The protective and pro-inflammatory mechanisms of MMP-8 may depend both on its temporal and spatial expression and on its origin (Dejonckheere et al. 2011).

Several studies have demonstrated that disturbed regulation and overexpression of MMP-8 is associated with atherosclerotic plaque formation, maturation and rupture.

The main MMP-8 substrates, fibrillar collagens, are the most numerous ECM proteins in the arterial walls, atherosclerotic plaques and the myocardium and important for the structural unity of these tissues and stability of the atherosclerotic plaque. (Kassiri & Khokha 2005, Stary 1994 and Stary et al. 1995). Collagen type I is the major load-bearing matrix protein of the fibrous cap (Herman et al. 2001). MMP-8 can also cleave many other proteins, such as proteoglycans, fibronectin, fibrinogen, angiotensin-I, and substance P (Laxton et al. 2009, Van Lint & Libert, 2006). Some of these proteins or their cleaved products, including angiotensin-II, have previously been connected with atherogenesis (Weiss et al. 2001).
In *ex vivo* studies, MMP-8 levels were higher in vulnerable plaques when compared to normal arteries and stable plaques (Galis *et al.* 1994, Herman *et al.* 2001). Dollery *et al.* (2003) showed in their study on aortic fragments from cardiac transplantation donors that MMP-8 is present in atherosclerotic plaques and is localized particularly in the shoulder areas. Their findings confirmed that the MMP-8 level is increased in atheromas, particularly in those prone to rupture. Naruko *et al.* (2002) demonstrated that neutrophil infiltration is actively associated with acute coronary events and that the release of MMP-8 may be linked to fibrous cap thinning and plaque rupture, which leads to transformation of stable into unstable lesions in clinical cardiovascular disease.

Later, the activity of MMP-8 in unstable plaques was confirmed in cohort studies with 159 patients (Molloy *et al.* 2004) and 150 patients (Sluijter *et al.* 2006). Molloy and co-authors also found that MMP-8 was present particularly in macrophage-rich areas of plaques, and possibly expressed by macrophages. Finally, it was shown by Peeters *et al.* (2011) that increased plaque MMP-8 levels of endarterectomy patients were associated with an increased risk of secondary cardiovascular event. The important role of MMP-8 in atherogenesis was confirmed by Laxton *et al.* (2009) in an MMP-8-deficient mouse model. The study showed that inactivation of MMP-8 caused a significant reduction in the extent of atherosclerosis in apoE-deficient mice fed a Western diet and that the atherosclerotic lesions in the mice contained fewer macrophages but higher collagen content.

**MMP-7**

MMP-7, also known as matrilysin-1, is the smallest molecule of all MMPs because it lacks the hemopexin domain. It is expressed by various cells such as macrophages and epithelial cells. The expression of MMP-7 is induced by microbial products. MMP-7 is capable of processing many cell surface proteins and activating proMMP-8, (Dozier *et al.* 2006) as well as proMMP-2 and proMMP-9 (Wilson *et al.* 1996).

MMP-7 can impact the EC function through modulating VEGF pathway (Huo *et al.* 2002). It is upregulated in atherosclerotic lesions (Halpert *et al.* 1996). In a recent study, MMP-7 was present especially in symptomatic atherosclerotic lesions, and high MMP-7 plasma levels were independently associated with total mortality (Abbas *et al.* 2014). In mouse studies, it has been shown that MMP-7
reduces the SMC content of atherosclerotic plaque but has no effect on its growth or stability due to the lack of collagenolytic functions (Dozier et al. 2006).

**MMP-13**

MMP-13, which is also known as collagenase-3, is a member of the same collagenase subfamily as MMP-1 and MMP-8. It was first found in human breast cancer tissue (Freije et al. 1994). The overexpression of MMP-13 is linked to various tissue-destructive pathologies, including periodontitis (Uitto et al. 1998). MMP-13 also plays an important role in skeletal biology (Stahle-Backdahl et al. 1997, Johansson et al. 1997) and is highly potent in cleaving type II collagen and thus suggested to be a key factor of arthritides (Knauper et al. 1996, Konttinen et al. 1999). It is expressed by endothelial cells, macrophage-like cells, fibroblasts, plasma cells, and osteoblasts (Hernandez et al. 2006, Nakamura et al. 2004, Rydziel et al. 2000). MMP-13 is present in many human tissues characterized by inflammation and ECM remodelling, such as skin cancer (Airola et al. 1997), head and neck carcinomas (Johansson et al. 1997), and periodontitis (Uitto et al. 1998). It is shown that increased collagenolysis in vulnerable plaques is partly mediated by MMP-13 (Sukhova et al. 1999, Libby 2013). According to mouse studies, MMP-13 is suggested to be a main collagenase in plaque progression (Quillard et al. 2014, Quillard et al. 2011). Contrary to MMP-8, the circulating levels of MMP-13 are very low or undetectable, which may complicate its use as a biomarker (Momiyama et al. 2010).

**Tissue inhibitor of metalloproteinases-1 (TIMP-1)**

The main inhibitors of MMPs are their specific tissue inhibitors, TIMPs. To date, four TIMPs have been identified, TIMPs -1, -2, -3 and -4. TIMPs and MMPs can form tight complexes with a 1:1 stoichiometry. TIMPs have a 2-domain protein structure, in which the N-terminal domain contains the inhibitory residues and can bind non-covalently to the catalytic site of MMPs (Nagase et al. 2006). All the TIMPs can prevent the conversion of proMMPs to active forms (Uitto et al. 2003). They are also capable of control autocatalytic activation of many proMMPs by producing complexes with proenzymes (DeClerck et al. 1991, Howard et al. 1991, Lambert et al. 2004). However, their inhibitory effect varies between different MMP molecules. For example, because of their missing hemopexin domain, MMP-7, -23 and -26 are poorly inhibited by TIMP-1 (Stetler-Stevenson 2008).
The expression of TIMPs is weak in healthy stable tissues but increases both in physiological tissue turnover and in pathological conditions (Brew et al. 2000, Beaudeux et al. 2004). In addition to their specific protease inhibition, TIMPs also have an ability to modify cell growth, migration and apoptosis, have growth factor-like characteristics affecting cell morphology, gonadal steroidogenesis, and stimulation of cell growth, and may affect MMP localization, transportation and stabilization. (Gomez et al. 1997, Kato et al. 2000, Lambert et al. 2004, Stetler-Stevenson 2008). The balance between ECM synthesis and degradation is tightly controlled and necessary for the integrity of normal tissues (Ryan et al. 1996). If the production of MMPs exceeds the production of their inhibitors, the disturbed MMP/TIMP-balance may lead to uncontrolled tissue destruction at acute inflammation (Sorsa et al. 2004) and may be a crucial factor in angiogenesis (Inokubo et al. 2001). It is suggested that proteinase inhibitory properties of TIMPs could be utilized for therapeutic benefit in degradative diseases (Baker et al. 2002).

TIMP-1 was found first in vitro in human fibroblasts (Bauer et al. 1975). A decade later, Gasson et al. (1985) investigated that it was identical to the erythroid potentiating activity protein. Like MMP-8, its main inhibitor TIMP-1 is expressed by several cell types, including macrophages, epithelial and endothelial cells, fibroblasts and many tumour cells (Welgus et al. 1983, Welgus et al. 1985, Cawston et al. 1986, Bord et al. 1999). In addition to MMP-8 inhibition, TIMP-1 is the main inhibitor of MMP-9 and MMP-12.

TIMP-1 may contribute to atherogenesis by inducing inflammation and SMC proliferation which can result in vascular wall damage (Akahane et al. 2004). High serum TIMP-1 levels may indicate plaque instability. Elevated serum TIMP-1 levels have been associated with MI (Cavusoglu et al. 2006) and death (Lubos et al. 2006), and plasma TIMP-1 concentration is increased in unstable CAD. Furthermore, elevated TIMP-1 levels may predict future CVD events (Tuomainen et al. 2007, Pussinen et al. 2013). Elevated MMP-8/TIMP-1 ratios are associated with ACS (Inokubo et al. 2001, Pussinen et al. 2013).

**Myeloperoxidase (MPO)**

Myeloperoxidase (MPO), the major mediator in innate immunity and defence against pathogens, is expressed mainly in neutrophils and, to a lesser extent, in monocytes (Klebanoff et al. 2013, van der Veen et al. 2009). Its primary function is to kill phagocytized microorganisms (Klebanoff 2005), but it also has a role in.
pathologic inflammatory conditions, for example, by regulating the activity of MMPs (Fu et al. 2003). MPO can activate proMMP-8 and -9, as well as inactivate TIMP-1 oxidatively by producing hypochlorous acid (HOCl) (Sorsa et al. 2006, Weiss 1989). In chronic inflammation and acute oxidative stress, it is released into the extracellular space where its oxidants may damage host tissues. (Weiss 1989, Klebanoff 2005, Sorsa et al. 2006) Clinical studies have suggested that MPO can serve as a sensitive predictor for myocardial infarction and that it is a potential biomarker for the cardiovascular risk (Brennan & Hazen 2003, Schindhelm et al. 2009), as well as a drug target (Malle et al. 2007). Heslop et al. (2010) have reported that measuring both MPO and CRP (C-reactive protein) provide added benefit in risk prediction compared to measuring CRP alone. In a study from our laboratory, it was suggested that the combination of a high MMP-8 and a low MPO level in serum, eventually reflecting selectively modified neutrophil degranulation, indicates an increased risk of arterial disease (Pradhan-Palikhe et al. 2010).

**Neutrophil elastase (NE)**

Neutrophil elastase, also known as PMN elastase, was first recognized and isolated by Janoff in 1973. NE is produced and stored by circulating polymorphonuclear (PMN) leukocytes. During acute inflammation, PMN leukocytes release elastase into the ECM space as a rapid primary line of defence against bacterial infections by neutrophil degranulation (Weiss 1989). Neutrophil elastase has a broad substrate specificity, and it is capable of both terminating pathogens and degrading host tissues during inflammation (Hiemstra et al. 1998, Shapiro 2002). It has parallel functions in ECM degradation as MMPs (Werb et al. 1989) and can regulate MMPs by activating proMMPs, including proMMP-9 and proMMP-13 (Okada et al. 1988, Ferry et al. 1997), and inactivating TIMPs (Okada et al. 1988, Itoh & Nagase 1995). NE plays a role in tissue destruction of many inflammatory diseases by cleaving collagen IV and elastin of the ECM. NE is present within atherosclerotic plaques (Dollery et al. 2003) and contributes to the processes of matrix degradation and weakening of the vessel wall responsible for the complications of aneurysm formation and plaque rupture (Garcia-Touchard et al. 2005). It is shown that serum elastase antigen concentrations are elevated in patients with coronary disease and are predictive of myocardial infarction (Smith et al. 2000). According to animal studies, NE is a promising therapeutic target in atherosclerosis (Henriksen & Sallenave 2008).
CRP

CRP is the most extensively studied proinflammatory molecule and circulating biomarker in atherosclerosis. In healthy individuals, only trace levels of CRP can be detected in the circulation, but under acute phases of infectious diseases and tissue destruction, the concentrations can multiply in a few hours. CRP is a widely used clinical marker because of its analytical stability and a good availability of commercial assays. Studies show that CRP may have direct proinflammatory effects and contribute to the initiation and progression of atherosclerotic lesions (Ridker et al. 2001, Heeschen et al. 2000, van Exel et al. 2002, Verma et al. 2006). Nevertheless, the predictive value of CRP in atherosclerosis is only moderate (Danesh et al. 2004), and a causal association between CRP and CAD has been questioned both in mouse and Mendelian randomization studies (Elliot et al. 2009, C Reactive Protein Coronary Heart Disease Genetics Collaboration 2011). As a non-specific acute-phase protein, CRP reflects general systemic inflammatory burden, and high CRP levels are recognized, for example, as a risk factor for depression (Howren et al. 2009, Vogelzangs et al. 2012), among many other conditions. Because of the low specificity, the use of CRP as a CVD risk marker can be discussed.

2.6 MMPs as therapeutic targets in CVD

2.6.1 Introduction

The pivotal role of MMPs in pathological conditions such as cancer cell migration and atherogenesis has encouraged the development of drug therapies targeting these proteases. (Hansson & Hermansson 2011) It has been suggested that pharmacological therapy addressed to restrain the disturbed MMP regulation may prevent atherogenesis and thereby reduce cardiovascular morbidity and mortality (Hopps & Caimi 2015).

MMP-inhibition can be selective or broad-spectrum. Until now, more than 50 synthetic MMP inhibitors have been considered for possible clinical development and several have made it through to phase III studies. They are targeted predominantly at the inhibition of tumour growth and metastasis or rheumatoid arthritis (Newby 2014). Moreover, selective inhibitors, especially monoclonal antibodies against MMPs, have given promising results in animal models, for example, in hepatic ischemia/reperfusion injury (Shirahane et al. 2006), cerebral
ischemia (Gu et al. 2005) and MI (Creemers et al. 2001). In addition to monoclonal antibodies, another group of selective inhibitors is mechanism-based inhibitors, which bind covalently in the active site of the MMP protein (Ikejiri et al. 2005). However, all these selective therapies are at the experimental level and need further investigation.

Broad-spectrum MMP inhibition, for example, by reducing cytokine and inflammatory response, might maximize the inhibition of ECM degradation. One promising drug group is Zn$^{2+}$ ion chelators, but their nonselective characteristic may cause too broad protease inhibition (Hopps & Caimi 2015). The complexity of MMP functions and regulation cause obstacles to the development and use of both selective and broad-spectrum inhibitors, as they prevent both matrix degradation and tissue repair functions of different MMPs. (Vandenbroucke & Libert 2014).

When targeting therapies at MMPs, it should be remembered that several current cardiac and antidiabetic treatment strategies have MMP-inhibiting features as part of their mode of actions. The MMP-regulating effect is shown in diuretics (Ceron et al. 2010), calcium channel blockers (Martínez et al. 2006, Marcal et al. 2012), angiotensin receptor blockers (Sasamura et al. 2006), statins (Koh et al. 2001, Crisby et al. 2001, Izidoro-Toledo et al. 2011), angiotensin converting enzyme (ACE) inhibitors (Yokota et al. 2014), and metformin (Hanefeld et al. 2011), among many others.

2.6.2 Anti-inflammatory effect of doxycycline in CVD

Golub et al. showed in the early 1980s that tetracyclines are capable of inhibiting MMP activity (Golub et al. 1983, Golub et al. 1985), and later they demonstrated that this feature was independent of antimicrobial properties (Golub et al. 1998).

Doxycycline, a commonly used second-generation member of tetracycline group, is a broad-spectrum antibiotic drug used in the treatment of infections caused by bacteria and protozoa. Doxycycline has superior pharmacokinetic properties and minor toxicity compared to first-generation tetracyclines (Smilack 1999), and it has both antiangiogenic and anti-inflammatory features (Sapadin & Fleischmajer 2006). Half-life of doxycycline is long, varying from 18 to 22 hours. A regular daily antimicrobial dose is 100–300 mg once or twice a day.

The smaller dosage, so-called low-dose, or sub-antimicrobial-dose-doxycycline (SDD), is 20–50 mg daily, and contrary to the regular antibiotic dose, administered over a longer period from three weeks up to two years.
Knowledge about biochemical mechanisms for the action of doxycycline is still lacking (Franco et al. 2006, Sapadin & Fleischmajer 2006.) It is thought that doxycycline has inhibitory effects on MMPs, probably because of its zinc-chelating properties (García et al. 2005). Both antimicrobial and anti-inflammatory qualities of doxycycline may be beneficial in the prevention of CVD (Brown et al. 2004, Kardara et al. 2006). The immune-modulatory effects are seen both in anti-inflammatory and regular doses. Doxycycline is capable of reducing the levels of MMP-7, MMP-8, MMP-8/TIMP-1-ratio, NE, MPO, and CRP and increasing the level of TIMP-1 (Brown et al. 2004, Lauhio et al. 1994, Golub et al. 1998, Abdul-Hussien et al. 2009, Gu et al. 2011, Gu et al. 2011b, García et al. 2005). SDD is approved as an adjunct anti-inflammatory therapy for the treatment of rosacea, acne and periodontitis by the FDA (Food and Drug Administration) in the United States, and it is also used for these indications in Europe. (Valentin et al. 2009, Caton & Ryan 2011). In clinical pilot trials, doxycycline has given contradictory results in chronic inflammatory conditions, such as AAA and reactive arthritis (Dodd & Spence 2011, Greenwald 2011).
3   Aims of the study

The general aims of this thesis project were to examine the role of MMP-8 and its regulators as inflammatory biomarkers and potential therapeutic targets in cardiovascular diseases. This thesis consists of four original studies.

The specific aims were:

1. To examine the role of MMP-8 and its regulators in CVD incidence.
2. To study clinical use of MMP-8 and TIMP-1 as biomarkers in both prevalent and incident cardiovascular outcomes.
3. To compare the commercial and in-house determination of MMP-8 serum concentrations and thus investigate a possible diagnostic method for ACS.
4. To examine the effect of prolonged systemic doxycycline therapy on circulating tissue-degrading proteinases in coronary bypass patients.
4 Study subjects and methods

4.1 Study populations and designs

4.1.1 The FINRISK97 cohort (Studies I and II)

The FINRISK chronic disease risk factor surveys have been carried out since 1972 every five years using independent, random and representative population samples from Finland. The survey was first carried out in the eastern Finnish North Karelia and Kuopio provinces in 1972 as the basis for the evaluation of the North Karelia Project (Puska et al. 2016). Since then, up to five geographical areas have been included in the surveys.

The FINRISK97 cohort comprised 8,446 individuals aged between 25 and 74 years and recruited through random sampling from the National Population Information System (Vartiainen et al. 2010). The survey included a mailed questionnaire and a clinical examination. Body mass index (BMI), systolic and diastolic blood pressure and waist/hip ratio were measured in the clinical examination, and years of education and smoking habits were recorded using self-administered questionnaires (Vartiainen et al. 2010).

The individuals with prevalent CVD or diabetes were identified on the basis of a self-reported doctor-diagnosed disease, as well as through national drug reimbursement records and hospital discharge register data. Prevalent CVD included both CAD and stroke events (excluding subarachnoid haemorrhage). CAD events included subjects with a history of MI, revascularization or percutaneous coronary angioplasty. The study includes up to 20 years of follow-up data.

The FINRISK97 study was approved by the Ethics Committee of the National Public Health Institute and carried out according to the recommendations of the Declaration of Helsinki. All individuals gave their informed consent.

Study I was a nested case-control study. From the main FINRISK97 cohort, persons with no CVD at the baseline were selected (n=8,090). During the median 10.2-year follow-up (years 1997–2007), 471 cases with incident CVD were ascertained on the basis of record linkages to the National Causes of Death Register and the National Hospital Discharge Register (Pajunen et al. 2005). At the end of the follow-up, up to three sex-, age- and area-matched controls were
selected for each case. The CVD events included MI, stroke, coronary revascularization, and CVD death.

Study II was a prospective cohort study with several endpoints (Figure 7). Similarly as in Study I, only subjects free of CVD (n=7,918) were selected for the study. During the median 13.8-year follow-up (years 1997–2012; IQR 0.115), the following endpoints were ascertained on the basis of record linkage to the National Causes of Death Register and the National Hospital Discharge register: cardiovascular disease events (CVD), including CAD events and stroke; coronary artery disease events (CAD), including MI, bypass surgery and angioplasty; myocardial infarction; stroke; and all-cause death.

Fig. 7. The study design of Study II. A total of 7,918 subjects of the FINRISK97 cohort were free of CVD at baseline. Individuals with prevalent CVD (n=518) were excluded. During the follow-up, 89.7% survived without registered endpoints, while 9.6, 6.7, 3.7, and 3.5% developed a CVD event, CAD event, stroke, or MI, respectively, and 10.4% died. Some participants with incident CVD suffered from multiple events classified as stroke, MI or CAD; thus, the number of subjects does not correspond to the number of incident CVD events.
4.1.2 The Corogene study (Study III)

The Corogene study population was a prospective cohort including 5,295 patients undergoing a coronary angiogram at the Helsinki University Central Hospital between June 2006 and March 2008. In Finland, coronary angiogram is performed to practically all patients assigned for invasive heart examination. The aim of the study was to recognize coronary disease risk factors and genetic factors predisposing to CAD. (Vaara et al. 2012). The study was approved by the ethical committee of the University of Helsinki and it complied with principles of the Declaration of Helsinki. A written informed consent was obtained from the subjects.

For the study, MMP-8 concentrations were analysed for all acute coronary syndrome patients (ACS; n=2,072), and randomly selected patients without significant coronary artery disease CAD (no-CAD; n=653) were used as a reference. The exclusion criteria were as follows: non-Finnish origin (for follow-up reasons), previous heart transplantation, low haemoglobin, or previous blood transfusion during the same hospitalization.

4.1.3 The Doxyspot trial (Study IV)

The Doxyspot trial included 31 non-smoking men aged 58±5 years. The study individuals had previously had coronary bypass surgery at the Helsinki University Central Hospital (HUCH) from 6 months to 5 years earlier. The patients were randomly assigned to have 100 mg of doxycycline (Orion Pharma, Espoo, Finland) (n=16) or placebo (n=15) daily for four months. The original goal was to reduce the risk of secondary cardiovascular events by eradication of persistent Chlamydia pneumoniae infection (Sinisalo et al. 1998). The study protocol was approved by the ethics committees of HUCH and the Finnish National Agency for Medicines, and all participants gave their written informed consent.

The exclusion criteria of the study were: diabetes or impaired glucose tolerance, smoking, heart failure, neoplasm, chronic infectious or inflammatory systemic disease, infection within six weeks with general symptoms or need of antimicrobial drugs, antacid use, BMI >32, hypolipidemic medication, and hypersensitivity to doxycycline.
4.2 Sample collection

In the FINRISK97 population (Studies I and II), the serum samples were collected at public health care centres or other survey sites at the baseline. In the survey invitation letter, the subjects were asked to fast at least for four hours and to avoid heavy meals during the day before the blood sampling. The baseline laboratory measurements included serum total and HDL cholesterol, triglyceride (TG), apoA-I, apoB, C-reactive protein (CRP), and \(\gamma\)-glutamyltransferase (\(\gamma\)-GT) concentrations (Pussinen et al. 2011). The samples were stored at -70°C for later measurements.

In the Corogene cohort (Study III), the blood samples for the serum determinations were drawn from the arterial line during the angiogram. The samples were handled according to the accredited laboratory standards of the Helsinki University Central Hospital. Serum was stored at -80°C for later laboratory determinations.

In the Doxyspot trial (Study IV), the serum samples were taken before the doxycycline treatment and at 2, 4 and 10 months after the beginning of the treatment. The samples were stored at -70°C until the analysis were performed.

4.3 Inflammatory biomarker detection methods

The laboratory methods used in the original articles are summarized in Table 2. The principal methods of the present thesis are also briefly described in this section.

4.3.1 MMP-8 time-resolved immunofluorometric assay (Studies I, II, III and IV)

The MMP-8 concentrations were measured from serum samples with a quantitative time-resolved immunofluorometric assay (IFMA) (Hemmilä et al. 1984, Holopainen et al. 2003). The monoclonal MMP-8-specific antibodies 8708 and 8706 (Medix Biochemica Ab Oy, Kauniainen, Finland) were used as catching and tracer antibody, respectively. The detection antibody was labelled using Europium-chelate (Hemmilä et al. 1984). Samples were diluted in the assay buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl\(_2\), 50 \(\mu\)M ZnCl\(_2\), 0.5% BSA, 0.05% sodium azide, and 20 mg/l DTPA, and incubated for one hour, followed by an incubation of one hour with the tracer antibody. Then
enhancement solution was added, and after five minutes, fluorescence was measured using a fluorometer. (Hanemaaijer et al. 1997).

4.3.2 Enzyme-linked immunosorbent assay (ELISA) / Enzyme immunoassay (EIA) (Studies I, II, III and IV)

Other biomarkers were measured according to the manufacturer’s protocol using commercial kits.

<table>
<thead>
<tr>
<th>Assay, specimen</th>
<th>Method</th>
<th>Manufacturer</th>
<th>Study</th>
<th>Inter-assay CV %</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-7, serum</td>
<td>ELISA</td>
<td>R&amp;D systems</td>
<td>I</td>
<td>9.3</td>
<td>0.016 µg/l</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>R&amp;D systems</td>
<td>IV</td>
<td>4.4</td>
<td>0.016 µg/l</td>
</tr>
<tr>
<td>MMP-8, serum</td>
<td>Time-resolved IFMA</td>
<td>Medix Biochemica, Kauniainen, Finland</td>
<td>I, II, III, IV</td>
<td>7.3</td>
<td>0.08 µg/l</td>
</tr>
<tr>
<td></td>
<td>dentoELISA</td>
<td>Dentognostics GmbH, Jena, Germany</td>
<td>II, III, IV</td>
<td>7.3</td>
<td>0.025 µg/l</td>
</tr>
<tr>
<td>MMP-13, serum</td>
<td>ELISA</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
<td>I</td>
<td>7.8</td>
<td>0.032 µg/l</td>
</tr>
<tr>
<td>MPO, plasma</td>
<td>ELISA</td>
<td>Medix Biochemica, Bensheim, Germany</td>
<td>IV</td>
<td>4.1</td>
<td>20.0 µg/l</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Immunodiagnostik AG, Vienna, Austria</td>
<td>I</td>
<td>7.9</td>
<td>1.98 µg/l</td>
</tr>
<tr>
<td>NE (PMN-elastase), serum</td>
<td>ELISA</td>
<td>Bender Medsystems, Vienna, Austria</td>
<td>I</td>
<td>7.9</td>
<td>1.98 µg/l</td>
</tr>
<tr>
<td>TIMP-1, serum</td>
<td>CMIA</td>
<td>Architect i2000, Abbot</td>
<td>I, II</td>
<td>3.1</td>
<td>1.25 µg/l</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>Amersham Biotrack, GE Healthcare, Buckinghamshire, UK</td>
<td>I, II, IV</td>
<td>13.1</td>
<td>1.25 µg/l</td>
</tr>
<tr>
<td>MMP-7/TIMP-1 ratio</td>
<td>Calculation*</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-8/TIMP-1 ratio</td>
<td>Calculation**</td>
<td>I, II, IV</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculation using MWs 28 and 28 g/mol for MMP-8 and TIMP-1
** Calculation using MWs 65 and 28 g/mol for MMP-8 and TIMP-1
The most common detection method of enzyme immunoassay (EIA) is enzyme-linked immunosorbent assay (ELISA), which was first reported by Engvall and Perlmann in 1971 (Engvall & Perlmann 1971) and is widely used as a diagnostic tool. Chemiluminescent microparticle immunoassay (CMIA) is the modified and advanced form of the ELISA technique.

DentoELISA (DentoGnostics Gmbh, Germany), which was used in Study III to determine the serum MMP-8 concentrations, is a diagnostic assay originally designed to measure biomarker levels from oral fluids. It is designed for rapid MMP-8 detection, applying a sandwich-based immunoassay system with two monoclonal antibodies.

### 4.4 Statistical analysis (Studies I, II, III and IV)

The statistical analyses of all the studies were performed using SPSS software package versions 15.0–22.0 (IBM, New York, NY, USA) and with R (URL www.R-project.org). In all the studies, p-values <0.05 were considered as statistically significant.

In Study I, the cases and controls were age-, sex-, and area-matched. Before the analysis, the variables with skewed distribution were normalized with a natural logarithm. The subjects with prevalent CVD were excluded from the analysis.

The significance of the differences in characteristics, as well as the determined serum proteinase concentrations between the cases and controls, were tested by using the Mann–Whitney U test. The significance of the differences in categorical variables was tested with Chi-squares tests.

The associations of the biomarker concentrations with incident CVD events were analysed using four separate conditional logistic regression models. Model 1 was unadjusted, Model 2 was adjusted for CVD risk factors, and Model 3 was additionally adjusted for CRP concentration. Model 4 included simultaneously the measured proteinase concentrations appearing statistically significant in Model 2 (MMP-7, MMP-8 and TIMP-1) adjusted for the CVD risk factors. The results were also interpreted as percentage increase in the odds of getting a CVD event per 1% increase in the non-transformed predictor, while holding all the other predictors fixed. Partial Pearson correlation coefficients were calculated after log or square root transformations adjusting for age, sex and study region.

In Study II, individuals with prevalent CVD were excluded from the analysis. CRP, MMP-8, MPO and MMP-8/TIMP-1 concentrations were transformed by
using a natural logarithm before the analysis due to the skewed distributions. The subjects with prevalent CVD were excluded. The significance of the differences in baseline characteristics of individuals with and without incident outcome was tested with the Mann–Whitney U test, and the differences between categorical variables were tested with Chi-square tests. The associations of selected biomarker concentrations with different endpoints were analysed using the Cox proportional hazards model and age as a time scale. The model was adjusted with Framingham CVD risk factors (D’Agostino et al. 2008), and additionally with the study area and education years (Havulinna et al. 2008, Laatikainen et al. 2000). Furthermore, the assumption of proportional hazards was assessed by extending the Cox model with a time by biomarker interaction factor.

The potential improvement of the Framingham cardiovascular prediction of the MI risk by MMP-8 and TIMP-1 was evaluated with C-index, integrated discrimination improvement (IDI) statistics, and net reclassification improvement (NRI) (Pencina et al. 2008). NRI was assessed both in categorical and in clinical category ranges. The categorical range included the entire study population by using the following four risk categories: <5%, 5% to 10%, 10% to 20%, and >20%, and clinical only the individuals in the intermediate risk range of 5% to 20% based on the reference (Tikkanen et al. 2013). For the 13-year absolute risk predictions, 10x validation was used to reduce over-optimism. The cross validation was stratified by sex. The 10x cross-validated model calibration was tested using the Hosmer–Lemenshow goodness-of-fit test.

In Study III, a log-transformation was performed on parameters with skewed distribution. A receiver operating characteristic (ROC) analysis based on logistic regression derived predictors, standardized to age, sex and smoking, was applied to evaluate the diagnostic sensitivity and specificity of ELISA in comparison to IFMA. A paired sample t-test and Spearman’s correlation was used to analyse correlation and significance of the differences between the two assays and subpopulations.

In Study IV, the significance of the differences between the characteristics of the doxycycline group and the placebo group was tested by the t-test or Mann–Whitney test, while proportions were tested by using the Chi-square or Fisher’s exact test. The significance of the differences in the mean change from the baseline in the serum concentrations between the groups was analysed by using the Mann–Whitney test.
5 Results and discussion

5.1 Characteristics of FINRISK 1997 cohort individuals (Studies I and II)

Studies I and II both comprised subjects from the population-based FINRISK cohort, which is representative of the Finnish population.

In Study I, the subjects with an incident CVD event during the follow-up had significantly higher BMI, systolic blood pressure, waist/hip ratio, and serum triglyceride, CRP, \( \gamma \)-GT, and apoB concentrations, as well as lower serum HDL and apoA-I concentrations compared to the controls. Furthermore, they were also more often smokers, less educated and diabetic. Because of the nested case-control study design, there were no differences in age and sex.

In Study II, the subjects with a CVD event during the follow-up had significantly higher age, BMI, systolic blood pressure and total cholesterol concentration, as well as lower serum HDL concentration. Furthermore, they were more often men, less educated and had more diabetes in all the endpoint groups. Smoking was significantly more common among those who died during the follow-up period. The characteristics of the studied subjects are in line with the earlier knowledge of the risk factors.

5.2 Serum MMP-8 level is increased in acute cardiac syndrome (Study III)

Study III shows that among coronary angiograph patients, significantly higher mean MMP-8 levels were seen in individuals with acute cardiac syndrome relative to ones with no coronary artery disease (\( p<0.001; \) 93.5 ng/ml and 47 ng/ml, respectively). The results are in agreement with earlier findings: In the study of Turu and colleagues, increased MMP-8 plasma levels were found in patients with hypoechogenic, unstable plaques (Turu et al. 2006). In another cohort study of 250 patients who had undergone angiography, plasma MMP-8 concentrations were significantly higher in CAD patients when compared to subjects without the disease (Kato et al. 2005). Furthermore, in a Japanese cohort, plasma MMP-8 levels were higher in patients with stable CAD than in the control subjects, and even higher in patients with UAP when compared to ones with CAD reflecting coronary plaque instability. (Momiyama et al. 2010). It has been shown
that a high serum MMP-8 concentration is clearly able to discriminate UAP and AMI patients from healthy controls. (Pussinen et al. 2013).

Mean MMP-8 concentrations of subjects without coronary artery disease (n=653) and subjects with acute coronary syndrome (n=2,072). The results were analysed with dentoELISA and IFMA assays. Patients with ACS had significantly higher serum levels of MMP-8 than patients without CAD (p<0.001). The values of the two different assays correlated significantly (r=0.881, p<0.001) and showed no statistical difference.

5.3 High MMP-8 is a risk marker for future CVD (Studies I and II)

In both FINRISK cohort studies, an increased MMP-8 level was associated with the future incident CVD outcome. The results suggest that high MMP-8 levels can be measured already at early stages of atherosclerosis, even years before the plaque rupture.
In Study I, subjects with a future incident CVD event had higher serum MMP-8 when compared to control subjects who remained CVD-free during the 10-year follow-up. In further calculations, elevated MMP-8 was a risk factor for CVD event independently of traditional risk factors. When examining the association of selected proteinases with CVD, the statistical significance was lost when CRP was used as an additional risk factor. In the same study, CRP had a positive association with MMP-8, as well as with its regulators MMP-7, TIMP-1 and NE. The results indicate that MMP-8 is associated with an increased CVD risk independently of classical risk factors but dependent on inflammation. (Table 4)

Parallel results were seen in Study II, which had a more detailed study design (Figure 7) with several endpoints and a longer, 13-year follow-up time. The serum MMP-8 concentration was associated with the increased risk of CAD events, MI and even all-cause mortality. Interestingly, the association was not seen in all the endpoint groups. There was no significant association between MMP-8 and stroke, or MMP-8 and CVD. The divergent results may reflect the different pathophysiology of these CVD manifestations. Although high circulating MMP-8 levels are associated with present and future CVD in many studies, there are contradictory results, too: Decreased MMP-8 plasma levels have been reported in patients with heart failure (Wilson et al. 2002) and cerebral ischemia (Lorenzl et al. 2003). These results may reflect a dissimilar aetiology of these conditions, but it is possible that the results arise from the small sample size with inadequate statistical power. However, when estimating the association of MMP-8 with stroke, it should be noted that in the present study, ischemic and haemorrhagic strokes were not separated in the data, which may confound the results.

The inflammatory process and endothelial dysfunction are features already seen in the early stages of atherosclerosis (Hansson et al. 2006). The results of this thesis confirm that high circulating MMP-8 levels can be measured even years before a plaque rupture. The results strengthen earlier findings: MMP-8 has a pivotal role at all stages of atherogenesis. MMP-8 is present during endothelial dysfunction and inflammatory changes associated with early atherosclerosis, as well as in plaque initiation and progression.

A high serum MMP-8 concentration indicates later plaque instability and tissue damage. Still, whether there is a causal relation between MMP-8 and CVDs is not clear. High serum MMP-8 levels may originate from atherosclerosis-induced tissue damage, or reflect systemic inflammatory burden. It has been suggested that in acute inflammation, MMP-8 is released from
polymorphonuclear granulocytes, but in chronic inflammation, it is synthetized and released from ECs, SMCs and macrophages as a response to a longer exposure to proinflammatory cytokines (Herman et al. 2001, Hansson et al. 2006). The PMN-type MMP-8 is more glycosylated and its molecule weight is larger compared to non-PMN-type MMP-8s (Hasty et al. 1986, Ding et al. 1997, Balbin et al. 1998). Still, it is not possible to distinguish these different forms or their sources because MMP-8 antibodies utilized in assays may detect molecule complexes including MMP-8 as well as MMP-8 fragments. (Leppilahti et al. 2011)

Table 3. Associations of MMP-8 and TIMP-1 with incident CVD events in 471 cases and 1,413 matched controls of Study I. MMP-8 and TIMP-1 are significantly associated with incident CVD event independently of traditional risk factors (Model 2) but dependently of systemic inflammation (Model 3). The percent increase represents the odds of getting CVD event per 1% increase in the absolute proteinase concentration, while holding all the other predictors fixed.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>OR (95% CI) / 1-SD increase</th>
<th>Percent increase (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-8</td>
<td>1.13 (1.01-1.25)</td>
<td>0.13</td>
<td>0.030</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.31 (1.17-1.47)</td>
<td>1.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>1.08 (0.97-1.20)</td>
<td>0.08</td>
<td>0.187</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-8</td>
<td>1.13 (1.01-1.26)</td>
<td>0.13</td>
<td>0.037</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.16 (1.02-1.31)</td>
<td>0.69</td>
<td>0.021</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>1.13 (1.00-1.27)</td>
<td>0.13</td>
<td>0.040</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-8</td>
<td>1.10 (0.98-1.24)</td>
<td>0.10</td>
<td>0.120</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.09 (0.96-1.24)</td>
<td>0.06</td>
<td>0.183</td>
</tr>
<tr>
<td>MMP-8/TIMP-1</td>
<td>1.09 (0.96-1.23)</td>
<td>0.09</td>
<td>0.186</td>
</tr>
</tbody>
</table>

*Unadjusted
**Adjusted for traditional risk factors
***Adjusted for traditional risk factors and CRP
Genetic factors might explain up to 40% of variation in the concentrations of systemic inflammatory biomarkers (Schnabel et al. 2009). In a recent large genome-wide association study (GWAS) on FINRISK1997 and Corogene populations, the increased levels of MMP-8 seemed to have genetic association. Interestingly, upregulation of MMP-8 was not associated with MMP-8 gene polymorphism, but in the complement system, especially with its alternative pathway. (Salminen 2016). This supports the conclusions of Studies I and II: MMPs seem to be associated with an increased CVD risk independently of the classical risk factors but dependent on the inflammation and immune system. As a conclusion, based on the present results, MMP-8 is not necessarily a causally associated risk factor for CVDs, but it is a promising risk marker for a future CVD event, especially for CAD and MI.

5.4 Serum TIMP-1 is a predictor of a future CVD (Studies I and II)

In both FINRISK cohort studies, a high TIMP-1 serum level was significantly associated with future CVD events, and in the more detailed examination performed in Study II, it was significantly associated with all the studied endpoints. These results are in agreement with earlier findings. In previous studies, a high TIMP-1 level has been associated with the risk of a future MI, stroke or death in CAD patients (West et al. 2008), as well as with CVD mortality both in healthy subjects and ACS patients. (Velageti et al. 2010, Cavusoglu et al. 2006, Lubos et al. 2006). The present studies show an association between high circulating TIMP-1 levels and CAD or CVD in healthy subjects.

It was thought earlier that an increased serum TIMP-1 level is an adaptive inhibitory response to MMP activity. Many of its functions seem to be independent of MMPs. Although TIMP-1 does not have proteolytic potential, it is capable of regulating many hallmarks of atherogenesis, such as angiogenesis, cell growth and migration, and apoptosis (Stetler-Stevenson 2008, Lambert et al. 2004, Spinale 2007). Still, it is possible that an increased TIMP-1 concentration may reflect the release of MMP-8 from PMN neutrophils during acute inflammation, but in some cases, there are disturbances in TIMP-1 level regulation. It is known that imbalance between pro- and anti-degradative factors can lead to plaque vulnerability. Imbalance between MMP-8 and TIMP-1 is associated with poor outcome in patients with ACS (Shah et al. 1995, Tuomainen et al. 2007, Pussinen et al. 2013). In Study II, an increased MMP-8/TIMP-1 ratio was associated with
the risk of MI and strengthened the role of this imbalance in incident CVD outcomes.

5.5 High systemic MMP-7 and MPO levels are associated with a future CVD

In Study I, high circulating MMP-7 levels were associated with incident CVD. Although MMP-7 upregulation has been showed in atherosclerotic lesions (Halpert et al. 1996), an association with a future CVD has not been demonstrated earlier. What has been shown earlier, however, is that MMP-7 is capable of activating proMMP-8.

In Study II, a high MPO concentration was associated with the risk of CVD and stroke. The result supports the earlier findings showing MPO as a risk factor for incident CVD events (Baldus et al. 2003, Brennan et al. 2003, Schindhelm et al. 2009, Scharnagl et al. 2014). MPO can contribute to vascular inflammation and initiation of atherosclerosis by oxidising proteins (Daugherty et al. 1994, Malle et al. 2000).

It is known that MMP-8 plays a key role in the atherosclerotic tissue-degrading cascade in concert with its activators MMP-7, NE and MPO. (Halpert et al. 1996, Dollery et al. 2003, D’Aiuto et al. 2005, Hansson et al. 2006, Kato et al. 2007). The results indicate that systemic collagenolytic MMP cascade contributes to atherosclerosis, and may be detected systemically in serum.

5.6 Serum MMP-8 level can be analysed reliably in hospital settings by using an inexpensive ELISA assay (Study III)

As shown earlier, MMP-8 is strongly associated with prevalent and incident CVDs and their complications, and measuring circulating MMP-8 is a promising diagnostic tool for CVD complications (Molloy et al. 2004, Sluijter et al. 2006, van den Borne et al. 2009, Sorsa et al. 2011). Until now, a laborious and expensive immunofluorometric assay (IFMA), which is only available in special laboratories, has conventionally been used to analyse MMP-8 levels in serum.

In Study III, we compared a new ELISA assay, MMP-8 dentoELISA, to IFMA in determination of serum MMP-8 levels. As a result, the ELISA assay proved to be diagnostically as sensitive and specific as the IFMA. ROC statistics showed highly similar areas under the curve for both assays. The AUC was 0.781 for ELISA (CI=0.761-0.801) and 0.778 for IFMA (CI=0.758-798). MMP-8
concentrations measured by using ELISA correlated strongly with concentrations determined with IFMA. (Figure 8)

5.7 Serum samples are suitable for the measurement of circulating MMPs and TIMPs (Studies I–IV)

A biomarker with great potential for improvement in risk prediction is useless if it is not possible to measure its reliably elsewhere. Because of that, the sample type for biomarkers has been discussed. It has been suggested that serum samples may be less suitable for measuring circulating MMPs and TIMPs (Jung 2008), and in some studies, there have been significant differences in the concentrations of MMP-9 (Jung et al. 1998) and TIMP-1 (Mannello et al. 2007) of plasma in contrast to serum samples. In their recent study, Jonsson and colleagues (2016) demonstrated higher levels of MMP-8 and other MMPs in serum samples compared to corresponding plasma samples. The reason for this is not fully understood, but it has been speculated that measurements in serum reflect a release of proteases from blood cells during the sample clotting process. The use of an anticoagulant in the collected blood may prevent this artefact (Zucker 1999), but it is also possible that MMPs are released from platelets and leukocytes during the blood collection process and centrifugation (Jung 2008). Therefore, it is suggested that future studies on MMPs as biological markers should consider the use of citrate plasma instead of serum in cancer (Jonsson et al. 2016) and atherothrombotic syndromes (Mannello 2008).

Because of this annotation, the difference between serum and plasma MMP-8 concentrations has been tested in our laboratory with two different methods, namely IFMA and commercial ELISA (Tuomainen et al. 2008). With both methods, serum MMP-8 concentrations were significantly higher than plasma concentrations. Still, there were significant positive correlations between serum and plasma IFMA concentrations and serum and plasma ELISA concentrations. That may indicate the influence of the different antibodies used in the assays and suggest the importance of the assay selection for the results.

As a conclusion, the aim of these studies was to investigate the association between serum MMP-8 and CVD irrespective of the origin of the protein, and it is most probable that the serum concentrations reflect the overall homeostasis of MMP-8. The results and conclusions related to using serum MMP-8 as a biomarker are well founded in several wide population-based cohorts (Tuomainen et al. 2007, Pussinen et al. 2013), and are further confirmed in this study.
5.8 Doxycycline may reduce the risk of secondary myocardial infarctions through inhibition of MMP-8 and MMP-7 (Study IV)

The purpose of Study IV was to investigate the effect of a four-month regular-dose doxycycline medication on serum MMP-7, MMP-8, TIMP-1, MPO, NE (PMN elastase) and CRP levels compared to placebo. The study subjects were non-smoking men with a history of cardiac bypass surgery. The operation had been performed at least six months before the trial, so the CHD status can be assumed to be stable. No differences existed between the groups, except for the triglyceride ($p=0.03$) and fibrinogen levels ($p=0.05$), but importantly, these differences remained the same throughout the trial (Sinisalo et al. 1998). The measured proteinases were selected because of their role as biomarkers and mediators of systemic inflammation. The special focus was on MMP-8.

After the four-month drug therapy, the MMP-7 and MMP-8 serum levels were significantly lower compared to the placebo group serum levels (Figure 9). A similar trend was seen in the MMP-8/TIMP-1 ratio, as well as in the NE level, but an opposite trend was observed in the TIMP-1 level, which increased in the doxycycline group subjects compared to the placebo subjects. In MPO and CRP levels, no considerable changes were seen. In earlier studies, MMP-8 has not been associated with an increased risk of secondary CVD endpoint (Tuomainen et al. 2007, Pussinen et al. 2013). On the contrary, increased acute phase and recovery period TIMP-1 concentrations are associated with poor secondary outcome, and it has been suggested that imbalance in TIMP-1 response to acute phase MMP-8 expression may explain that (Pussinen et al. 2013). Unfortunately, due to the small sample size, the statistical power of Study IV was low and no significant changes could be seen in the TIMP-1 concentration or MMP-8/TIMP-1 ratio.
Serum MMP-8 level changes (presented as percentages) of 4-month placebo and regular-dose doxycycline groups during a 10-month follow-up. The concentrations were measured by using IFMA. A significant difference (p<0.05) between the groups is seen after the 4-month doxycycline therapy (marked with *).

The results are in line with previous studies demonstrating the anti-inflammatory effect of doxycycline: Doxycycline therapy has been demonstrated to reduce the serum MMP-8 level and MMP-8/TIMP-1 ratio (Payne et al. 2011). Doxycycline is able to inhibit both conversion of latent proMMP-8 to active form as well as activated MMP-8. (Brown et al. 2004, Guentsch et al. 2008, Pussinen et al. 2013) The inhibition of MMP-7 seen in our results may have corresponding effects, as MMP-7 is capable of activating proMMP-8 (Dozier et al. 2006).

Doxycycline is probably the most investigated anti-proteolytic drug in CVDs. Still, the dose and duration of the drug therapy, as well as study designs, vary widely. As the daily doxycycline dose in the present study was 100 mg because of its original goal to eradicate persistent Chlamydia pneumoniae infection, the results are not fully comparable with the results of the earlier subantimicrobial-dose doxycycline (SDD) studies. In SDD formulation (10–20 mg twice a day), the serum peak levels of doxycycline are up to 0.3 – 0.7 μg/mL, which is considered too low to have an antibacterial effect (Golub et al. 1998, Gu et al. 2011. It has been shown that SDD does not induce the development of antibiotic-resistant bacteria (Walker et al. 2005, Thomas et al. 2000) or other side effects even after two-year administration (Payne et al. 2011, O’Dell et al. 2006). Regular-dose doxycycline is well tolerated in clinical trials but may have some adverse effects, including diarrhoea, fungal infections and antibiotic resistance.
(Smith & Leyden 2005). In the present study, patients did not report any unfavourable side effects (Sinisalo et al. 1998).

The results of Study IV show that doxycycline has a capability to reduce the systemic inflammatory biomarkers. Thus, doxycycline may provide a systemic anti-proteolytic and anti-inflammatory protection and promote plaque stability and affect the outcome of patients with CVD favourably. Unfortunately, there is no follow-up data about the study subjects after 10 months, and therefore the clinical effect of the therapy on possible CVD outcomes is not known.

Hence, with cautions concerning comparability learnt from the earlier studies, the findings of the study provide motivation to implement a comprehensive clinical trial to further investigate the anti-proteolytic and anti-inflammatory efficacy of doxycycline on CVDs. The results can also enlighten the usability of MMP-8 as a predictive biomarker. A better understanding of underlying pathological mechanisms of CVD and further advancements in pharmacogenetics can also provide new background for tailored anti-inflammatory doxycycline therapies in the prevention of CVDs.

5.9 Practical perspectives on the study findings

The aim of translational medicine is to convert laboratory findings of basic research into clinical applications, ‘from bench to bedside’ (Littman et al. 2007), and thus to create new tools for diagnosis, clarify prognosis of patients, improve therapies, and possibly help developing individualized treatment.

In this thesis, it is shown that a high MMP-8 serum level is significantly associated with present (III) and future (I, II) CVD and TIMP-1 with future CVD (I, II). Furthermore, we demonstrate that serum MMP-8 can be measured reliably in hospital settings (III), and that the systemic levels of MMP-8 and MMP-8/TIMP-1 can be modified by using doxycycline therapy.

Regardless of its use a biomarker is functional in clinical practice, provided that it is accurate, reproducibly obtained in a standardized fashion, acceptable to the patient, and easy to interpret by clinicians, it has high sensitivity and high specificity for the outcome it is expected to identify, it explains a reasonable proportion of the outcome independent of established predictors consistently in multiple studies, and there are data to suggest that knowledge about biomarker levels can change management of disease. (Vasan 2006)

When evaluating MMP-8 and TIMP-1 as biomarkers of CVD, MMP-8 fulfils at least some of these conditions for a diagnostic biomarker. Still, further
calculations and studies are needed to assess discrimination or cut-off limits for clinical purposes. It has been shown that MMP-8 measurement can be utilized in the diagnostics of prevalent periodontitis and intra-amniotic infection (Leppilahti et al. 2015, Myntti et al. 2016). There are point-of-care (POC) immunoassays for periodontitis diagnostics, and corresponding assays are being developed to utilize these biomarkers in CVD (Sorsa et al. 2011).

However, finding a prognostic or predictive biomarker is even more difficult than finding one for diagnosing a prevalent condition. The CVD pathophysiology is intricate, its different outcomes have diverse aetiologies, and levels of biomarkers may be changeable during the progression of disease. It is possible that MMP-8 and TIMP-1 are not risk factors, or causally related to disease, but as shown in Study II, they may still serve as risk markers because they may help in risk stratification or identifying individuals who are more responsive to therapies (Ridker et al. 2008, Wilson et al. 2008, Pencina et al. 2010).

CVD is a complex multifactorial disease, and despite intense research, its basic mechanisms are not fully understood. Biomarker research is important for increasing our knowledge about the pathogenesis of the disease. Although findings of this study provide new information about MMP-8 and its regulators in CVD, it is credulous to think that detection of a single biomarker from a labyrinthine network of inflammatory cascade could be a breakthrough in CVD research. In addition to MMP-8 and TIMP-1, many other MMPs, TIMPs and other inflammatory markers promote atherogenesis. Secondly, there is no certainty which part of the destructive imbalance of inflammatory markers is explained by genetic factors and which part as a response to pathogens or environmental factors.

5.10 Future directions

In the future, a ‘multi-marker’ risk stratification strategy including biochemical, genetic and individual lifestyle characteristics should be utilized in clinical practice (Zaiou & el Amri 2016).

Novel risk stratification scores including clinical variables have provided improved results in screening the future CVD risk (Johansson et al. 2016), and it has been shown that adding inflammatory biomarkers in a risk prediction model may improve the differentiation (Ridker et al. 2008, Kaptoge et al. 2012), which is demonstrated in Study II, too. So far, as detection of circulating MMP-8 and TIMP-1 concentrations is relatively quick and inexpensive by using in-house
assays, they are more cost-effective than genetic biomarkers. It should be remembered, however, that as CVDs are extremely prevalent, even small improvement in primary prevention might result in numerous saved lives.
6 Conclusions

The present study allowed the following conclusions to be made from Studies I–IV:

1. High serum MMP-8 concentration indicates an acute cardiac condition and predicts a future CVD event.
2. TIMP-1 and an imbalance between MMP-8 and TIMP-1 are implicated in the pathophysiology of atherosclerotic diseases.
3. Measurement of serum MMP-8 concentration is a reliable, anti-invasive and inexpensive test, which can also be done in hospital settings.
4. Regular-dose doxycycline decreases the systemic inflammatory burden in patients with earlier myocardial infarction. It inhibits circulating MMP-7, MMP-8 and the MMP-8/TIMP-1-ratio. Doxycycline is a promising anti-inflammatory therapy in the prevention of CVDs with relatively minor side effects.
5. MMP-8 and TIMP-1 can be considered promising risk markers both for diagnostic and for screening purposes.
List of references


Original publications


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TRANSLATIONAL PERSPECTIVES ON MATRIX METALLOPROTEINASE 8 AND OTHER INFLAMMATORY BIOMARKERS IN CARDIO-VASCULAR DISEASES