Studies on matrix metalloproteinases in atherosclerosis and coronary heart disease

by

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This work is dedicated to Sara, Andrea and Oliver

Always in my heart.

Table of contents

1. Acknowledgements……9

2. Abbreviations and glossary……11

3. Papers included……13

4. Introduction……15

4.1. Atherosclerosis……15

4.2. Coronary Heart Disease……19

4.3. Metalloproteinases……20

4.3.1. In general…… …20

4.3.2. Metalloproteinase 9/

Tissue inhibitor of metalloproteinase-1 and –2……21

4.3.3. EMMPRIN……22

4.3.4. Pregnancy-associated plasma protein A……24

4.4. Interventions……25

4.4.1. Diet and omega-3 fatty acids……25

4.4.2. Percutaneous coronary intervention – stent implantation……26

4.4.3. Autologous bone marrow stem cell transplantation……26

5. Aims of the study……27

6. Materials and methods……28

6.1. Study subjects and design……28

6.1.1. The Diet and Omega-3 Intervention Trial (DOIT) –study

(Paper I and II)……28

6.1.2. Profile of inflammatory markers in acute myocardial

infarction: The PIMI Trial (Paper III)……29

6.1.3. Autologous stem cell transplantation in acute myocardial infarction: The ASTAMI trial (Paper IV)……...30

6.2. Ethics……32

6.3. Intervention principles……33

6.3.1. Diet and omega-3 fatty acids……...33

6.3.2. PCI……...33

6.3.3. Stem cell transplantation……33

6.4. Laboratory methods……34

6.4.1. Blood collection……..34

6.4.2. Enzyme immunoassays ……..35

6.4.3. RNA isolation and gene expression ……...35

6.4.4. Ultrasonography ……35

6.4.5. Magnetic Resonance Imaging ……36

6.5. Statistics……37

7. Summary of results……..38

7.1. Paper I……...38

7.2. Paper II……38

7.3. Paper III……39

7.4. Paper IV……39

8. Discussion……41

8.1. Methodological considerations……41

8.2. Association between the measured variables and disease entities……...42

8.3. Effects of long term diet and/or omega-3 fatty acids intervention……..44

8.4. Importance of clinical cardiovascular outcome……45

8.5. Time profiles of MMP-9, TIMPs and PAPP-A……..46

8.6. Effects of mBMC transplantation on MMPs……47

8.7. Association between the measured biomarkers and myocardial injury and infarct size……50

9. Conclusions……52

References……55

PAPER I

PAPER II

PAPER III

PAPER IV

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Oslo, June 2014 Eline B. Furenes

2. Abbreviations and glossary

ACS	acute coronary syndrome
AMI	acute myocardial infarction
AP	angina pectoris
AP-1	activator protein-1
CABG	coronary artery bypass graft
CAM	cellular adhesion molecule
CCR2	chemokine receptor type 2
cDNA	complementary DNA
CK	creatine kinase
CKMB	creatine kinase myocardial band
COLD	chronic obstructive lung disease
CV	coefficient of variation
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DNA	deoxyribose nucleotid acid
ELISA	enzyme linked immunosorbent assays
EMMPRIN	extracellular matrix metalloproteinase inducer (CD147)
EPA	eicosapentaenoic acid
GP	glycoprotein
HDL	high density lipoprotein
HT	hypertension
IGF	insulin growth factor
IL-1	interleukin 1
IL-6	interleukin 6

IL-10	interleukin 10
LDL	low density lipoprotein
LVEF	left ventricular ejection fraction
mBMC	bone marrow-derived mononuclear cells
MCP-1	monocyte-chemoattractant protein-1
MI	myocardial infarction
MMP	matrix metalloproteinase
mRNA	messenger ribose nucleotid acid
MRI	magnetic resonance imaging
n-3 PUFA	n-3 polyunsaturated fatty acids
NF-κB	nuclear factor-κB
NSTEMI	non ST-elevation myocardial infarction
oxLDL	oxidized low density lipoprotein
PAPP-A	pregnancy associated plasma protein A
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
RNA	ribose nucleotid acid
SPECT	single photon emission computed tomography
STEMI	ST-elevation myocardial infarction
TGFβ	transforming growth factor β
TIMP-1	tissue inhibitor of metalloproteinases 1
TIMP-2	tissue inhibitor of metalloproteinases 2
TCSF	tumor cell-derived collagenase stimulatory factor
Th2	T helper cell type 2
TNFα	tumor necrosis factor α

3. Papers included

Paper I: **Furenes EB**, Seljeflot I, Solheim S, Hjerkinn EM, Arnesen H. *Long-term influence of diet and/or omega-3 fatty acids on matrix metalloproteinase-9 and pregnancy-associated plasma protein-A in men at high risk of coronary heart disease. Scand J Clin Lab Invest* 2008; 68: 177-184

Paper II: Weiss TW, **Furenes EB**, Trøseid M, Solheim S, Hjerkinn EM, Seljeflot I, Arnesen H. *Prediction of cardiovascular events by MMP-9 in elderly men. Thromb Haemost* 2010; 103:679-81

Paper III: **Furenes EB**, Arnesen H, Solheim S, Grøgaard HK, Hoffmann P, Seljeflot I. *The profile of circulating metalloproteinases after PCI in patients with acute myocardial infarction or stable angina*. *Thromb Res* 2009; 124: 560-564

Paper IV: **Furenes EB**, Opstad, TB, Solheim S, Lunde K, Arnesen H and Seljeflot I. *The influence of autologous bone marrow stem cell transplantation on matrix metalloproteinases in patients treated for acute ST-elevation myocardial infarction.* Submitted 2014

4. Introduction

In developed countries, cardiovascular disease (CVD) is the leading cause of morbidity and death (1). Ischemic heart disease is often a late and dreaded complication of atherosclerosis, a key process in myocardial infarction (MI) and most strokes (2;3).

The early and late mortality of AMI is declining. Nevertheless, despite an increasing knowledge about risk factors for acute myocardial infarction (AMI) which may lead to congestive heart failure or cardiac death, CVD still affects worldwide.

Inflammation is considered to be an important prosess for development of atherosclerosis and this includes a number of cellular and molecular responses resulting in plaque formation (2;4). The atherogenesis includes excessive subendothelial lipid accumulation in macrophages, which transform into foam cells that further secrete numerous of cytokines leading to an inflammatory state.

4.1 Atherosclerosis

The atherosclerotic lesion (atheroma) is seen as an asymmetric focal thickening of intima, the innermost layer of an arterial wall. Accumulation of lipids and components of extracellular matrix combined with inflammation, causes atherosclerosis, and early stages are especially characterized by subendothelial lipid accumulation. Inflammation is a key process for the development of atherosclerosis and is associated with activation of endothelial cells, macrophages and T-lymphocytes, and proliferation of smooth muscle cells (2;3).

Upon stimulation by oxidized LDL the endothelial cells express increased amounts of different proinflammatory substances. Different inflammatory cytokines and chemokines play central roles in all phases of atherosclerosis from the fatty streaks to advanced atherosclerotic plaques (2;3;5) (Figure 1).

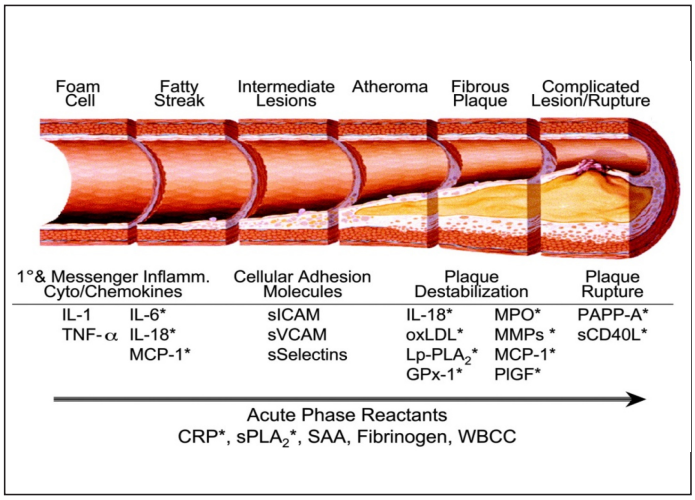

Figure 1. Adapted from Koenig W, ATVB 2007;27. (8) The developing process of atherosclerosis, from fatty streaks to advanced plaques and plaue rupture.

The endothelium serves as a selective permeable barrier between the vessel wall and the circulating blood. Normally it provides a nonthrombogenic and anti-inflammatory surface towards circulating blood leukocytes and platelets (6). On the endothelial surface cellular adhesion molecules (CAMs), which play a central role in the inflammatory process are expressed (7), contributing to adhesion and migration of circulating cells to the intima (8) (Figure 2). In the context of monocyte recruitment in the atheroma, the vascular cell adhesion molecule 1 (VCAM-1) plays a major role. By adhesion to the activated endothelial layer, the monocytes change their phenotype-and penetrate into tunica intima. Different chemokines contribute in this process, escpecially the interaction between monocyte-chemoattractant protein-1 (MCP-1) and its receptor chemokine receptor type 2 (CCR2). Monocytes migrated

into the intima get the character of tissue macrophages, express scavenger receptors which bind lipoproteins modified by oxidation or glycation, among others.

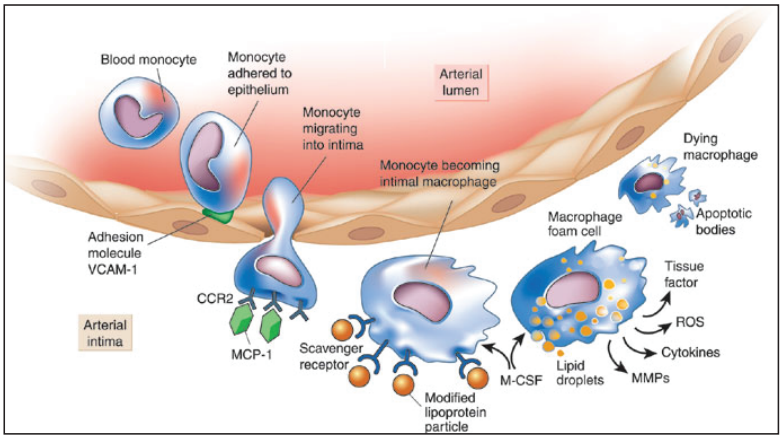

Figure 2. Adapted from Libby P. *Nature.* 2002;420:868-74 (8). The transformation process of blood monocytes into foam cells, with contribution of chemokines, and migration from arterial lumen into the intima.

These processes, as result of accumulation of lipid droplets inside the cytoplasm, create the arterial foam cells. Monocyte-derived macrophages and foam cells as well as invaded T-cells, involving both the innate and the adaptive immune system have several functions related to the development of atherosclerosis and its complications. They secrete pro-inflammatory cytokines which enhances the local inflammatory response in the deposit layer (9)

As the atherosclerotic lesion develops, also smooth muscle cells migrate to the intima in response to growth factor released by activated macrophages and endothelial cells. Various growth factors in the intima cause smooth muscle cell proliferation and the lesion develops to a fibrotic plaque. The smooth muscle cells change their phenotype and become secretory and pro-inflammatory (9).

The activated macrophages also play a key role in the thrombotic complications in atherosclerosis and plaque rupture, by producing matrix metalloproteinases (MMPs) degrading the extracellular matrix which gives strength to the fibrous cap of the plaque *(vide infra)* (10) (Figure 1 and 2). When the plaque ruptures as a consequence of this process, the blood comes in contact with other products of the macrophages like the potent pro-coagulant protein, tissue factor, the important initiator of the coagulation cascade.

Early activation of endothelial cells may be seen as beneficial due to elimination of accumulated lipids by the recruited phagocytizing cells to the intima. In atherosclerosis, however, the continous excessive lipid accumulation within the macrophages, transform them into lipid rich foam cells that secrete numerous proinflammatory cytokines, leading to a chronic low graded inflammatory state.

The activation of endothelial cells may occur in response to different stimuli related to traditional risk factors for CVD, such as hypercholesterolemia, a diet high in saturated fat, lack of physical activity, obesity, hyperglycemia, insulin resistance, hypertension, smoking, shear stress and infectious agents. They are all stimuli which mediate migration of monocytes and lymphocytes to the intima, thus potentiating the atherosclerotic process (11).

The two major causes of coronary thrombosis are plaque rupture and endothelial erosion (6). The immune cells are activated, and they produce numerous inflammatory substances and proteolytic enzymes. These, in turn, can transform the plaque into an unstable structure that may rupture and induce thrombosis, which may lead to an AMI. The different inflammatory mediators play central roles in all phases of the disease (Figure 1). Cytokines

like TNFα, interleukin-1 (IL-1) and interleukin-6 (IL-6) promote inflammation, whereas proteases seem to be of greater importance for plaque destabilization.

Circulating levels of several pro-inflammatory markers of inflammation have been shown to be predictive for cardiovascular events (12-14), of which CRP, IL-6 and IL-18 are the most studied markers. On the other hand, activation of the anti-inflammatory cytokines like IL-10, TGFβ and others are produced from Th2 cells contribute in inhibiting the atherosclerotic process and thereby act as "good guys" (7;15).

Although the involvement of inflammation in the development and progression of atherosclerosis is undebatable (2), there are still many questions to be answered to recognize and define the key players and also to understand the regulatory mechanisms behind. These inflammatory players might probably reflect an imbalance between "good and bad guys".

4.2. Coronary Heart Disease

Ischemic coronary heart disease refers to failure of coronary circulation to supply adequate circulation to cardiac muscle and surrounding tissue, with a reduced oxygen supply and thereby an imbalance between oxygen demand and supply. This is most commonly equated with atherosclerotic coronary artery disease. It is the most common form of disease affecting the heart and is an important cause of premature death in the developed world.

Angina pectoris (AP) is a clinical syndrome, diagnosis based on clinical and laboratory recordings. Symptoms are most common characterized by central retrosternal discomfort, but can also be accompanied by radiating pain in arms, shoulder regions, upper back, or lower jaw or teeth, or simply dyspnoe. AP is caused by myocardial ischemia as a result of an imbalance between myocardial oxygen demand and supply, most often caused by atherosclerosis and reduced arterial luminal area. The reduced blood flow causes symptoms

especially during physical activity or emotional stress when the demand of oxygen rich blood is increased. The pain caused by ischemic myocardium is mediated by stimulation of angiotensin I receptors on cardiac nerve endings. The discomfort will typically be relieved by rest and/or use of nitroglycerine.

Acute myocardial infarction (AMI) is caused by ischemia, usually due to formation of an occlusive thrombus in a coronary artery and thereby cell death in the myocardium (16). The AMI can be visualized and diagnosed by ECG: ST-elevation myocardial infarction (STEMI) may indicate an occlusive thrombus in a central artery with indication for prompt revascularization by either acute percutaneous coronary intervention (PCI) or thrombolysis. Patients with chest pain without ST-elevation in the ECG; Non-ST-elevation myocardial infarction (NSTEMI) are normally treated with anti-ischemic and anti-thrombotic therapy often followed with coronary angiography later on.

4.3. Metalloproteinases

4.3.1. In general

Matrix metalloproteinases (MMPs), initially described in 1962 by Gross et. al (17), are proteinases that participate in extracellular matrix degradation through regulation of their transcription, activation of the precursor zymogens, interaction with extracellular matrix components and inhibition by endogenous inhibitors, TIMPs (18). Metalloproteinases are a class of at least 25 zinc-dependent endopeptidases (collagenases, gelatinases, stromelysins and membrane type MMPs), and are found in most tissues (19). They are physiological regulators of the extracellular matrix, and participate in vascular remodeling, plaque instability by degrading the fibrous cap (vide supra), and ventricular remodeling after cardiac injury (4;20).

4.3.2. Metalloproteinase 9 / Tissue inhibitor of metalloproteinase-1 and -2

MMP-9, also named gelatinase B (Figure 3) (18), belongs to the group of metalloproteinases called gelatinases which means that their substrate is denatured collagens, gelatins (21). These enzymes have three repeats of a type II fibronectin domain inserted in the catalytic domain, which bind to gelatin, collagens and laminin. In humans the gene for MMP-9 is found on the chromosome 20q11.2-q13.1 (18).

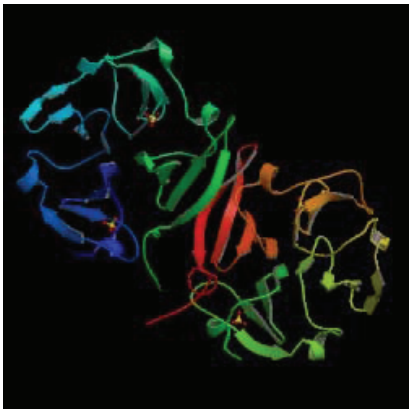

Figure 3. The strucure of MMP-9 (18).

The structure of the MMP-9 prodomain consists of three α-helices and connecting loops where the first loop between helix 1 and 2 is a protease-sensitive region, and with a cleft of catalytic domain containing zinc as the active site.

Most MMPs, including MM-9, can be activated by cytokines, proteinases, nitrogen oxide (NO), heat, low pH and by various chemical agents and growth factors in vitro (4;20-24). Most proMMPs are secreted from cells and activated extracellularly (18), and all vascular cells including endothelial cells and macrophages secrete MMPs. The regulation of the MMPs activation includes genetic regulation, secretion of pro-enzymes that require activation, and inhibition by tissue inhibitor of metalloproteinases (TIMPs) (25). In a stepwise activation

mechanism it has been shown that proMMP-9, mostly secreted from cells, can be activated extracellularely by plasmin, and the zinc-containing site (also called hemopexin) binds to the C-terminal of tissue inhibitor of metalloproteinase 1 (TIMP-1). In addition, several MMPs can contribute to activation of other MMPs, also MMP-9, in a positive feedback mechanism (23). In atherosclerotic vascular tissue MMP-9 is localized at the shoulder of the plaque. This area is known to be most vulnerable to plaque rupture. It is reported that the activity of MMP-9 differ at various stages of plaque progression (25). The role in atherosclerosis is however still not fully understood. The MMP-9 is regulated by specific endogenous inhibitors (TIMPs), by transcription and by certain precursors, i.e when pro-MMP-9 binds to TIMP-1 (23). The ratio between MMP-9 and TIMP-1 may thus be of importance. TIMP-1 is secreted as a soluble protein, and its activity is associated with inflammatory cytokines (26). Tissue inhibitor of metalloproteinase 2 (TIMP-2) is also a member of the TIMP gene family and a natural inhibitor of metalloproteinases. In addition, it plays a unique role in its ability to directly suppress the proliferation of endothelial cells, and might therefore be critical to the maintenance of tissue homeostasis (27).

4.3.3. EMMPRIN

Extracellular matrix metalloproteinase inducer (EMMPRIN, CD147), which is a member of the immunoglobulin superfamily, has lately been discussed to be involved in both expression and release of MMP-9 (29), and may therefore play a regulatory role in atherosclerosis, plaque rupture and CVD. EMMPRIN was originally discovered on the surface of solid tumor cells (30) and therefore named tumor cell-derived collagenase stimulatory factor (TCSF). Later, it has also been shown to be expressed in atherosclerotic plaques as well as in cell types like monocytes, macrophages and platelets (31-35). With the ability to induce expression of

various matrix metalloproteinases it was renamed EMMPRIN. The structure of EMMPRIN consists of two extracellular domains including three glycosylation sites, a transmembrane and a short cytoplasmatic domain as shown in Figure 4 (36). The stimulation of MMPs depends on the glycosylation state of these sites, because unglycosylated EMMPRIN is unable to stimulate MMP induction (37). This site where EMMPRIN binds to the platelet-specific collagen receptor glycoprotein (GP) VI, is expressed in various cell types, including leukocytes and platelets (38). With subsequent activation of MMP-9 it may contribute to plaque instability in human atherosclerosis and may therefore be important in events like acute coronary syndrome (ACS) and AMI (39-41). Circulating levels of EMMPRIN are limited explored in humans.

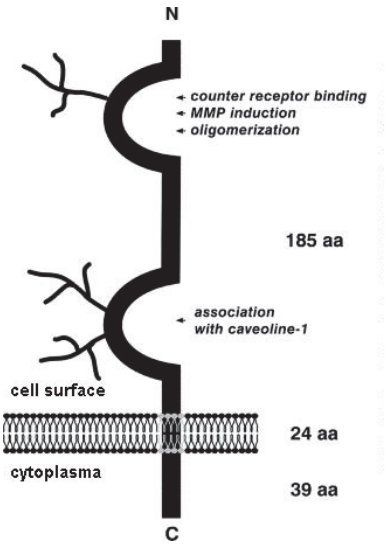

Figure 4. The structure of EMMPRIN (36).

4.3.4. Pregnancy-associated plasma protein A

Pregnancy-associated plasma protein A (PAPP-A) was first recognized in pregnant women as a high-molecular weight constituent associated with Down syndrome. More recently it has been shown that PAPP-A also is present in unstable carotid and coronary atherosclerotic plaques and with increased circulating concentrations in individuals with ACS (42). The molecular composition in the two different organs are different. Whereas PAPP-A in pregnancy exists as a heterotetrameric complex with the proform of eosinophil major basic protein, PAPP-A in coronary syndromes has been found to be a protease specific for insulin-like growth factor-binding protein 4 and –5 (43). In a variety of biological systems, insulin growth factor-I (IGF-I) and –II play important roles in promoting cell differentiation and proliferation. Therefore, increased bioavailability of IGFs through PAPP-A-mediated proteolysis could play a crucial role in the progression of coronary atherosclerosis and thus also as a prognostic factor in patients with unstable coronary artery disease (44).
PAPP-A, as a glycoprotein and an insuline-like growth factor metalloproteinase (4), is produced by different cell types, including fibroblasts and vascular smooth muscle cells. PAPP-A is disrupting the integrity of the protective cap in the atheroma, and release of PAPP-A occurs during atherosclerotic plaque disruption (45). Thus, PAPP-A is regarded as proatherosclerotic and may therefore play an important role in AMI. (45;46). The exact mechanism of PAPP-A in atherosclerosis is, however, not ruled out.

The relationship between MMP-9, TIMP-1, EMMPRIN and PAPP-A and the pathogenesis of different phases of atherosclerosis and CHD are still not known in details. Evaluation of the circulating levels as well as gene regulatory studies, may contribute to further understanding their interactions and role in cardiovascular risk.

4.4. Interventions

4.4.1. Diet and omega-3 fatty acids

From epidemiological and clinical studies the importance of certain dietary patterns with regard to cardiovascular disease seems obvious. Especially, dietary fat and fatty acids which affect plasma lipids and lipoproteins and thus are linked to atherosclerosis, are of importance. Several large observational studies have focused on 'the Mediterranean diet' with increased intake of fruit, vegetables, fish and cereals, and reduced amounts of saturated fat (47). The results of this dietary pattern have shown to reduce all-cause mortality from coronary heart disease (48;49).

Polyunsaturated fatty acids (PUFA) are fatty acids containing two or more double bonds and classified as n-3 fatty acids (n-3 PUFA) and n-6 fatty acids depending of the localization of the first double bond from the methyl end of the molecule. Usually, diet contains both acids, dependent of the diet composition. The most common n-6 fatty acid is arachidonic acid which metabolize to prostaglandins, leukotrienes and other lipoxygenase or cyclooxygenase products, and thereby function as proinflammatory, atherogenic and pro-thrombotic substituents (50). In contrast, the typical marine n-3 fatty acids like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (used in the DOIT study) (*vide infra*) are competitive for the arachidonic acid metabolism.

The clinical benefits of n-3 PUFA of marine origin are well recognized (51). The exact mechanism by which n-3 PUFAs exert their cardioprotective effect is, however, still not fully understood. In addition to substantial reduction in serum triglyceride level, they have been shown to be antithrombogenic, antiarrhythmic, antiinflammatory and also to improve endothelial dysfunction (51).

4.4.2. Percutaneous coronary interventions (PCI)

Coronary arteries with occlusion or significant stenosis with lumen diameter reduction of > 50% are dilated with a transluminal balloon catheter to regain normal blood flow (via arteria radialis or arteria femoralis). The earliest methods used plain balloon angioplasty. Bare metal stents or drug eluting stents as appropriate, are now in most cases implanted during the PCI procedure. Several studies have shown that stent implantation prevent arterial recoil and restenosis and thereby reduce the need for repeated revascularization (52).

4.4.3. Autologous bone marrow stem cell transplantation

Despite well documented treatment of AMI survivors, both medicationally and by PCI, some patients either don't receive this treatment, don't respond satisfactory, or develop congestive heart failure despite treatment.

Several studies have shown that bone marrow stem cells trans differentiate to cardiomyocytes when infused into the affected myocardium (53;54), and paracrine mechanism is one of the methods (55). Treatment with autologous stem cells from bone marrow has been suggested to reduce myocardial damage in patients with AMI. Results from clinical trials are, however, conflicting with regard to improvement of left ventricular ejection fraction (56-62) in patients with ST elevation myocardial infarction (STEMI). Possible mechanisms by which autologous bone marrow stem cells act are discussed to be cardiac transdifferentiation, paracrine effects, angiogenesis, and reduced apoptosis.

5. Aims of the study

The overall aim of the present investigation was to explore any importance of circulating levels of MMP-9, TIMP-1 and -2, EMMPRIN and PAPP-A in atherosclerosis and ischemic coronary heart disease.

Specific aims were:

- to study MMP-9, TIMP-1 and -2 and PAPP-A in a cohort of patients at high risk for CHD as related to different disease entities and risk factors in the population (Paper I)
- to study the effects of diet and/or n-3 PUFA intervention for 3 years on these markers (Paper I)
- to study MMP-9 and TIMP-1 as predictors of clinical cardiovascular outcome in the same population (Paper II)
- to study the time profile of circulating MMP-9, TIMP-1 and -2, and PAPP-A in patients with acute STEMI, revascularized with PCI and stent implantation as compared to patients with stable angina, electively treated with the same PCI procedure (Paper III).
- to evaluate the influence of intracoronary injections of mBMC on selected metalloproteinases in patients with STEMI undergoing PCI. Circulating levels as well as genetic expression in circulating leukocytes at different time points were evaluated (Paper IV).
- to investigate any association between the measured proteinases and infarct size assessed by myocardial biomarkers and imaging methods like MRI (Paper III) and SPECT (Paper IV) (method description; vide infra).

6. Materials and methods

6.1. Study subjects and design

6.1.1. The Diet and Omega-3 Intervention Trial (DOIT) –study (Paper I and II)

The basis for recruitment into the DOIT study was a long term follow-up of the participants from the diet- and anti-smoking part of the Oslo study, comprising 1232 men (born 1923-1932) with high risk of CVD (63).

Subjects in this cohort were originally included in 1972 if they had serum cholesterol concentrations of 6.9-9 mmol/L and systolic blood pressure < 150 mmHg.

In 1997 the 910 survivors were contacted to participate in the DOIT study (64). Altogether 655 subjects attended a screening visit, and 563 were enrolled in the final DOIT-study. The study has a 2x2 factorial design, and the participants were randomly assigned to receive n-3 PUFA placebo capsules (corn oil) and no dietary advice (control group); dietary advice and n-3 PUFA placebo capsules; no dietary advice and n-3 PUFA capsules; and finally dietary advice and n-3 PUFA capsules combined (Figure 5).

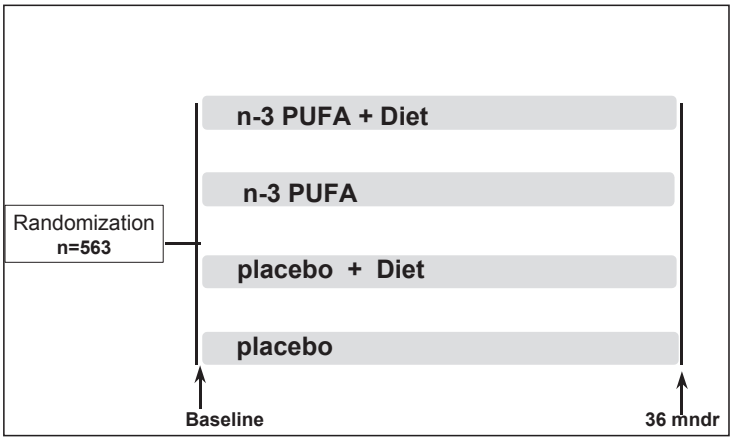

Figure 5. DOIT study design.

Definitions of clinical sub-groups and end-points

CVD at inclusion was defined as patient history of myocardial infarction, performed PCI, coronary artery bypass graft (CABG), aortic dissection, cerebral infarction or claudicatio intermittens (all according to hospital records). Diabetes was defined as treated diabetes or fasting glucose > 7 mmol/L, and hypertension as treated hypertension and/or systolic/diastolic blood pressure >140 / >90 mmHg.

Cardiovascular events, recorded after 3 years, were a composite of fatal and nonfatal CVD, defined as myocardial infarction, revascularization procedures, aortic aneurism, peripheral arterial occlusive disease and cerebrovascular events, according to medical records and the official death certificates held by Statistics Norway.

6.1.2. Profile of inflammatory markers in acute myocardial infarction: The PIMI trial (Paper III)

The study design is described in details in Paper III. In brief; it comprises men and women between 30 and 75 years with acute ST-elevation myocardial infarction (n = 20) or stable angina pectoris (n = 10) admitted to Ullevaal University Hospital, Oslo, Norway. All were treated successfully with PCI in a central coronary artery obtaining normal blood flow. Patients in the stable angina pectoris (AP) group should have symptoms consistent with stable AP and angiographically proven coronary artery disease with indication for PCI. Exclusion criteria in both groups were previous transmural infarction, cardiogenic shock and considerable co-morbidity (malignancy, stroke, inflammatory diseases, endocrinological disturbances and lung disorders). The blood sampling procedure is shown in Figure 6.

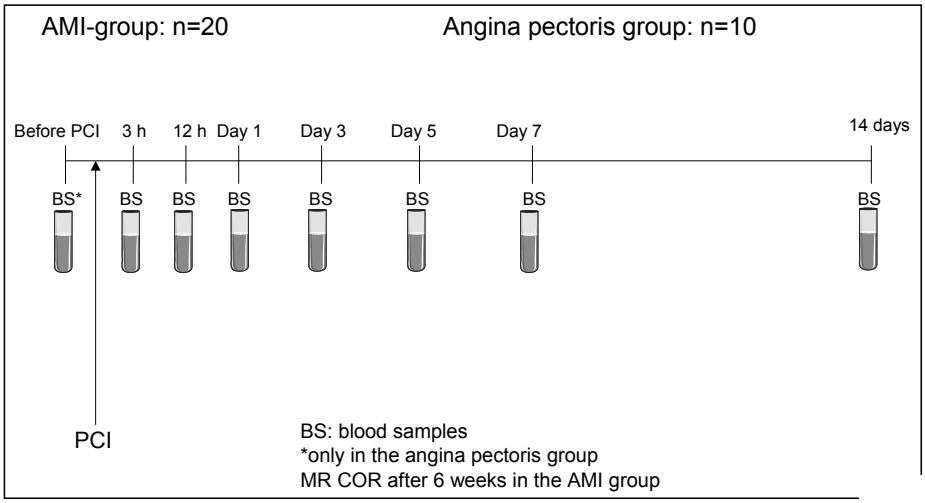

Figure 6. PIMI blood sampling procedure.

6.1.3. Autologous stem cell transplantation in acute myocardial infarction: The ASTAMI trial (Paper IV)

The ASTAMI study is decribed in details elsewhere (65). It was a randomized open-labelled two-arm study, where one arm was intracoronary treatment with mBMC and the other controls. This was performed as a permuted block randomization stratified in two centers, Rikshospitalet University Hospital and Ullevål University Hospital, Oslo, Norway.

One hundred patients, both gender were included.

Baseline recordings were performed during day 5-7 after AMI, whereas MRI was performed after 2-3 weeks and after 6 months.

Inclusion criteria:

- Age between 40 and 75 years
- Acute anterior wall myocardial infarction with a history > 3 hours and < 12 hours
- ST elevation myocardial infarction (WHO ECG criteria)

- Angiographic criteria: occlusion of proximal LAD (ie proximal to second diagonal branch)

- Successful primary rescue PCI (TIMI flow 2 or better)

- Echocardiographic criteria: Evidence for anterior wall infarction as judged by hypo- or akinesia in more than 2 adjacent anterior wall segments (66)

- Enzymatic criteria: CKMBmass > 3 times upper normal value

Exclusion criteria:

- Previous myocardial infarction with established significant Q waves on ECG

- Cardiogenic shock

- Hemodynamic instability neccessitating intraarterial baloon pump treatment

- Permanent pacemaker or other contraindications to MRI

- Stroke with significant sequelae

- Short life expectancy due to extra cardiac reason, ie. COLD, dissaminated malignant disease, or other reason

- Uncontrolled endocrinological disturbance

- Anamnestic indications for significant mental disorder, including dementia

- Established HIV or hepatitis B positivity

- Any condition which interfers with patients possibility to comply with protocol

The ASTAMI blood sampling procedure is shown in Figure 7 and described in chapter 6.4.

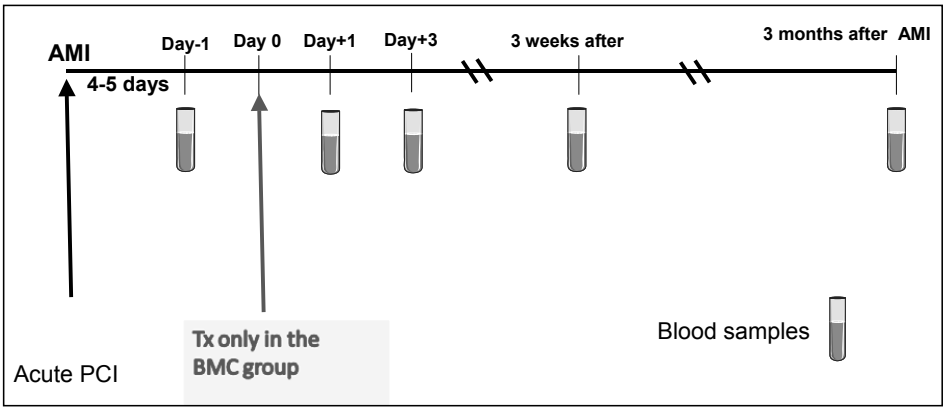

Figure 7. ASTAMI blood sample procedure. SPECT was performed in addition at day 4-5.

6.2. Ethics

The DOIT, PIMI and ASTAMI study protocols, including the biobanks were approved by the Regional Committee for Medical Research Ethics and all patients gave written, informed consent to participate. The DOIT and ASTAMI studies are registered at www.clinicaltrials.gov, NCT number 00764010 (DOIT) and NCT 00199823 (ASTAMI).

6.3. Interventions

6.3.1. Diet and omega-3 fatty acids

Details of the intervention are given in Paper I. Briefly, the dietary advice was individually given by a clinical nutritionist, based on a food frequency questionnaire. The subjects were supported with a margarine rich in polyunsaturated fatty acids (based on sunflower and rapeseed oil) and vegetable oils (rapeseed oil), free of cost. In addition, to decrease the use of meat, advices to increase intake of vegetables, fruit and fish, targeting at energy percents from fat 27-30 %, protein 15-18 % and carbohydrate 50-55 % (a "Mediterranean type" diet), were given. The n-3 PUFA capsules (Pikasol®, Lube, Denmark) used, contain about 60 % n-3 PUFA, mainly eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) in a ratio 2:1, and 3.4 mg/g tocopherols to avoid peroxidation in the capsules. The placebo capsules (corn oil) contain 56 % linoleic acid (18:2 n-6), 32 % oleic acid (18:1 n-9), 10 % palmitic acid (16:0) and 3.0 mg/g tochopherols. Two capsules twice daily, corresponding to a daily intake of either 2.4 g n-3 PUFA or 2.4 g corn oil, were given.

6.3.2 PCI

The percutaneous coronary intervention with stent implantations of choice as appropriate according to patient situation was preformed according to routine procedures in our hospital, as was also use of medication per- and post procedure.

6.3.3. Stem cell transplantation

The patients were treated in accordance with current guidelines (67) and were randomized 1:1 to receive mBMC or to a control group. Only the mBMC group was aspirated for 50 ml of bone marrow from the iliac crest 4-7 days (baseline) after the acute PCI. The bone marrow was treated as previously described in details (65). The next day, a median of 5 days

(interquartile range 5 to 6) after AMI (Day 0), 10 ml of the mBMC suspension was injected in the infarct related coronary artery. The control group was treated with PCI with stent in the infarct related coronary artery at admission, but was not aspirated for bone marrow and did not go through any further coronary intervention. Blood sample procedure is shown in Figure 7.

6.4. Laboratory methods

Details on the laboratory measures used are given in each paper. Briefly:

6.4.1. Blood collection

In the DOIT-study blood samples were drawn between 8 and 10 am after an overnight fasting (≥10 hours) at the time of randomization (baseline) and after 3 years intervention.

In the PIMI-study blood samples were collected immediately before PCI in the AP group, and 3 and 12 hours, 1, 3, 5, 7 and 14 days after PCI in both groups (Figure 6). From day 1 all samples were obtained in fasting state and before intake of any medication.

In the ASTAMI-study blood samples were collected in fasting condition between 08.00 and 10.00 am the day before transplantation in the mBMC group (day-1), the day after (day 1) and further day 3, and after 2-3 weeks and 3 months (Figure 7). The same time interval was used for the control group, except baseline sampling (day-1) which was drawn median 4 days after PCI compared to median 5 days in the mBMC group.

All blood samples, except for routine analyses, were processed and kept frozen at -80°C in a biobank until analysed. PaxGene tubes for gene expression in circulating leukocytes were also collected, kept at room temperature for at least two hours before freezing.

6.4.2. Enzyme immunoassays

For analyses of MMP-9, TIMP-1, PAPP-A and EMMPRIN commersial enzyme linked immunosorbent assays (ELISA) were used on serum samples.

For analyses of MMP-9, TIMP-1 and EMMPRIN kits from R&D Systems Europe (Abingdon, Oxford, UK) and for PAPP-A kits from DRG Instruments GmBH (Germany) were used. The interassay coefficients of variation (CV) were 7.4 % for MMP-9, 4.4 % for TIMP-1, 8.7 % for TIMP-2, 5.4 % for EMMPRIN and 6.9 % for PAPP-A.

6.4.3. RNA isolation and gene expression

Isolation of RNA from PaxGene tubes was performed according to the manufacturers instruction (PreAnalytix, Qiagen GmbH, Germany). The quality and quantity were evaluated by use of NanoDrop 1000 (Saveen Werner, Stockholm, Sweden). The procedure is described in details in Paper IV. Briefly, the mRNA content was achieved by inversely transcribing total RNA in the samples. The genetic expression of the variables was performed by use of real-time PCR on the ViiA^{TM} 7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and quantified in a relative manner (the $\Delta\Delta$Ct method (68)). β-2 macroglobulin was chosen as house-keeping gene.

To analyze mRNA expression derived from the PaxGene tubes, reverse transcription (RT) followed by polymerase chain reaction (PCR) was used. The mathematical model is described by Pfaffl (69).

6.4.4. Ultrasonography

Intima media thickness (IMT) measurements were used to determine the anatomical structural changes in the carotid artery and was performed in a supine position with an ultrasound scanner (Acuson 128, Mountain View, CA, USA) with a 7.0 MHz linear array transducer as

described in Paper I. All scans were performed by the same sonographer and the ultrasound images were captured and recorded on video tapes for off-line analyses. The analyses were performed by experienced personnel (the Ultrasound Laboratory, Clinical research unit, Department of Medicine, Malmø University Hospital, Sweden), according to the leading edge principle, using a computer assisted image analyzing system. At the position of the thickest part in a 10 mm long segment of the far wall of the common carotid artery (CCA) (visually judged), three end-diastolic images were obtained. The mean IMT value was used for statistical analyses.

6.4.5. Magnetic Resonance Imaging and SPECT

Cardiac magnetic resonance imaging (MRI) was provided 6 weeks after PCI in the AMI-group in the PIMI-study using a 1.5 T whole body scanner (Philips Intera, Best, The Netherlands) (Paper III). The analysis was performed on View Forum workstation (Philips Medical Systems). Short axis images were aquired for left ventricular volume and ejection fraction analyses. Infarct size by MRI was determined with the gadolinium late contrast enhancement technique (70).

Single photon emission computed tomography (SPECT) (GE Medical Systems with 4D-MSPECT software) was used to determine left ventricular ejection fraction (LVEF) and infarct size in the ASTAMI trial (Paper IV). Infarct size was expressed as percentage of the LAD-area.

6.5. Statistics

Details on statistical analyses used are given in each paper.

MMP-9, TIMP-1/-2, PAPP-A and EMMPRIN levels were all skewly distributed and non-parametric statistics were therefore used throughout. Median values and 25, 75 percentiles are given, except for baseline characteristis in Paper I and IV. For group comparisons the Mann-Whitney test was used for continous data, and Chi square test was used for categorial data. Friedman test was performed to analyze for differences between any time points within groups. For changes within groups (Paper I, III and IV) Wilcoxon test was used, only when Friedman test was significant. Multiple linear regression models were used to adjust for covariates on logtransformed data when appropriate (Paper I and II). Spearman's rho was calculated for correlation analysis. The SPSS software package version 14.0-18.0 was used throughout.

7. Summary of results

7.1. Paper I

In the DOIT-study, a population at high risk for atherosclerosis and CHD (n = 563), results cross sectionally at baseline showed that smokers had significantly higher levels of MMP-9 ($p < 0.0001$) and MMP-9/TIMP-1 ratio ($p < 0.0001$). TIMP-1 levels were lower in subjects with previous AMI (n = 101) ($p = 0.021$). MMP-9 was significantly correlated to LDL-cholesterol and inversely correlated to high density lipoprotein cholesterol (HDL-C) (both $p < 0.0001$). There were no significant correlations between the measured variables and IMT.
Dietary- and/or n-3 PUFA intervention did not influence the measured markers. Significant reductions in the levels of MMP-9 and PAPP-A after 36 months intervention were, however, found in all study groups ($p<0.0001$, all).

7.2. Paper II

In the DOIT-study, cardiovascular (CV) events were recorded in 68 individuals. Higher levels of MMP-9 ($p = 0.046$) but not triglycerides, total cholesterol, HDL, LDL or oxidised LDL were associated with CV events. Univariate regression analysis revealed a significant association between higher MMP-9 levels (> 75th percentile; 543 ng/ml) and CV events (OR 1.93; CI 1.13-2.30; $p = 0.016$). Analysing MMP-9 combined with lipid levels, it appeared that elevated MMP-9 levels predicted CV events significantly in individuals with hypertriglyceridaemia (>1.7 mmol/l) (OR 3.69; CI 1.67-8.19; $p = 0.001$). The prediction of CV events by MMP-9 was still significant in patients with hypertriglyceridaemia after adjustment for relevant covariates (OR 3.17; CI 1.33 - 7.55; $p = 0.009$).

7.3. Paper III

We investigated the profile of MMP-9, TIMP-1, TIMP-2 and PAPP-A after PCI in patients with AMI and stable angina pectoris (AP). In AMI patients (n = 20) levels of MMP-9 and the MMP-9/TIMP-1 ratio decreased significantly 1 day after PCI ($p < 0.01$ for both), sustaining during 14 days. Both were higher compared to the AP group (n = 10) at 3 hours ($p=0.062$ and $p<0.01$, respectively). A similar pattern was observed in PAPP-A levels being significantly reduced after 12 hours ($p < 0.01$). TIMP-1 levels increased significantly after 12 hours ($p < 0.001$), sustaining thereafter.

In the AP group only small changes after PCI were observed, except from a significant increase in PAPP-A levels from before PCI to 3 hours ($p < 0.0001$), followed by significant reduction.

No significant correlations were found between any of the measured biomarkers and the infarct size, either evaluated in the acute phase or after 6 weeks.

7.4. Paper IV

We observed no differences in MMP-9, TIMP-1 or EMMPRIN between the mBMC group and the control group during the study period. There was a significantly more pronounced increase in MMP-9 from before stem cell transplantation (baseline) to 2-3 weeks ($p = 0.03$) and 3 months ($p = 0.05$), compared to the control group. EMMPRIN levels were significantly reduced from baseline to 2-3 weeks and 3 months in both the mBMC group and the control group ($p < 0.0001$, all), with no difference in changes between the groups. We observed no changes in TIMP-1 levels in either groups.

MMP-9 and EMMPRIN gene expression levels were significantly reduced from baseline to 3 months in the mBMC group ($p < 0.0001$ and $p = 0.002$ respectively). This could not be demonstrated in the control group.

When defining baseline mRNA level (RQ-values) in the total population to 100 %, there was a 20 % reduction in MMP-9 gene expression from baseline to 2-3 weeks, and a 50 % reduction after 3 months in the mBMC group. A similar pattern was seen for EMMPRIN, with a 20 % reduction after 2-3 weeks and 60 % reduction after 3 months in the mBMC group.

We observed a significant correlation between MMP-9 and EMMPRIN ($p = 0.011$) at baseline. Peak levels of CK correlated significantly to both MMP-9 and EMMPRIN ($p = 0.005$ and $p < 0.001$, respectively). MMP-9 and EMMPRIN were significantly correlated to infarct size measured by SPECT ($p = 0.018$ and $p = 0.008$ respectively), and EMMPRIN was inversely correlated to LVEF at baseline ($p = 0.002$).

8. Discussion

8.1. Methodologic considerations

The DOIT study was an extension of the Oslo Diet and Anti-smoking study from 1972, with a predefined population of caucasian male subjects. This is a quite homogenous population of long time survivors from a high-risk cohort, and survivor bias coud be a possibility that should be taken into account.

In this study the follow up time frame was three years, which results in longer storage time in the freezer for the earliest blood samples collected than the latest. This could influence the results of the analyses. However, tests (unpublished data) have shown that some relevant markers are stable at -80°C for a longer period, thus we assume no such influence on the results. Anyway, between group differences in the intervention trial would not have been influenced by the storage time.

In the PIMI study we did not manage to collect blood samples before PCI in the AMI group as the subjects were acutely admitted to the hospital. We therefore have no information about the levels of the measured biomarkers during the first early period of the acute phase. The number of patients enrolled were rather limited, thus we cannot rule out statistical type II errors. Another limitation is that the included patients in this study had rather small myocardial infarcts, and a more profound expression of the measured variables might be seen with larger infarcts.

In the ASTAMI study bone marrow aspiration and placebo injection was not performed in the control group due to ethical reasons. Although baseline characteristics did not differ between the goups, these procedures are considered traumatic, and might therefore influence the outcome of some of the variables. Furthermore, gene expression of the selected

variables was performed in circulating leukocytes, which might not be the main source of proteases in the current disease state.

The biomarkers used for measurement of infarct size were CK and CK-MB. The reason for not using troponin, was that the two hospitals including patients in the ASTAMI study (Rikshospitalet University Hospital and Ullevål University Hospital) were using different types as standard biomarker, troponin I and troponin T, respectively, and thereby complicating the statistics.

8.2. Association between the measured variables and disease entities

MMP-9 and TIMP-1 were both partly associated with traditional risk factors, significantly correlated to LDL-C, inversely to HDL-C, but not to triglycerides. We are not aware of other reports on such associations, although Nakamura et al. (71) showed no correlations between LDL-C and MMP-9 and TIMP-1 levels in their smaller population of patients with type 2 diabetes. More recent research, both gene expression studies (72) and on circulating levels (73;74) has contributed to the assumption that higher levels of MMP-9 and/or lower levels of TIMP-1 is associated with LDL cholesterol.

Conflicting data regarding the expression pattern of the vascular MMP/TIMP system in human hypertension have been reported (75;76). Associations between hypertension and elevated levels of MMP-9 have been shown and also a reduction in MMP-9 levels after treatment of hypertensives (28;77-79), and also a reduction in blood pressure after inhibiting MMP-9 (80;81). For TIMP-1 levels, both increased and decreased levels have been reported (28;82;83). In our study population, no association between hypertension and MMPs were observed which might be due to the fact that about 30 % of the cohort already was treated for their hypertension. A slight inverse correlation between PAPP-A and SBP was recorded,

however, not significant after adjustment for covariates. To our knowledge, results on this item have not been reported elsewhere.

No significant differences in MMP-9 or TIMP-1 in diabetics, mostly type 2 diabetes, versus non-diabetic subjects, were observed. This is partly in line with the study by Lee et al. (84) showing no difference in MMP-9. They could, however, demonstrate significantly higher levels of TIMP-1 in type 2 diabetics. Our group have recently also shown in a larger study of stable CAD patients no differences in MMP-9 or TIMP-1 in diabetics (type 2) vs non-diabetics (85).

It has, however, been shown that type 1 diabetic subjects exhibit significantly higher circulating levels of both MMP-9 and MMP-9/TIMP-1 ratio (19). The latter fits in with the observation that high glucose has been shown to induce expression and activity of MMP-9 from monocyte-derived macrophages without affecting TIMP-1 expression (24).

The smokers had significantly higher levels of MMP-9 and MMP-9/TIMP-1 ratio compared to the non-smokers. This difference was still significant after adjusting for covariates. A stimulating effect of smoking on MMP-9 secretion has been shown in mice (86) and also in cultures of human endothelial cells (87). This observation could contribute to the understanding of the proneness to AMI in smokers, and to the best of our knowledge, this was the first report on this associations in humans. More resent publications have confirmed that MMP-9 levels are elevated in both previous (88) and current smokers (89). Heavy smoking has also been shown to increases MMP-9 gene expression in saphenous vein conduits (90). TIMP-1 levels were significantly lower in subjects with previously AMI, and might contribute to our hypothesis of the proneness to rupture of plaques by reduced inhibition of MMP-9, and thereby degrading of the fibrous cap.

8.3. Effects of long term diet and/or omega-3 fatty acids intervention

One of the main findings in this study was no effect of intervention with n-3 PUFA and/or diet on levels of MMPs compared to controls. Conflicting results on this item have been reported. The results by Ercan et al. with diet intervention (91) are in accordance with our study, although with a shorter follow-up time of 2 months. Also in line with our results, a study of 12 weeks intervention with either 6.6 grams or 2.0 grams n-3 PUFA per day showed no significant changes in serum levels of MMP-9 or TIMP-1 (92). The latter dose is similar to the dosis used in our study. Others have shown reduction in MMP-9 levels in a short-term (3 weeks) intervention with diet combined with exercise in patients with the metabolic syndrome, and thus to some degree comparable to our study population (70).

We observed an overall correlation between the change in MMP-9/TIMP-1 ratio and the change in IMT, the latter being significantly influenced by dietary intervention (93). This might indicate the importance of MMP-9 for the progression of atherosclerosis, although no correlation with soluble MMP-9 could be detected. Our results are also partly in line with the study by Aarsetøy et al. (94), who found a non-significant reduction in MMP-9 in patients with AMI treated with n-3 PUFA supplementation for 12 months. However, they could demonstrate a significant increase in PAPP-A levels in their population with AMI, but only in subjects younger than 65 years, a population different from ours. It has been demonstrated in animal models, that n-3 PUFA exerts a beneficial effect on MMP-9, i.e. in accordance with our hypothesis (95). However, in our study, all groups showed significant reduction in MMPs after 3 years. The reduction in MMP-9, MMP-9/TIMP-1 ratio and PAPP-A during the study period might be explained by increased use of medications or higher age. Both statins and ACE-inhibitors have been shown to reduce MMP-9 levels (96), and in our population both medications were used in increased amounts throughout the study period. Previous studies have shown that statins inhibit secretion of MMPs (91;97) without affecting TIMP-1, possibly

contributing to the plaque-stabilizing effects of statins. However, we could not demonstrate lower levels of MMP-9 in statin users at baseline (Paper I).

8.4. Importance for clinical cardiovascular outcome

We investigated the hypothesis that an interaction between MMPs and plasma lipoproteins may be associated with cardiovascular risk. The 68 CV events recorded during the three-year study period were weakly associated with elevated MMP-9 levels compared to event-free subjects. This is to some degree in line with the results of Blankenberg et al. (98), showing a significant association with cardiovascular mortality.

In our study, especially individuals in the upper quartile of MMP-9 had a significantly elevated risk for CV events, and even more pronounced risk when present in combination with hypertriglyceridaemia. Thus, the association between MMP-9 and CV events was partly dependent on high triglyceride values, despite no correlation between MMP-9 and triglycerides (Paper I). High triglyceride values in our study was defined as the upper quartile in the population, which is in accordance with the clinical definition of hypertriglyceridemia (1.7 mmol/L), thus this cut-off level should reflect a biological treshold. The exact biological mechanism behind this interaction remains unknown, but hypertriglyceridaemia may introduce a proinflammatory state that contributes to the deleterious effect of MMP-9. MMP-9 was strongly correlated to LDL-C as shown in Paper I, however, no further increase in event rate was observed with the combination of high MMP-9 and LDL-C levels, indicating VLDL-cholesterol to be more important regarding this interaction.

Our results indicate that MMP-9 might be a predictor in identifying elderly men at risk for CV events, also previously reported in another population (98). The non-independency shown in our study is in accordance with the study by Jeffris et al (99), showing MMP-9 to

predict clinical outcome in univariate analysis, however, not as a separate and independent predictor as adjustment for cigarette smoking attenuated the odds ratio.

8.5. Time profiles of MMP-9, TIMPs and PAPP-A in STEMI and AP

The overall change in metalloproteinases and their inhibitors in the acute phase after AMI until 14 days, shown in Paper III, indicate that these markers are either involved in the process of the acute event *per se* or might only be solely markers of an acute phase reaction. The PCI procedure *per se* seems to influence the variables to a limited degree.

As we observed significantly lower levels of circulating MMP-9 in stable AP patients compared to AMI patients, it seems that the acute vs stable coronary syndrome correlates to the release of MMPs. This is in accordance with previous observations showing plaque rupture *per se* to be associated with increased MMP levels (100). MMP-9 is secreted from macrophages and is also released from T-lymphocytes, which is highly present in the ruptured plaque area and might therefore also be important sources for the MMP-9 obtained during an AMI (101;102). In addition, generation and secretion of MMP-9 have been shown when activated platelets adhere to the endothelium, an active process during an AMI (101). In animal models it has also been shown that MMP release occurs in the early phase after an acute MI (103).

The role and regulation of TIMP-1 and -2, both synthesized by smooth muscle cells and macrophages, has not been established. Johnson and co-workers showed that overexpression of TIMP-2, but not TIMP-1, inhibits plaque development and destabilisation (105) which can be discussed along with our findings of TIMP-2 levels being higher in the AP-group compared to the AMI group throughout.

Elevated PAPP-A levels have been suggested to predict ACS (106), and have also been shown to be a marker of plaque instability (107). The steep reduction observed in our

AMI-population from three hours to all later time points, support the notion that PAPP-A is an important marker, although the levels before PCI are unknown. Our results can also be discussed in line with a previous report showing an early peak in PAPP-A during the first 12 hours from symptoms onset, followed by a rapid normalization after 48 hours (108).

The limited changes in the stable AP patients indicate that the PCI-procedure itself does not induce release of MMP-9 and TIMP-1 and -2. Interestingly, we observed a significant rise in PAPP-A three hours after the procedure. PAPP-A is present in all human fibroblasts and is released during atherosclerotic plaque disruption, and therefore expected to be increased also in the AMI group. It might be speculated that the increase shown very early after PCI in the AP-group, is a result of procedure related release to the circulation from smooth muscle cells in the atherosclerotic plaque, which probably are masked in the AMI patients in which the levels before the procedure are unknown, but thought to be at a high level. It should, however, be emphasized that heparin induces release of PAPP-A (109;110), which may be relevant for the increase observed after 3 hours. The same heparinization procedure was, however, used in both groups, but the lack of increase in the AMI-group might be related to binding of heparin in the acute phase of an AMI (111).

The early peak and thereafter a marked reduction in the MMP-9, MMP-9/TIMP-1 ratio and PAPP-A levels shown in the AMI group when compared to patients with stable AP, both treated with PCI, indicates that the metalloproteinases are involved in the early phase of the plaque rupture process, with limited influence of the PCI procedure.

8.6. Effects of mBMC transplantation on MMPs in STEMI

In this sub-study of ASTAMI limited influence of intracoronary injection of mBMC transplantation after AMI was observed on circulating levels of MMP-9, TIMP-1 and

EMMPRIN. There was, however, a more pronounced increase in MMP-9 after 2-3 weeks and 3 months in the mBMC group. EMMPRIN levels were reduced during the study period in both groups.

Our hypothesis in this part of the project was that treatment with mBMC would reduce the circulating levels of the selected biomarkers based on results from both *in vitro* and *in vivo* studies showing stem cell transplantation to reduce MMPs after AMI and also to improve ventricular remodeling (112).

The underlying mechanisms for the influence of stem cells on MMPs are not clarified, and a conclusion regarding type of stem cells for the purpose of autologous transplantation has also not yet been made. In cell culture of cardiac fibroblasts Wang et al. (113) could demonstrate that the protein expression and activity of MMP-2, but not MMP-9, were increased in response to hypoxia, and decreased when co-cultured with mesenchymal stem cells. It has also been demonstrated that early endothelial progenitor cells increased MMP-9 expression *in vitro*, whereas MMP-2 was increased in outgrowth endothelial cells (114).

As discussed in Paper IV, the type of stem cells seems to be of importance regarding the degree of influence on MMPs (115). A study on rat hearts with AMI, treatment with modified mesenchymal stem cells (116), showed a reduction in MMP-9 levels. The results can not be compared to ours, as mesenchymal stem cells are multipotent stromal cells with potential for differention into a variety of cell types (117). In this particular study the stem cell injection was performed only one hour after AMI compared to 5 days in our study, and the timing of the transplantation is still also a debatable question.

We found a significantly more pronounced increase in MMP-9 levels in the mBMC group compared to controls, and in accordance with these findings, Roderfelt et al. demonstrated a transient inflammatory response and upregulation of MMP-9 activity after bone marrow transplantation in mice (118). As we previously have shown that MMP-9 levels

are reduced already 1 day after AMI (Paper III), we assume that the levels were normalized when baseline sampling in the this study was performed, and therefore limited influenced by the acute phase reaction.

Studies have shown that invasive intervention principles to some extent affects the release of MMPs (119;120), although we found limited effects of the PCI-procedure *per se* (Paper III). Bone marrow aspiration was not performed in the control group in the ASTAMI study. This procedure which itself is a trauma, could influence the release of inflammatory markers and contribute to the elevated levels in the mBMC group. Accordingly, baseline measures which was performed before the stem cell aspiration, showed no significant differences between the groups. A similar mechanism might explain the increase in MMP-9 levels shown in traumatic injury (121), and that elevated levels of MMP-9 may delay wound healing (122) and are associated with coronary artery in-stent restenosis (123).

MMP-9 expression is a crucial pathogenic feature in a range of conditions and disease states, also other than CVD (124-126), in which treatment with stem cells have been shown to suppress or down regulate the MMP-9 expression (126) and thereby improving the current condition. The importance of EMMPRIN as an inducer of MMP-9 has been explored only to a limited extent in humans. In our study circulating levels of MMP-9 and EMMPRIN were significally correlated, indicating a common regulatory pathway. In the work by Reddy et al, both EMMPRIN and MMP-9 expression in primary cardiomyocytes were induced by the pro-inflammatory cytokine interleukin-18. EMMPRIN was induced via JNK/Sp1 signalling and MMP-9 was induced both via EMMPRIN and through the transcription factors AP-1 and NF-κB activation (127). Other studies have shown a common inhibition of MMP-9 and EMMPRIN (128;129), and gene silencing of EMMPRIN was shown to reduce upregulation of MMP-9 in cell culture (130).

The significant reduction in genetic expression of MMP-9 seen at 3 months might be discussed as compensatory to the increase observed in the circulating levels. Circulating levels as well as genetic expression of EMMPRIN were significally reduced along with the increase in MMP-9 which might be discussed as a negative feed back mechanism. Expression of the EMMPRIN-gene in circulating leukocytes, also reported by Xu et al. assessed by flow cytometry (131), may indicate that the leukocytes contribute to the circulating levels, although no correlation between circulating levels and gene expression levels was observed in our study. The reduction over time seen in EMMPRIN expression, with subsequent reduction in MMP-9 gene expression, contribute to the assumption that EMMPRIN is an inducer of MMP-9.

8.7. Association between the measured biomarkers and myocardial injury and infarct size

In Paper III, no significant correlations between MMP-9, TIMP-1/2 and PAPP-A and infarct injury measured by troponin T and CK-MB, or infarct size measured by MRI were found. This is in accordance with others (104), and may be discussed to be due to the relatively small infarcts in the population studied.

In the ASTAMI population (Paper IV), we observed significant correlations between both MMP-9 and EMMPRIN, and myocardial injury assessed by biomarkers (CK) as well as by infarct size measured by SPECT. In this population the infarcts were larger, which may explain the different results. In experimental AMI, MMP-9 has been shown to increase infarct size and left ventricular fibrosis (20), in accordance with our findings. As for EMMPRIN, this has been sparsely explored in humans, however, it has been shown that EMMPRIN is enhanced in cardiomyopathies and therefore proposed as a marker for cardiac inflammation (132). We also observed an inverse correlation between EMMPRIN and LVEF. An

association between EMMPRIN and the degree of myocardial injury and LVEF was also reported in the work by Nie et al (133), but this was a post mortem immunohistochemistry study which showed a strong increase in EMMPRIN around the zone of necrosis in the AMI group. As to our knowlegde, such association has not previously been reported in clinical studies with survivers after myocardial infarction. Our results contributes to the suggestion that the expression of EMMPRIN is a decisive factor in regulating MMP-9 activity, and thereby being involved in myocardial remodeling.

MMP-9, and also EMMPRIN as an inducer of MMP-9 have in several contexts been discussed to be 'bad guys', thus an attempt to reduce such levels would be beneficial. In our work focusing atherosclerosis and CHD, intervention with diet, marine n-3 PUFA, or transplantation with autologous bone marrow stem cells, failed to reduce the circulating levels or gene expression of our selected markers in our populations of human subjects.
The anti-TNF-α antibodies infliximab and etanercept have been shown to reduce the serum levels and expression of MMP-9 (134;135) in human subjects with rheumatoid arthritis, and also in an animal model. A future goal in preventing atherosclerosis and its serious complications, therefore, might be in line with these results, with more pin pointed influence on the inflammatory process

9. Conclusions

In the present work on matrix metalloproteinases, aimed to investigate any importance of the MMPs in atherosclerosis and coronary heart disease, we could demonstrate:

- a highly significant correlation between MMP-9 and serum lipids, elevated MMP-9 levels in smokers, and reduced TIMP-1 levels in subjects with previous AMI
- individuals with high levels of MMP-9 had a significantly higher risk of cardiovascular events, especially in combination with hypertriglyceridaemia
- intervention with diet and/or n-3 PUFA supplementation for 3 years did not influence the levels of MMP-9, TIMP-1 or PAPP-A
- patients with ST-elevation myocardial infarction treated with PCI, presented initially with high levels of MMP-9, followed by an early reduction of both MMP-9, MMP-9/TIMP-1 ratio and PAPP-A, compared to patients with stable angina pectoris treated with the same procedure
- limited effects of intracoronary injection of mBMC transplantation on circulating levels as well as gene expression of MMP-9 and EMMPRIN in patients with STEMI treated with PCI
- both MMP-9 and EMMPRIN, were significantly correlated to myocardial injury and infarct size in STEMI patients with larger infarcts, indicating that the regulation of metalloproteinases is important in the process of an AMI.

Interventions with diet, n-3 PUFA or autologous bone marrow stem cell transplantation did not influence the levels of the selected matrix metalloproteinases in our populations. Nevertheless, these results might strengthen the hypothesis that metalloproteinases are involved in atherosclerosis, and might be useful tools in identifying individuals at risk for

cardiovascular events. Furthermore, the results contribute to the understanding of the pathophysiology of metalloproteinases in AMI, but in the treatment with autologous bone marrow stem cells, further investigations are needed regarding influence of timing and type of cells.

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