Leukocyte-derived matrix metalloproteinase-9 in patients with coronary artery disease.

Associations with psychological stress and glucocorticoid sensitivity

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“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.”
— Albert Einstein
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Original publications

Paper I
Increased levels of leukocyte-derived MMP-9 in patients with stable angina pectoris.

Paper II
Overexpression of MMP-9 and its inhibitors in blood mononuclear cells after myocardial infarction--is it associated with depressive symptomatology?
Jönsson S, Lundberg AK, Jonasson L.

Paper III
The glucocorticoid receptor alpha isoform is overexpressed in blood mononuclear cells from patients with coronary artery disease - Evidence for increased glucocorticoid sensitivity.
Simon Jönsson, Anna K Lundberg, Lena Jonasson
Submitted

Paper IV
Inflammatory response to acute mental stress is associated with altered cortisol reactivity and telomere shortening in patients with coronary artery disease
Anna K Lundberg*, Simon Jönsson*, Helene Zachrisson, Margareta Kristenson, Lena Jonasson
Submitted
Abstract

Inflammation is closely associated with development of atherosclerosis. The proteolytic enzyme matrix metalloproteinase (MMP)-9 is considered to play a prominent role in this process. MMP-9 has also been introduced as a marker for plaque vulnerability. Still, the possible mechanisms behind altered levels of MMP-9 and its tissue inhibitors (TIMPs) in patients with atherosclerotic disease remain unclear. The general aim of this thesis was to compare leukocyte-derived MMP-9 and TIMPs in patients with coronary artery disease (CAD) and healthy controls and to further relate the findings to psychological stress and glucocorticoid sensitivity.

Levels of leukocyte-derived MMP-9 and TIMP-1 showed a significant difference between CAD patients and controls. Neutrophils in CAD patients were more prone to release MMP-9 and furthermore, PBMCs in patients expressed higher levels of MMP-9 and TIMP-1 and -2 mRNA than PBMCs in controls while there were no differences in plasma or serum levels. The increase in leukocyte-derived levels of MMP-9 and TIMPs indicate the presence of preactivated leukocytes in CAD.

Inflammation has been proposed as a mechanistic link between cardiovascular risk and depressive symptoms. We investigated whether the overexpression of leukocyte-derived MMP-9 and TIMPs in CAD patients was associated with psychological factors. Patients exhibited sustained elevations in depressive symptoms, however, these symptoms were not related to any MMP-9 or TIMP variables. The findings suggest that overexpression of leukocyte-derived MMP-9 and TIMPs and elevated depressive scores represent two parallel phenomena in CAD.

Chronic inflammation may be associated with reduced glucocorticoid sensitivity. We found that PBMCs in CAD patient expressed significantly increased levels of glucocorticoid receptor (GR)-α mRNA, whereas GR-β mRNA levels did not differ between patients and controls. Moreover, in ex vivo assays, dexamethasone efficiently suppressed MMP-9 and TIMPs equally or even more in patients compared to controls. The findings provide evidence for enhanced glucocorticoid sensitivity in CAD patients and also suggest that a state of relative hypocortisolism may contribute to the overexpression of leukocyte-derived MMP-9 and TIMPs.

Lastly, we explored the release of MMP-9, TIMPs and cortisol in response to acute mental stress in CAD patients. Patients who exhibited a significant stress-induced increase in serum MMP-9 also exhibited an altered cortisol response. Moreover, the susceptibility to stress-induced increase in serum MMP-9 was associated with shorter leukocyte telomere length and atherosclerotic plaque burden. The findings highlight the existence of a high-risk group which may be in need of improved diagnostic and therapeutic strategies.
Populärvetenskaplig sammanfattning


Vi såg tydliga skillnader mellan kranskärllssjuka och friska individer. Vita blodkroppar hos patienter hade ökad produktionskapacitet av MMP-9 och TIMP och dessutom frisatte celler från patienter mer MMP-9 när de utsattes för stress (i provröret). De cirkulerande halterna av MMP-9 och TIMP var däremot inte förhöjda hos patienterna. Att enbart mäta cirkulerande halterna av MMP-9 och TIMP kan således ge en missvisande bild.

Det finns ett samband mellan stress och kranskärlssjukdom och en förklaringsmodell är att stress ger upphov till inflammation i kärlväggen. Vi fann att många patienter som tidigare haft hjärtinfarkt led av bestående nedstämdhet. Det fanns dock inget samband mellan ökad nedstämdhet och ökade halter av MMP-9 och TIMP i vita blodkroppar.

Kortisol är kroppens egen starka inflammationshämmande substans. Vi undersökte därför om kortisol kunde hämma MMP-9 och TIMP i vita blodkroppar och om detta i så fall fungerade sämre hos patienter som haft hjärtinfarkt. Kortisol visade sig vara en stark hämnmare av MMP-9 och TIMP. Vita blodkroppar från patienter var dessutom känsligare för kortisol än vita blodkroppar från friska. Detta kan bero på att patienternas celler upplever en bristande tillgång på kortisol i kroppen.

Abbreviations

ACS          Acute coronary syndrome
ACTH         Adrenocorticotropic hormone
CAD          Coronary artery disease
CRH          Corticotropin-releasing hormone
CES-D        Center for Epidemiologic Studies - Depression Scale
CorC         Coronary Computed Tomography
CVD          Cardiovascular disease
ECM          extracellular matrix
DC           Dendritic cell
GC           Glucocorticoid
GR           Glucocorticoid receptor
HPA          Hypothalamus-pituitary-adrenal
HSD          Hydroxysteroid dehydrogenase
IFN          Interferon
IL           Interleukin
IL-1Ra       IL-1 receptor antagonist
IMT          Intima-media thickness
LDL          low density lipoproteins
LPS          Lipopolysaccharide
TL           Telomere length
MI           Myocardial infarction
MIMMI        Mental – Immune Interactions in Myocardial Infarction
MMP          Matrix metalloproteinase
MPO          Myeloperoxidase
NF-κB        Nuclear factor-κB
NK cells     Natural killer cells
NSTEMI       Non-ST elevation myocardial infarction
PBMC         Peripheral blood mononuclear cells
PCI          Percutaneous coronary intervention
qPCR         quantitative polymerase chain reaction
STEMI        ST elevation myocardial infarction
Th1          T helper 1
TNF          Tumor necrosis factor
TIMP         Tissue inhibitor of metalloproteinase
Introduction

**Coronary artery disease**

It is estimated that around 17 million people die from cardiovascular diseases (CVD) each year, equivalent to 30% of all deaths worldwide being the most common cause of death globally. However, 80% of the CVD deaths occur in low and middle-income countries, while CVD mortality has declined in Western Europe and North America, partly due to successful therapy and prevention strategies. Nonetheless, CVD remains the most common cause of death in developed countries. The most common CVDs are coronary artery disease (CAD) and stroke, which accounted for 7.3 and 6.2 million deaths respectively in 2008 (1).

Traditional risk factors for acute myocardial infarction (MI) have been evaluated in the large case-control study INTERHEART with participants from 52 countries representing all inhabited continents. In this study comprising 15152 cases and 14820 controls, 9 risk factors emerged: raised apoB/apoA1 ratio (odds ratio 3.25), smoking (2.87), psychosocial factors (2.67), diabetes (2.37), history of hypertension (1.91), abdominal obesity (1.12), regular alcohol consumption (0.91), regular physical activity (0.86) and daily consumption of fruits and vegetables (0.70). These associations were found in men and women, old and young and in all regions over the world (2). Many of these risk factors have been associated with low-grade chronic inflammation which, as will be further described below, may play a fundamental role in the development of the disease.

A large number of population-based prospective studies over the years have also investigated the predictive value of circulating inflammatory markers. These studies present strong evidence that markers such as C reactive protein (CRP) and interleukin (IL)-6 are independent predictors of developing CAD (3, 4).

Stable CAD is characterized by a reversible state of myocardial ischemia, which is usually induced by exercise or stressful events, but also occurs spontaneously. The occurrence of ischemia is often associated with transient chest pain (angina pectoris) but symptoms such as dyspnea, nausea and weakness also occur. The most common underlying mechanism is a progressive narrowing of a coronary artery due to an atherosclerotic plaque, which limits the blood flow and causes an insufficient oxygen supply for the heart’s metabolic demand (5).
CAD patients can remain asymptomatic or with stable symptoms for a lifetime, however serious events occur when the atherosclerotic plaque undergoes destabilization resulting in erosion or rupture. This life-threatening condition is called acute coronary syndrome (ACS) and includes unstable angina pectoris and MI. Unstable angina is defined as repeated episodes of unexpected chest pain while resting or rapid aggravation of effort-induced angina, but without any objective signs of myocardial damage. The damage occurs when the deficit of oxygen induces cell death in the myocardium and causes myocardial necrosis, which is the definition of a MI. Myocardial infarction is further divided in ST elevation myocardial infarction (STEMI) and non-ST elevation myocardial infarction (NSTEMI) depending on the ST segment changes on the electrocardiogram. An elevation of the ST segment is associated with complete occlusion of a coronary artery and needs immediate revascularization to salvage myocardium, such as thrombolysis or percutaneous coronary intervention (PCI), whereas NSTEMI is a partial occlusion of the coronary artery not in need of immediate revascularization. Instead, NSTEMI cases receive antiplatelet/anticoagulant therapy often followed by coronary angiography within 1-2 days (6).

**Atherosclerosis**

Some decades ago, atherosclerosis was viewed as a lipid disease due to a passive build-up of cholesterol in the artery wall. In the 1960s and 1970s, i.e. the modern era of cell biology, the focus was on the proliferation of smooth muscle cells as the core of the atherosclerotic plaque. However, during the last three decades, the concept of inflammation has gained more and more acceptance for its fundamental role in atherogenesis (7).

The chronic low-grade inflammation of the arterial lesion is initiated by the retention of low density lipoproteins (LDL) to proteoglycans in the extracellular matrix (ECM). A modification (or oxidation) of retained LDL particles is carried out by reactive oxygen species through attacking double bonds in unsaturated fatty acids, which in their turn mediate posttranslational alteration of proteins triggering an immune response. Furthermore, a number of enzymes can catalyze LDL oxidation, such as myeloperoxidase (MPO) and phospholipase A₂. A local cellular response involves the upregulation of leukocyte adhesion molecules on
the endothelial lining of the artery and release of chemokines from the vascular cells. These signals attract and facilitate the infiltration of circulating monocytes to the innermost layer of the artery, the tunica intima. This infiltration and migration of leukocytes has also been shown to depend on matrix metalloproteinases (MMP), especially MMP-9 (8, 9). Subsequent to extravasation, the monocytes differentiate into macrophages, which is associated with upregulation of the scavenger receptor that mediates the internalization of modified LDL particles. The phagocytosis of LDL causes an accumulation of cholesteryl ester droplets in the cytosol eventually leading to the formation of lipid-laden foam cells. As the disease progresses, efferocytosis becomes defective which give rise to an accumulation of apoptotic bodies and necrotic debris, forming a necrotic core. In turn, the inadequate engulfment and digestion of dead cells may result in secondary necrosis and further activation of the immune system, driving the progress towards a vulnerable plaque (10-12).

While a stable plaque is characterized by a relatively thick fibrous cap, high content of collagen and smooth muscle cells, low proinflammatory activity and a small necrotic core, a vulnerable plaque may be described as the opposite. It has a relatively large lipid-rich necrotic core which is covered by a thin fibrous cap. Activated macrophages and T cells are abundant. Other signs are increased proteolytic activity by MMP, particularly MMP-9 and MMP-8, decreased production of collagen by smooth muscle cells and intraplaque hemorrhage and neovascularization from vasa vasorum (13). When the plaque ruptures, the subendothelial space is exposed and tissue factor, collagen and von Willebrand factor come in contact with the blood stream. This starts off the coagulation cascade and recruitment and activation of platelets, thus producing an intraluminal thrombus that will completely or partly obstruct the blood flow (13).

To conclude, the progression of atherosclerosis involves two distinct processes: a chronic phase, during which the plaque slowly grows by accumulation of lipids in the artery wall thus narrowing the lumen (the classical concept of atherogenesis) and an acute phase when the plaque ruptures and causes an event of ACS.
Leukocytes in atherosclerosis

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) comprise T cells, B cells, natural killer (NK) cells, monocytes and dendritic cells (DCs). The proportions of each subset vary between individuals, but within the PBMC population 49-77% are T cells, 6-17% are B cells, 7-40% are NK cells, 6-12% are monocytes and ~1% are DCs. PBMCs are often studied as one group due to its accessibility by density centrifugation isolation, a process in which red blood cells and polymorphonuclear granulocytes are removed from whole blood. Gene expression profile analyses of isolated PBMCs have demonstrated an association between a number of genes involved in the inflammatory response and CVD. PBMC accumulation in advanced atherosclerotic lesions, visualized by in vivo imaging techniques, also correlates with the severity of CVD.

Monocytes constitute the main component of innate immunity and are responsible for counteracting exogenous pathogens mainly by phagocytosis. They are also involved in endogenous inflammatory activity such as elimination of modified/damaged cells and molecules. Monocytes are subdivided into “classical” monocytes characterized by a high expression of CD14 and lack of CD16 (CD14++CD16-), which represents about 80-85% of circulating monocytes. These cells are considered inflammatory and form the predominant subpopulation in atherosclerotic lesions. The “non-classical” monocytes have a lower expression of CD14, while CD16 is highly upregulated (CD14+CD16++) and are believed to patrol the vasculature, respond early to infection and inspect endothelial integrity. Also, an intermediary population has been described with a high expression of CD14 and low expression of CD16 (CD14++CD16+). The importance of circulating monocyte subsets in CVD is not fully clarified. The intermediate population was recently found to independently predict cardiovascular death, MI and stroke in 951 patients undergoing elective coronary angiography. In a general population, the classical CD14++CD16- monocytes predicted cardiovascular events over a 15 year follow-up independent of age, sex and classical cardiovascular risk factors.

The monocyte migration to the intima and subsequent differentiation into macrophages induces the production and release of a multitude of cytokines,
including tumor necrosis factor (TNF), IL-1\(\beta\) and IL-6, but also several MMPs. As will be described below, differentiated macrophages produce and release a wide range of MMPs, including MMP-1, -2, -3, -8, -9 and -14 (18). Macrophages are considered to comprise two subsets, the M1 type, which is abundant in progressing plaque and secretes pro-inflammatory cytokines, such as IL-1\(\beta\), IL-12 and TNF, thus promoting a T helper 1 (Th1) response. The other type, M2, is present in regressing plaques and considered to be anti-inflammatory by releasing IL-1 receptor antagonist (IL-1Ra) and IL-10. The M2 cell also participates in tissue remodeling and wound healing (19, 22).

Direct involvement of NK cells in human atherosclerosis seems to be scant, although their presence in lesions has been reported (23, 24). Patients with CAD have lower numbers of circulating NK cells compared to healthy subjects (25), a characteristic that is shared by other chronic inflammatory diseases. The role of NK cells in atherosclerosis is still unclear.

The processing and proper presentation of putative plaque antigens is important for initiating the inflammatory cascade in atherogenesis. DCs are professional antigen-presenting cells and populate most tissues where they serve to recognize infectious or injured components. Like macrophages, DCs have the capacity to internalize oxidized LDL and become foam cells.

A large body of evidence points towards the central role of adaptive immunity in the progression of atherosclerosis. In 1986, T cells in plaques were visualized for the first time in human atherosclerotic lesions (26). The T cell response in the atherosclerotic lesion is polarized to a Th1 cell cytokine secretion pattern including interferon (IFN)-\(\gamma\) and TNF, both cytokines associated with disease progression and plaque rupture (11). The Th1 polarization is also associated with activation of MMPs, especially MMP-9, thereby contributing to the thinning of the fibrous cap.

The antibody production towards modified LDL may also be important in atherogenesis, suggesting a role for B cells (27). The presence of antibodies within the atherosclerotic plaques as well as in the circulation is prominent with both natural antibodies of the IgM isotype and specific IgG, directed at antigens including oxLDL and heat shock proteins. Natural antibodies, derived from B1 cells, are considered as anti-inflammatory and anti-atherogenic while IgG is suggested to be pro-atherogenic (28).
**Neutrophils**

Polymorphonuclear neutrophils (or just neutrophils) represent 50-60 % of circulating leukocytes and have an essential role in innate immunity. Like monocytes, neutrophils are derived from myeloid progenitors, but in contrast to monocytes, they have a very short lifetime (1-2 days), although at sites of inflammation their life span can be extended.

Neutrophil granules are formed during the differentiation into mature neutrophils. The first type of granules which appear are azurophilic (or primary) granules containing MPO, therefore also called peroxidase-positive granules. Peroxidase-negative granules are subdivided into specific (secondary) and gelatinase (tertiary) granules based on time of appearance and contents. Specific granules have a high content of lactoferrin and low content of gelatinase, while the opposite is seen in gelatinase granules. Lastly, there are secretory vesicles, most likely formed by endocytosis since they contain the plasma protein albumin (29-31). However, neutrophil granules are not only the storage of secretory proteins, but also reservoirs of membrane proteins that are incorporated in the surface membrane of the cell when granules fuse and release their contents. Many of these membrane proteins are involved in the early inflammatory response, such as β2-integrin, the lipopolysaccharide (LPS) receptor CD14 and the FcγIII receptor CD16 (30).

Three different MMPs have been identified in the granules of neutrophils. MMP-9 is predominantly present in the gelatinase granules, while the collagenase MMP-8 mainly resides in the specific granules. MMP-25 (leukolysin) is largely located in gelatinase granules, but also found in specific granules, secretory vesicles and in the plasma membrane of resting neutrophils (29). The MMPs are stored as inactive proforms and undergo proteolytic activation following secretion, as described more in detail below (29).

Increased numbers of neutrophils in the circulation is a well-documented finding in CAD patients. However, neutrophil research in the atherosclerosis field has lagged behind probably due to difficulties in identifying their presence in lesions. The development of antibodies recognizing CD66b, a specific marker for neutrophils, changed this and according to recent studies, neutrophils as well as neutrophil proteases, including MMP-9 and -8, are present in rupture-prone plaque areas (32). Neutrophils have also been identified in lesions from patients with unstable angina, but not in lesions from patients with stable angina (33).
mice, the lesion size is associated with circulating neutrophil counts and the
depletion of neutrophils leads to reduced lesion size, providing evidence for their
involvement in the development of atherosclerosis (34). The neutrophils are
suggested to migrate from the lumen into large arteries (34), but also via
adventitial or intimal microvessels (35). They are captured by selectins and roll
on the endothelium where integrins mediate a firm arrest. Chemokines from the
lesion or from platelets interacting with the endothelium contribute to the
recruitment, firm adhesion and migration of neutrophils into the vessel wall (36).

Matrix metalloproteinases and their endogenous inhibitors

The MMP family consists of at least 23 members sharing a zinc-binding motif
and a conserved methionine that is located on the C-terminal side to the zinc-
ligands. Together, all MMPs can cleave and degrade most ECM proteins and are
categorized depending on different structure or preferred substrates into
collagenases, gelatinases, stromelysins, matrilysins and membrane-bound
MMPs. Several of these MMPs have been implicated in atherosclerosis. In this
thesis, special focus has been laid on MMP-9 and MMP-8 and they will therefore
be described more in detail below.

MMP-9 (also known as gelatinase B) mainly degrades gelatin and collagen type
IV, but is also able to process non-ECM proteins, such as growth factors,
cytokines (pro-TGF-β1, pro-TNF and pro-IL-1β), chemokines (CXCL8), cell
receptors, serine protease inhibitors and proforms of MMPs (pro-MMP-2, -MMP-9 and -MMP-13), thereby regulating the activity of these proteins (37). The
regulation of MMP-9 is complex and may occur on both the transcriptional and
post-transcriptional level. The transcription of MMP-9 in leukocytes is activated
by cytokines, viruses, bacterial products and plant lectins. Cell-cell interactions
between T cells and monocytes also activate MMP-9 gene transcription. The
activation of pro-MMP-9 requires physical delocalization of the prodomain from
the catalytic site. There are two main mechanisms to activate pro-MMP-9; 1) by
proteolytic cleavage of the prodomain, e.g. by MMP-3 or serine proteases or 2)
by allosteric displacement of the prodomain without cleavage, e.g. by neutrophil
gelatinase-associated lipocalin (38). MMP-9 is produced by several cell types
although neutrophils, macrophages, foam cells and smooth muscle cells are
considered major sources.
MMP-8 (also known as neutrophil collagenase) was first detected in neutrophils. In vitro measurements have shown a 100-fold higher level of MMP-8 in neutrophils compared to macrophages (39). It is released as a proenzyme and activated by reactive oxygen species, proteases like cathepsin G, chymotrypsin or other MMPs (-3, -7, -10, -14). The activated MMP-8 mainly degrades collagen type I, II and III, but also has the potential to activate the chemokine CXCL8 as well as other MMPs (-2, -3 and -9) (40).

An important mechanism for regulation of MMPs is the binding of tissue inhibitor of matrix metalloproteinase (TIMP)-1 to -4. All TIMPs can bind most MMPs, however their affinity differs. TIMP-1 is the main inhibitor of MMP-9, although TIMP-2 has been shown to be an important inhibitor as well (38, 41, 42). In addition, TIMPs have several functions including pro-MMP activation, cell growth promotion, matrix binding, inhibition of angiogenesis and induction of apoptosis.

**MMPs and TIMPs in atherosclerosis - findings from clinical studies**

In normal arteries, only pro-MMP-2, TIMP-1 and TIMP-2 can be detected and there is no MMP activity. On the other hand, several MMPs are found in atherosclerotic plaques. The levels of MMP-1, MMP-8, MMP-9 and MMP-13 are significantly higher in vulnerable plaques in comparison to fibrous plaques (43-46). Increased levels of MMP-3, MMP-11, MMP-14 and MMP-16 have also been detected in rupture-prone regions of the plaque (47).

Observational studies have reported an increase in both plasma and serum levels of MMP-9 in patients with stable CAD (48, 49), unstable angina (50-53) and MI (54). Circulating levels of MMP-9 have also been shown to predict cardiovascular events in population-based cohorts (55, 56) as well as in CAD patients after revascularization (57). Furthermore, plasma MMP-9 has been associated with the presence of carotid stenosis and rapid progression of CAD (58, 59). However, the source of circulating MMP-9 is not clarified. Some suggest that it reflects a leakage from the plaque whereas others propose that it is released from the damaged cardiac tissue. Other potential sources are circulating leukocytes, in particular neutrophils. As described above, neutrophils store large amounts of MMP-9 in granules to be released when the cells are recruited to inflamed tissues (29). Interestingly, increased expression of MMP-9 in circulating
neutrophils has been reported in CAD patients compared to controls (60). PBMCs, in particular monocytes, have also shown to be a source of MMP-9 (61, 62). Since increased number of circulating leukocytes in CAD patients has been depicted as a hallmark for this malady, their contribution to the pool of MMP-9 in the circulation is of particular importance (63).

Although MMP-8 is less studied than MMP-9 in atherosclerosis, a few studies have identified this protease in vulnerable carotid plaques where it colocalizes with macrophages (44, 64). Patients with hypoechoic carotid plaques (i.e. plaques with high lipid content) show increased levels of plasma MMP-8 and furthermore, asymptomatic patients with carotid plaque progression have increased intraplaque MMP-8 levels compared to asymptomatic patients without plaque progression (65). Plasma levels of MMP-8 have also been associated with the presence and severity of CAD (66). Finally, in a population-based study with a follow-up time of 10 years, serum MMP-8 levels were associated with MI and cardiac death in men where the highest risk was found in men with carotid subclinical atherosclerosis at baseline (67).

TIMP-1 levels have been shown to be increased in the circulation in both stable and unstable CAD, while TIMP-2 levels have been reduced (48, 50, 52). However, one previous study has reported elevated levels of circulating TIMP-2 in stable CAD patients (49).

**Hypothalamus-pituitary-adrenal axis**

The hypothalamus-pituitary-adrenal (HPA)-axis is tightly regulating the glucocorticoid secretion (Figure 1). Corticotropin-releasing hormone (CRH) is secreted from neurons in hypothalamus directly into the hypophyseal-portal blood vessels to the anterior pituitary gland, where it stimulates the release of adrenocorticotropic hormone (ACTH). ACTH in turn acts on the zona fasiculata in the adrenal glands inducing the production and secretion of glucocorticoids. The secreted glucocorticoids then act in a classical negative feed-back loop by inhibiting further release of both CRH and ACTH. Glucocorticoids follow a diurnal rhythm with the highest levels about 30 minutes after awakening and the lowest levels at 3-4 AM. The HPA-axis interacts with the immune system and is activated in response to inflammatory cytokines, mainly IL-1, IL-6 and TNF. Upon activation of the HPA axis, glucocorticoids are rapidly released from the
adrenal glands to restore homeostasis. Disruption of HPA axis has been shown to be associated with chronic stress as well as with abdominal obesity, dyslipidemia, insulin resistance and hypertension (68, 69). A dysregulated HPA axis is also considered as a sign of increased allostatic load (discussed more below).

**Figure 1.** Schematic picture of the hypothalamus-pituitary-adrenal (HPA) axis. TNF, IL-1 and IL-6 stimulate release of CRH from hypothalamus, which in turn acts on the pituitary gland to release ACTH into the systemic circulation. ACTH binds to receptors on the adrenal glands where it stimulates cortisol secretion. Cortisol suppresses the inflammatory activity and inhibits further activation of the HPA-axis (dotted lines).

**Glucocorticoids and glucocorticoid receptors**

In humans, cortisol is the predominant glucocorticoid (GC). Cortisol is also the active form while cortisone is the inactive precursor. GCs act on nearly every tissue and organ in the human body playing an essential role in the regulation of immune functions, metabolism, blood pressure and adaptive response to physical and psychological stress. Due to their powerful immunosuppressive and anti-inflammatory actions, GCs have been, and still are, indispensable in the treatment of inflammatory and autoimmune diseases, such as asthma, allergy, and rheumatoid arthritis, but also to prevent organ transplant rejection. Long-term treatment with GCs however, is associated with adverse side effects such as
osteoporosis, skin atrophy, diabetes, abdominal obesity and hypertension. Additionally, long-term treated patients may develop a tissue-specific resistance to GCs (70, 71).

The GC actions are mediated via the glucocorticoid receptors (GR), which are expressed by almost all cells. The GR is a member of the nuclear receptor family and encoded by *NR3C1*, which consist of 9 exons. These 9 exons are subjected to alternative splicing giving rise to GR-α, -β, -γ, -A and -P isoforms, although research has so far mainly been focused on GR-α and -β. GR-α exerts the actions of cortisol by affecting gene expression while GR-β, not capable of binding cortisol, has been suggested to repress the effects of GR-α (72, 73). In the absence of cortisol, the GR resides in the cytoplasm bound to chaperones and immunophilins in a transcriptional inactive conformation, but with high affinity to cortisol (74). A majority of cortisol in the circulation is bound to corticosteroid-binding globulin. Only free cortisol can passively diffuse over the plasma membrane and interact with the GR. Upon ligation to cortisol, the GR is rapidly translocated to the nucleus where it binds directly as a homodimer to GC-response elements with a subsequent induction or repression of a plethora of genes (75, 76). In addition to genomic actions, GCs also exert non-genomic effects. The latter are defined as effects detected within 5-15 min, while genomic effects will appear first after 15 min. The mechanisms for non-genomic actions are still not clarified but membrane-bound GRs are possibly mediating the effects (70).

The major anti-inflammatory actions of the GR involve the transrepression of transcription factors activator protein-1 and nuclear factor (NF)-κB, both key players in the proinflammatory signaling cascade (77, 78). Another GC-mediated action is the recruitment of histone deacetylase-2, an important pre-transcriptional mechanism that modifies DNA accessibility, thus inhibiting transcription of pro-inflammatory genes by deacetylation and recondensation of histones (79). Furthermore, the induction of annexin 1 and inhibition of cyclooxygenase 2 prevent the formation of arachidonic acid, the precursor of prostaglandins and leukotrienes (80).

Cortisol may regulate MMP activity in a direct manner or indirectly by inhibiting Th1-activation and subsequent cytokine secretion. Dexamethasone, a synthetic GC, has been shown *in vitro* to strongly decrease the secretion of MMP-9 and TIMP-1 in PBMCs (81). The GC-mediated suppression of MMP-9 is also prominent in other cell types (82-84). Moreover, an intravenous injection of
methylprednisolone was shown to decrease cerebrospinal fluid levels of MMP-9 in patients with multiple sclerosis, as measured by zymography (85).

**Glucocorticoid sensitivity**

The biological effects of GCs are determined by the output of the HPA-axis, but also by the GC sensitivity in the affected tissue. Impaired GC sensitivity is associated with enhanced inflammatory activity. This has been demonstrated in both animal models and patients with chronic inflammatory diseases (70, 71). There are several factors influencing GC sensitivity, such as the bioavailability of cortisol, GR number and affinity, transcriptional activity of GR, post-translational modifications of GR and different gene polymorphisms (70). The number of GRs has been associated with GC sensitivity (86, 87), but upregulation of GR-β and a decreased ratio of GR-α/GR-β is also discussed as a potential mechanism of reduced GC sensitivity. The upregulation of GR-β in human cell lines by IL-1 and TNF correlates with reduced GC sensitivity (88). In chronic inflammatory diseases such as rheumatoid arthritis, asthma and inflammatory bowel disease, increased gene expression of GR-β or lower ratio of GR-α/GR-β has been associated with GC unresponsiveness in vivo (89-92). However, the latter is still a matter of debate since other clinical studies have not been able to detect any differences in GR-β expression (93-95).

The effect of released GCs also depends on the bioavailability of free/active cortisol, which is regulated by both cortisol-binding globulin, binding more than 90 % of circulating GCs, and the enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 and -2. Inactive cortisone is converted to active cortisol by 11β-HSD-1 while 11β-HSD-2 has the opposite effect.

Polymorphisms in the GR gene have been associated with both increased and reduced GC sensitivity. The Asn363Ser and BclI polymorphisms are associated with an increased GC sensitivity and features similar to Cushing syndrome. The polymorphisms ER22/23EK and 9β are instead associated with reduced GC sensitivity, ER22/23EK with reduced transactivation effectiveness and 9β with stabilization of the dominant negative GR-β isoform (70).
Psychological stress and its relation to inflammation and HPA axis

Chronic stress

Several studies have shown a relation between psychosocial stress and CAD. A variety of psychological stress factors including depression, cynicism, hostility, vital exhaustion and lack of mastery (or coping ability) have been related to increased cardiovascular risk. Depressive symptoms, hostility and cynicism are all negative emotions while mastery is defined as perceived control over a situation and resilience to prolonged exposure to stress. The worldwide case-control study INTERHEART, confirmed that psychosocial stress including depressive symptoms was a significant risk factor for myocardial infarction (96). Underlying mechanisms are still not clarified but inflammation has been proposed as a link between psychological stress and CAD. Population-based studies have shown that individuals experiencing psychosocial stress, in particular depression, have increased levels of inflammatory markers such as CRP and IL-6 in the circulation (97, 98). Increased levels of IL-6 and CRP have also been reported in CAD patients with depression compared to non-depressed CAD patients (99). Interestingly, in a middle-aged population in Sweden, MMP-9 in plasma was associated with psychosocial factors such as depression, cynicism and lack of coping, after adjustment for traditional risk factors (100). Moreover, MMP-9 in plasma was introduced as a strong marker of major depression in a large study investigating biomarkers of psychiatric disorders by using protein profiling (101) and increased levels of MMP-9 have been found in tumor-associated macrophages of depressed ovarian cancer patients (102).

The cortisol output in chronic psychological stress has been extensively studied with contradictory results reporting both reduced and increased levels (103). Vital exhaustion has been associated with higher baseline cortisol and an attenuated response to acute stress (104). In a middle-aged population, depressive mood and cynicism were associated with a flattened diurnal cortisol slope, i.e. a smaller difference between cortisol samples collected in the morning and in the evening (105).
Acute stress

Acute emotional stress, e.g. outburst of anger or excessive anxiety, may be a trigger of cardiac events (106, 107). An acute trigger is defined as an event occurring immediately or in the hours before the onset of MI. Mechanisms may involve factors like hemodynamic stress, arrhythmia, vasoconstriction and platelet activation but it is also discussed that activation of the immune system has a role.

The inflammatory response to acute mental stress has been investigated in several studies. In healthy individuals, increased levels of circulating cytokines, particularly IL-6, are detected 45-120 min after the stressful task (108). Moreover, Fagundes et al. (109) showed that the increase in IL-6 after mental stress was associated with the magnitude of depressive symptoms. In addition to circulating cytokine levels, increased NF-κB activity and IL-1β expression in PBMCs have been detected after stressful tasks (110, 111). Also, stress-induced mobilization and activation of neutrophils has been reported (112, 113).

An adequate stress-induced response of the HPA-axis with a subsequent release of cortisol is essential to maintain the homeostasis. Thus, an impaired cortisol response may fail to counteract the inflammatory activity during acute mental stress. In line with this, an inverse association between cortisol responsiveness and IL-6 levels or NF-κB DNA binding activity after stress has been seen in healthy individuals (114, 115).

Dysregulation of HPA axis activity in CAD patients

The HPA axis function in CAD patients has so far been sparsely investigated. However, there is some evidence for a dysregulated HPA axis in CAD patients. An earlier study of CAD patients showed that patients with ‘inappropriately’ normal morning cortisol production had high IL-6 levels (116). A later study of CAD patients showed an increased total release of cortisol per day as well as significantly higher levels of evening cortisol compared to healthy controls, the latter indicating a flattened cortisol diurnal rhythm (117, 118). Interestingly, the increased levels of evening cortisol also correlated with basal morning levels of
CRP, IL-6 and MMP-9 (102, 119, 120). Moreover, CAD patients were found to release significantly less cortisol than controls when they were exposed to acute physical or psychological stress. The blunted cortisol response was associated with increased levels of CRP the day after stress and also, there was evidence for dysregulated post-stress levels of MMP-9 in the patient group (119, 120). Similarly, a blunted cortisol response to stressful stimuli has been demonstrated in chronic inflammatory diseases such as rheumatoid arthritis and allergic asthma (121, 122). Altogether, the studies hitherto indicate that a dysregulated cortisol response in disease is associated with a reduced capacity to neutralize the inflammatory process. This may also reflect a state known as allostatic load, a concept first coined by Sterling and Eyer in 1988 (123). Allostatic load refers to an imbalance in the systems which promote adaptation to adverse psychological or physiological situations. These systems involve the HPA axis, the autonomic nervous system, the metabolic systems including thyroid axis, insulin, glucagon, and the gut, as well as the immune system. The imbalance can be a result of a too large burden of prolonged/repetitive stress, failure to shut down involved systems and/or failure to respond adequately to the stressful experience. However, it is a challenge to measure or define allostatic load and the use of a panel of biomarkers including neuroendocrine, metabolic and cardiovascular functions has been recommended (124).

Telomeres and stress

In 1978, Elisabeth Blackburn made the discovery of telomeres from ciliated protozoan called *Tetrahymena thermophile*, which consisted of a simple sequence repeat of TTGGGG (TTAGGG in humans (125)) and protected the chromosomes from degradation (126). A few years later, telomerase was discovered and described as the enzyme that elongates telomeres and compensates incomplete replication of telomere ends (127). It has later been shown that telomeres are protein-DNA complexes which form capping ends of the chromosomal ends, thus being crucial for genomic integrity. Their protective role is essential in many aspects of cell physiology, e.g. by avoiding cell senescence (128).

A recent systematic meta-analysis of prospective and retrospective studies found an inverse association between leukocyte telomere length (TL) and risk of CAD after adjustment for traditional cardiovascular risk factors (129). Shorter leukocyte TL has also been associated with high-risk plaque morphology (130).
In addition, telomere disruption in monocytes leads to pro-inflammatory activity as assessed by increased secretion of monocyte chemoattractant protein-1, IL-6, and IL-1ß and oxidative burst in vitro (130). In a recent study of healthy elderly individuals, short TL in PBMCs, combined with high telomerase activity, was associated with allostatic load, involving a blunted post-stress recovery in systolic blood pressure, heart rate variability and monocyte chemoattractant protein-1 as well as reduced responsivity in diastolic blood pressure, heart rate, and cortisol during stress. Moreover, shorter TL with high telomerase activity was associated with reduced social support, lower optimism, higher hostility, and greater early life adversity, though only in men (131). Also, chronic psychological stress in other settings has been related to higher oxidative stress and shorter leukocyte TL. Leukocyte TL has therefore been introduced as a molecular marker of allostatic load (132).
Aims

General aims

The overall aim of this thesis was to compare the levels of leukocyte-derived MMPs and TIMPs in patients with CAD and healthy subjects and to further investigate whether the gene expression and secretion of leukocyte-derived MMPs and TIMPs were influenced by psychological stress and altered glucocorticoid sensitivity.

Specific aims

- To compare the expression and secretion of MMP-9, MMP-8, TIMP-1 and TIMP-2 in neutrophils and PBMCs from CAD patients and healthy controls
- To evaluate whether PBMC-derived levels of MMP-9, TIMP-1 and TIMP-2 were associated with psychological risk factors including depressive symptoms and cynical hostility in CAD patients and healthy controls
- To evaluate whether elevated PBMC-derived levels of MMP-9, TIMP-1 and TIMP-2 were associated with reduced sensitivity to glucocorticoids ex vivo in CAD patients and healthy controls
- To evaluate the expression of GR-α and GR-β in PBMCs as a mechanism for altered sensitivity to glucocorticoids in CAD patients and healthy controls
- To investigate whether stress-induced rapid release of MMP-9, MMP-8, TIMP-1 and TIMP-2 was associated with stress-induced cortisol response in CAD patients
- To study whether stress-induced release of MMP-9, MMP-8, TIMP-1 and TIMP-2 was associated with leukocyte TL and carotid atherosclerotic burden in CAD patients
Methodological considerations

Study populations

Study participants were recruited in two different projects, named CorC (Coronary Computed Tomography) (paper I) and MIMMI III (Mental – Immune Interactions in Myocardial Infarction) (paper II-IV) (Table 1). In the CorC study, 44 patients with stable angina referred to elective coronary angiography at the Department of Cardiology, Linköping University Hospital were included in the study. They had angina symptoms graded as Canadian Cardiovascular Society class II and III (133) as well as positive exercise tests or myocardial scintigrams. CAD was verified in all patients by angiography. Heparinized peripheral blood and serum with activation clot were drawn in the morning after 12 h fasting and always performed before coronary angiography.

In the MIMMI III study, 64 patients with a recent index event, i.e. NSTEMI and/or coronary revascularization procedure (PCI or coronary artery bypass graft surgery) were consecutively included from the Outpatient Cardiology Clinic at the University Hospital in Linköping, Sweden. In paper II and III, only patients with a prior MI (n = 57) were included. Heparinized peripheral blood and serum with activation clot were drawn between 6 and 18 months after the index event, when patients were in a stable metabolic state.

The exclusion criteria were: age > 75 years, severe heart failure, immunologic disorders, neoplastic disease, evidence of acute or recent (< 2 months) infection, recent major trauma, surgery or revascularization procedure, or treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin). In papers II-IV, major clinical depression was added to the exclusion criteria.

Forty-seven clinically healthy controls with equivalent age and gender distribution were recruited in the CorC study and 41 controls were included in MIMMI III. They were randomly selected from a population register representing the hospital recruitment area. Use of statins or antihypertensive drugs for primary prevention was allowed.
Table 1. Clinical and laboratory characteristics of CAD patients and controls in paper I-IV.

<table>
<thead>
<tr>
<th></th>
<th>Controls Paper I n = 47</th>
<th>Controls Paper II and III n = 41</th>
<th>Patients Paper I n = 44</th>
<th>Patients Paper II and III n = 37</th>
<th>Patients Paper IV n = 64</th>
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</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63 (58-71)</td>
<td>67 (66-72)</td>
<td>63 (57-71)</td>
<td>66 (61-72)</td>
<td>66 (63-72)</td>
</tr>
<tr>
<td>Male/female</td>
<td>34/13</td>
<td>23/7</td>
<td>34/10</td>
<td>46/11</td>
<td>51/13</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>8 (18)</td>
<td>6 (12)</td>
<td>5 (7.8)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (16)</td>
<td>12 (21)</td>
<td>11 (17)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>0 (0)</td>
<td>5 (17)</td>
<td>32 (73)</td>
<td>27 (49)</td>
<td>33 (52)</td>
</tr>
<tr>
<td>Statin, long-term treatment, n (%)</td>
<td>0 (0)</td>
<td>4 (13)</td>
<td>36 (86)</td>
<td>56 (98)</td>
<td>63 (98)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.7 (4.9-6.6)</td>
<td>5.5 (4.5-5.9)</td>
<td>4.6 (4.0-5.4)</td>
<td>3.9 (3.3-4.3)</td>
<td>3.9 (3.3-4.2)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.6 (2.8-4.2)</td>
<td>3.2 (2.5-4.1)</td>
<td>2.4 (2.1-3.4)</td>
<td>2 (1.7-2.5)</td>
<td>2 (1.6-2.4)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.5 (1.2-1.7)</td>
<td>1.7 (1.4-1.8)</td>
<td>1.2 (1.1-1.4)</td>
<td>1.1 (0.95-1.4)</td>
<td>1.2 (1.0-1.3)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.1 (0.93-1.6)</td>
<td>1.2 (0.81-1.5)</td>
<td>1.4 (1.1-1.7)</td>
<td>1.2 (0.91-1.8)</td>
<td>1.2 (0.9-1.8)</td>
</tr>
</tbody>
</table>

Isolation of PBMCs and neutrophils

In the CorC study, PBMCs and neutrophils were isolated by density centrifugation using Lymphoprep and Polymorphprep, respectively (Axis-Shield PoC AS, Oslo, Norway). Lymphoprep was first layered on Polymorphprep and blood was layered on top. After centrifugation (420g, 40 min, room temperature (RT)), one band of PBMCs and one band of neutrophils were obtained. PBMCs were resuspended in phosphate buffered saline (PBS) with 0.1% fetal bovine serum (FCS) (PAA Laboratories GmbH, Pasching, Austria) and washed twice by centrifugation, 400g, 10 min, 4°C. The cells were then collected in RPMI-1640 media supplemented with L-glutamine (Gibco by Invitrogen, Carlsbad, CA, USA), 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco by Invitrogen) to a concentration of 5×10⁶ cells/ml.

The neutrophils were collected, resuspended and washed in PBS and NaCl. Remaining erythrocytes were lysed with a hypotonic solution and neutrophils were washed with Krebs-Ringers glucose (KRG) without Ca²⁺ (0.1 mol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 2 mmol/l KH₂PO₄, 8 mmol/l Na₂HPO₄ and 10 mmol/l glucose). The neutrophils were kept on ice at 5×10⁶ cells/ml before stimulation.

In the MIMMI III study, Ficoll Paque was used to isolate PBMCs by density centrifugation (400g x g for 40 min at RT). PBMCs were washed twice in PBS with 0.5% FBS and freshly isolated cells were either snap frozen in liquid nitrogen and stored at -80°C or resuspended in RPMI 1640 medium supplemented
with L-glutamine, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin to a concentration of 10⁶ cells/ml.

**Stimulation of PBMCs and neutrophils**

In the CorC study, PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma -Aldrich Corporation, St Louis, MO, USA) 25 ng/ml and ionomycin (Calbiochem, Darmstadt, Germany) 1 mg/ml in 37°C, 5% CO₂ for 4 hours. Supernatants were collected and cells were then washed three times and stored at -80°C. Neutrophils were stimulated in a 37 °C water bath with either PMA 25 ng/ml or IL-8 (Sigma -Aldrich Corporation, St Louis, MO, USA) 10 ng/ml for 10 min. Supernatants and cells were collected and stored at -80°C.

In the MIMMI III study, PBMCs were stimulated with *E. coli* lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, MO, USA) 100 ng/ml, with or without dexamethasone (Sigma-Aldrich, St Louis, MO, USA) at concentrations of 10⁻⁷ and 10⁻⁸ mol/l for 19 h in humidified atmosphere with 5 % CO₂ at 37 °C. Cell supernatants and cells were collected and stored at -80°C.

**ELISA and Luminex**

In the CorC study, ELISA (enzyme-linked immunosorbent assay; Quantikine immunoassay, R&D systems, Minneapolis, MN, USA) was used to determine concentrations of MMP-9, MMP-8, MPO, TIMP-1 and TIMP-2 in plasma, serum and cell supernatants. ELISA is a quantititative sandwich enzyme immunoassay where a capture antibody is coated onto a microplate. Samples are added to the wells and the antigen is bound by the immobilized antibody. Unbound sample is washed away and a detection antibody, conjugated with an enzyme, is added. A substrate is then added which change the color in proportion to the amount antigen that is bound in each well. The color development is stopped and color intensity is measured by spectrometer. The antigen concentration is quantitated by a standard of known antigen concentration which is generated for each assay run. The interassay coefficients of variation (CV) for all measured proteins were always less than 7%.

In the MIMMI III study, MMP-9, MMP-8, MPO, TIMP-1 and TIMP-2 were analysed by using Luminex Performance Assay (R&D Systems, Minneapolis
MN, USA) instead of ELISA due to higher throughput. The correlation between the two methods has been evaluated by the company with a slope of 0.9-1.1 and an R² value greater than 0.9. In Luminex assays, color-coded microparticles are used to multiplex up to 100 analytes within a single sample. The microparticles are coated with antigen-specific antibodies and samples are added. Unbound sample is washed away and a cocktail of biotinylated antibodies, specific to the analyte, is added. To detect each analyte, streptavidin-phycoerythrin (streptavidin-PE) conjugate is used, which binds to the captured biotinylated detection antibodies. The microparticles are then detected in a flow cytometry-based Luminex analyzer. One laser detects the microparticle, while the other laser detects phycoerythrin. The signal from phycoerythrin is proportionate to the amount of bound analyte and quantitated by a standard of known analyte concentrations, which is generated for each assay run. The interassay CVs were 13% for MMP-9, 6.8% for MMP-8, 6.5% for TIMP1, 14% for TIMP-2 and 5% for MPO.

**Real time quantitative polymerase chain reaction (PCR)**

Real-time quantitative PCR allows a sensitive, specific and reproducible quantitation of DNA and RNA. It is a powerful tool in molecular biology which can be used in a wide range of applications including the analysis of gene expression and gene regulation, determinations of the effects of variations in genetic composition, and the identification and quantification of microorganisms and viruses. For gene expression assays, RNA is first reversed transcribed to complementary DNA (cDNA), which is thereafter used in quantitative PCR (qPCR). Through incorporation and activation of a fluorescent probe in the copied sequence each cycle will increase the fluorescent intensity proportionally with each amplified DNA copy. Fluorescence is measured during all cycles and plotted over time. During the exponential phase it is assumed that a doubling of fluorescence in one cycle is directly proportional to a doubling of amplicons. This makes it possible to quantitate the amount of starting material by relating it to a standard curve.

To compensate for variation between samples, an endogenous control, 18S ribosomal RNA which is expressed in all cells with low variation, was used.
RNA isolation

In the CorC study, total RNA was isolated from both PBMCs and neutrophils with RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions and quantified by optical density at 260 nm. Using the high capacity cDNA reverse transcription kit with an RNase inhibitor (Applied Biosystems, Foster City, CA, USA), 0.73 µg was reversed transcribed as recommended by manufacturer. In the MIMMI III study, total RNA was isolated with MagMAX-96 Total RNA Isolation Kit (Life Technologies, Carlsbad, California, USA) from both freshly isolated PBMCs and cultured PBMCs, according to manufacturer’s instructions. 66 ng RNA was reversed transcribed using the same method as stated above.

Quantitative PCR

TaqMan Gene Expression Assay kits (Applied Biosystems) were used (Table 2) together with sample cDNA and the PCR-buffer, TaqMan Fast Universal PCR Mastermix (Life Technologies), in 96-well plates and amplified on an ABI 7500 Sequence Detector with SDS 1.3.1 software. Additionally, in the MIMMI III study, a Custom TaqMan assay for GR-α were used, (forward primer 5’ GAAGGAAACTCCAGCCAGAA 3’ and reverse primer 5’ CAGCTAACATCTCGGGGAAT 3’) (134). In all experiments, samples, non-template control and standard or calibrator were loaded in duplicates.

Table 2. TaqMan Gene Expression Assay kits (Life Technologies) used in the different papers.

| Table 2. TaqMan Gene Expression Assay kits (Life Technologies) used in the different papers.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>MMP-9</td>
<td>Hs00957562_m1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Hs01029057_m1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Hs00171558_m1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Hs00234278_m1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GR-α</td>
<td>Custom TaqMan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR-β</td>
<td>Hs00354508_m1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>4352930E</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Calculation of relative quantification values

The expression of MMP-9, MMP-8, TIMP-1, TIMP-2, GR-α and GR-β was related to the expression of 18S in each sample. For MMP-9, MMP-8, TIMP-1 and TIMP-2, standard curves were generated to quantify the expression according to the standard curve method in User bulletin no 2 (Life Technologies). Due to a low expression of GR-β, it was not possible to generate a standard curve and instead the ΔΔCT method was used, for both GR-α and GR-β, according to the User bulletin no 2. For detection of GR-β, 6 µl instead of 1 µl was needed. Still, the CT values for GR-β were much higher than CT values for GR-α, 33.6 (33-34.5) vs 27.3 (26.7-28.2).

Flow cytometry

Flow cytometry is used to characterize cells by labelling them with fluorescent antibodies. The cell-bound antibodies are then excited by lasers to emit light in different wavelengths as they flow pass in a liquid stream. Thousands of cells can be analyzed each second for relative granularity, size and fluorescence intensity. The optical signals are converted to electronic data for each cell based on fluorescent and light scattering properties.

In the MIMMI III study, whole blood was stained with the T cell marker CD3-APC/H7 clone SK7 (BD Biosciences, San José, CA, US) and the monocyte marker CD14-PeCy7 clone M5E2 (Nordic Biosite, Täby, Sweden) for 15 min, RT. BD FACS lysing solution (BD Biosciences) was used to lyse erythrocytes (10 min, RT) and at the same fixate white blood cells. Subsequent permeabilization was performed with Permeabilizing Solution 2 (BD Biosciences) for 30 min at RT and unspecific binding was blocked with 10 % FBS. After permeabilization, cells were incubated with MMP-9 and TIMP-1 antibodies conjugated with FITC and PE respectively, (RnD Systems) or rabbit anti-human GR-α and -β antibodies (ThermoFischer, Waltham, MA, USA; PA1-516 and PA3-514 respectively) for 30 min at 4°C. Only for GR-α and -β, a following incubation with a F(ab')2 fragment goat anti-rabbit IgG (Life Technologies; A10542), conjugated with PE for 30 min at RT. All cells were then washed and resuspended in PBS with 0.5 % FCS prior to analysis on the Beckman Coulter Gallios (Beckman Coulter, Miami Lakes, Florida, US). Obtained data were analyzed and visualized with Kaluza 1.2 software (Beckman Coulter).
In the MIMMI III study, cultured PBMCs were also analyzed by flow cytometry. 1 × 10^6 cells/ml were first washed in PBS with 0.5 % FCS and stained with CD14-PeCy7 clone M5E2 for 15 min at RT. Following a second wash step, cells were permeabilized for 30 min at 4°C with Permeabilizing Solution 2 and washed with Permeabilization buffer. Antibody staining and analysis for GR-α and GR-β was performed as in the whole blood protocol described above.

**Gelatin zymography**

Gelatin zymography is an excellent method for the detection and characterization of proteases with gelatin as a substrate, i.e. MMP-2 and MMP-9. By separating them with electrophoresis, both proforms and active forms can be detected on the gel. Both active and activated proteases degrade the incorporated gelatin in the gel. After staining with Coomassie blue, the proteolytically degraded sites show as clear bands on a blue background.

In the MIMMI III study, we used a subgroup of patients and controls to verify the presence of MMP-9 in PBMC supernatants. The samples were mixed with native Tris-Glycine sample buffer (Invitrogen) and loaded on 10 % SCD-polyacrylamide gels (Invitrogen) containing 0.1 % gelatin (Sigma). A molecular weight ladder was added (Precision Plus Protein All Blue Standards; Bio-Rad). The electrophoresis was run for 2.5 h at 125 V in 4°C and thereafter, proteins in the gel were renatured for 30 min with subsequent activation of the zymogens for 24 h at 37°C. In a last step, the gel was stained with Coomassie Brilliant Blue for 20 min. The gels were analyzed in Adobe Photoshop CS5 by measuring the optical density of the gelatinolytic bands.

**Carotid B-mode ultrasound**

In the MIMMI III study, intima-media thickness (IMT) and plaque occurrence were measured in both carotid arteries by B-mode ultrasound using a 9-18 Mhz linear 2D transducer (ACUSON S2000 TM ultrasound system, Siemens Medical Solutions USA, Inc.). The common carotid artery was assessed for IMT 1 cm below the bifurcation of the external and internal carotid arteries. Two repeated measurements were performed and a mean value of the IMT was determined. The
carotid arteries were scanned transversely and longitudinally for the occurrence of plaques. Mannheim Consensus (135) definition of plaques were used, stating that a plaque is a focal structure 50% thicker than IMT in the focal area or a thickness > 1.5 mm.

**Psychological factors**

In the MIMMI III study, persistent depressive symptoms, cynicism and coping were evaluated. Center for Epidemiological Studies – Depression scale (CES-D) was used for assessing depressive symptoms (136). The questionnaire was developed to capture the major components of depressive symptomatology i.e. diffuse and unspecific state of sadness, worthlessness and hopelessness in the population. However, it was not designed to identify clinical depression. CES-D has shown acceptable test-retest repeatability ($r = 0.54$ in a normal population) (136). It constitutes 20 items with 4 answer categories, giving a range of total score from 0 to 60.

The Cook-Medley Hostility Scale was used to evaluate cynical hostility (137). This survey was designed to capture a negative view on mankind, viewing people in general to be unworthy, dishonest and egoistic. Cynical hostility is considered to be formed during a long period of time, thus being a stable trait ($r = 0.84$ for 4 years follow-up and $r = 0.74$ for 10 years follow-up). It constitutes 12 items with 5 answer categories, giving a range of total score from 12 to 60.

The questionnaire used for assessing mastery (coping) was developed by Pearlin in 1978 (138) and aims to capture the ability to cope with stressful events in everyday life. Mastery is a behavior that buffers social experiences and protects against potential negative stressors. The protective function works by eliminating or modifying conditions that give rise to problems, by perceived control over the situation and by keeping emotional consequences manageable. Mastery is a stable trait and the test-retest stability is $r = 0.85$. The questionnaire has 7 items with 4 answer categories, giving a range of total score from 7 to 28.

Patients were asked to fill in the questionnaires for CES-D and cynical hostility on 3 separate occasions; 4 weeks, 12 months and 18 months after the index event, while mastery was evaluated after 4 weeks and 12 months. In healthy controls, measurements of CES-D and cynical hostility were performed on one occasion.
Psychological stress test

Patients included in MIMMI III underwent a standardized psychological stress test (119) with two different psychological stressors. The first stressor was an “anger recall” when the patient was asked to recall an event that had made him/her angry, frustrated or upset and had 6 min to relate what had happened and how he/she had felt during the event. The next stressor was an arithmetic test, starting 2 min after the first stressor, allowing time for instructions. The patient was instructed to count backwards from 700 to 0 with steps of 7 in 4 min as quickly and correctly as possible, being told it was an easy task. They had 20 min after the second stressor to recover.

Blood pressure and heart rate were measured every second minute throughout the test. Salivary and blood samples were collected just before the start of the test and after 34 min (Figure 2).

Figure 2. Psychological stress test protocol.
Cortisol analysis

Patients included in MIMMI III were also instructed to collect saliva on 3 consecutive days. The first sample was in the morning, 30 min after awakening and the second just before bedtime. Saliva was collected with Salivette cotton swabs (Sarstedt, Nümbrecht, Germany). Samples were analyzed at an accredited laboratory at the Department of Clinical Chemistry, Karolinska University Hospital, Sweden. Levels of free cortisol were determined by a commercial radioimmunoassay assay, CORT-CT2 (Cisbio Bioanalyser, Codolet, France). According to repeatedly performed quality assessments, the interassay coefficient of variance was less than 10 %.

In short, the cortisol assay is based on a competitive radioimmuno-principle. In tubes coated with anti-cortisol antibodies, the sample is added to a known amount of $^{125}$I labeled cortisol. Labeled and unlabeled cortisol are then competing for the limited number of binding sites of the anti-cortisol antibodies. Unbound antigen is washed away and the samples are measured in a gamma counter. The amount of labeled cortisol is inversely proportional to the amount of cortisol in the sample. The sample concentrations of free cortisol are obtained with a standard curve.

Telomere length measurement assay

In the MIMMI III study, DNA samples prepared from whole blood using Maxwell 16 blood DNA purification kit (Promega Biotech, Stockholm, Sweden) were sent to the Blackburn laboratory at the University of California for leukocyte telomere length determination, as previously described (139). A ratio is measured by dividing the telomere product (T) with a single copy gene (S). The single copy gene is used to normalize the amount of DNA input. The ratio (T/S) reflects the length of the telomeres. The primers used for the telomere PCR are tel1b [5'-CGGTTTT(GTTTGG)5GTT-3'] (final concentration of 100 nM), and tel2b [5'-GGCTTG(CCTTAC)5CCT-3'] (final concentration of 900 nM). The primers for the single-copy gene (human beta-globin) PCR are hbg1 [5' GCTTCTGACACAATGTGTTTCATAGC-3'] (final concentration of 300 nM), and hbg2 [5'-CACCAACTTCATCCACGTTCACC-3'] (final concentration of 700 nM). DNA from Hela cancer cells was used as reference and included in
each PCR run. The quantity of each sample is determined relative to a standard curve and the same reference DNA is used in all PCR runs.

Ethics statement

Written informed consent was obtained from all patients and controls in the CorC and MIMMI III studies. The studies were performed in accordance to the Declaration of Helsinki and the Ethical Review Board of Linköping University approved the research protocol.

Statistics

Data were analyzed using IBM SPSS statistics 21. Unless otherwise stated, data are presented as median with interquartile range. Mann-Whitney U-test was used to detect a statistically significant difference between two groups, whereas chi-square test was used for nominal data. For comparisons between more than two repeated observations on the same subject, Friedman’s test or one-way ANOVA was used. Wilcoxon signed-ranks test was used for pair-wise comparisons and for correlation analyzes, Spearman's rank correlation coefficient was used. Graphpad prism 5 was used to produce graphs. A p-value < 0.05 was considered statistically significant.
Results and Discussion

**Neutrophils as a source of MMPs and TIMPs**

The mRNA expression and protein secretion of MMP-9 and MMP-8 were compared in PBMCs and neutrophils (Paper I). Not unexpectedly, neutrophils were the main source of MMP-9 and MMP-8 proteins. Previous studies have shown a rapid release of both specific and gelatinase granules, containing MMP-8 and MMP-9 respectively, upon stimulation with bacterial toxins or pro-inflammatory cytokines such as TNF and IL-8 (140-142). After 10 min of culture, the spontaneous release of MMP-9 tended to be higher in neutrophils from stable CAD patients in comparison to neutrophils from controls (Table 3). In response to IL-8, a mild physiological stimulus, the neutrophils from patients secreted significantly larger amounts of MMP-9. The results are in agreement with a study by Tayebjee et al. (60) who showed increased levels of MMP-9, assessed by flow cytometry intracellular staining, in neutrophils from stable CAD patients compared to neutrophils from healthy controls. The rapid release of MMP-9 has been proposed as a sensitive marker of early neutrophil activation (141). Our findings may thus indicate a primed state of neutrophils in stable CAD, implicating that the neutrophils are more prone to release their granule contents upon stressful stimuli. However, when we used PMA, which strongly activates cells by directly activating protein kinase C leading to maximal degranulation, the neutrophils from healthy controls released larger amounts of MMP-9 than neutrophils from patients. This indicated a reduced maximal capacity in response to a non-physiological strong stimulus. In line with this, earlier studies have shown an impaired response to intense stimulation of neutrophils from stable CAD patients, defined as reduced capacity to generate reactive oxygen species (143, 144). Stable CAD patients are often extensively treated with statins, which may, at least in part, explain a suboptimal response to strong stimuli. For example, it has been shown that simvastatin attenuates the oxidative burst in neutrophils aggravated by endotoxin (145). The release of MMP-8 in neutrophils did not differ between patients and controls regardless of stimuli. The mRNA levels of MMP-9 and MMP-8 were negligible, which is in line with the concept that neutrophils produce granule contents during their development in bone marrow and exhibit low transcriptional activity after entering peripheral blood (30). We found that plasma and serum levels of MMP-9 correlated more strongly with
neutrophil counts than with monocyte counts, and not at all with other leukocyte subsets, thus supporting that neutrophils are the major source of MMP-9 and MMP-8 among leukocytes. The release of TIMP-2, on the other hand, was low and TIMP-1 was not even detectable in neutrophil supernatants. In line with this, it has been previously shown that inflammatory neutrophils, in contrast to monocytic and fibrosarcoma cell lines, release MMP-9 free of TIMP inhibitors (146).

Table 3. MMP-9, MMP-8, TIMP-1 and TIMP-2 levels (ng/ml) in neutrophil supernatants (n = 10 for both patients and controls).

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Patients</th>
<th>Controls</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous release</td>
<td>8.5 (4.6-10.4)</td>
<td>7.3 (5.9-9)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-8-induced release</td>
<td>10.0 (5.7-15.4)</td>
<td>8.9 (7-10.3)</td>
<td>NS</td>
</tr>
<tr>
<td>PMA-induced release</td>
<td>142 (96-192)</td>
<td>174 (133-190)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous release</td>
<td>126 (100-184)</td>
<td>93 (90-142)</td>
<td>0.07</td>
</tr>
<tr>
<td>IL-8-induced release</td>
<td>306 (214-333)</td>
<td>208 (175-289)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>PMA-induced release</td>
<td>1261 (1104-1441)</td>
<td>1557 (1362-1708)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous release</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>IL-8-induced release</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PMA-induced release</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td><strong>TIMP-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous release</td>
<td>1.3 (1.2-1.5)</td>
<td>1.3 (1-1.4)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-8-induced release</td>
<td>2.4 (2-2.9)</td>
<td>2.2 (2-2.4)</td>
<td>NS</td>
</tr>
<tr>
<td>PMA-induced release</td>
<td>10.2 (8-11.6)</td>
<td>11.1 (10-11.7)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are given as median (i-q range) (ng/ml).
ND = Non detectable
Blood mononuclear cells as a source of MMPs and TIMPs

In PBMCs, incubated in medium for 4 h, MMP-9 mRNA expression was found to be significantly increased in CAD patients compared to controls (Paper I). This was confirmed using freshly isolated PBMCs in a larger cohort of study participants (Paper II; Figure 3). The overexpression of MMP-9 mRNA in patients with stable CAD indicates the presence of “primed” mononuclear cells, i.e. cells which may have a higher propensity to adhere and migrate into tissues. Increased expression of MMP-9 mRNA in PBMCs or monocytes has been reported in unstable conditions of CAD and advanced carotid stenosis whereas stable CAD has not been previously associated with increased MMP-9 expression in circulating mononuclear cells (61, 147, 148). On the other hand, increased MMP-9 mRNA expression in blood mononuclear cells seems to be a shared feature with other chronic inflammatory diseases such as multiple sclerosis (149) and systemic lupus erythematosus (150). MMP-8 mRNA was not detectable in short-term cultured PBMCs (Paper I). Other studies have reported that MMP-8 mRNA is expressed in freshly isolated PBMCs. However, after short-term culture the expression of MMP-8 mRNA was reduced (62, 151), which may be in line with our results. Mainly due to the negative findings in study I, we did not perform measurements of MMP-8 mRNA in freshly isolated PBMCs.
As has been previously described, monocytes constitutively express and secrete TIMP-1 and, to a lesser extent TIMP-2 (62, 147, 152, 153). In PBMCs cultured in medium for 4 or 19 h, we found that TIMP-1 mRNA levels were significantly increased in patients and also, TIMP-2 mRNA levels tended to be increased in patients after 4 h (Paper I). The data are summarized in Table 4. On the other hand, when analyzing freshly isolated PBMCs, TIMP-1 mRNA levels tended to be elevated in patients while TIMP-2 mRNA levels showed a significant increase (Figure 3). The increase in TIMP mRNA levels was not unexpected since the inhibitors of proteases in many physiological settings are concomitantly upregulated with the proteases. This has been described earlier for MMP-9 and TIMP-1 (152) and as we show in paper II, MMP-9, TIMP-1 and TIMP-2 were strongly intercorrelated regarding both mRNA expression and protein secretion in PBMC. Increased expression of TIMP-1 mRNA tightly follows an increased expression of MMP-9 mRNA, has also been demonstrated in monocytes from patients with multiple sclerosis (62, 149). It is known that monocytes rapidly
upregulate MMP-9 as they migrate into tissues and differentiate into macrophages (152, 154).

Table 4. mRNA expression of MMP-9, TIMP-1 and -2 in PBMCs cultured for 4 h (Paper I, 10 patients, 10 controls) and 19 h with or without LPS (Paper II, 55 patients, 30 controls). Values, expressed as arbitrary units for mRNA expression are given as median (inter-quartile range). Please note that the values of mRNA expression between paper I and paper II are not comparable due to methodological differences.

<table>
<thead>
<tr>
<th>PBMC stimulation</th>
<th>Patients</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA Paper I 4-h culture, medium only</td>
<td>4.3 (3.6-5.2)</td>
<td>2.2 (1.5-4.2)</td>
<td>0.018</td>
</tr>
<tr>
<td>Paper II 19-h culture, medium only</td>
<td>298 (149-516)</td>
<td>249 (90-350)</td>
<td>0.17</td>
</tr>
<tr>
<td>Paper II 19-h culture, LPS</td>
<td>143 (96-248)***</td>
<td>147 (100-209)*</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA Paper I 4-h culture, medium only</td>
<td>4.8 (4.2-11)</td>
<td>2.0 (1.5-3.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>Paper II 19-h culture, medium only</td>
<td>14 (10-22)</td>
<td>8.7 (6.3-12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Paper II 19-h culture, LPS</td>
<td>17 (10-24)</td>
<td>14 (7.8-22)*</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>TIMP-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA Paper I 4-h culture, medium only</td>
<td>41 (23-83)</td>
<td>21 (12-39)</td>
<td>0.07</td>
</tr>
<tr>
<td>Paper II 19-h culture, medium only</td>
<td>491 (344-760)</td>
<td>429 (347-634)</td>
<td>0.35</td>
</tr>
<tr>
<td>Paper II 19-h culture, LPS</td>
<td>197 (134-324)***</td>
<td>290 (175-392)***</td>
<td>0.069</td>
</tr>
</tbody>
</table>

*= P < 0.05, vs 19-h culture, medium only
*** = P < 0.001, vs 19-h culture, medium only

We did not find any differences in ex vivo secretion of MMPs and TIMPs between patients and controls (Table 5). As demonstrated in paper II, stimulation of PBMCs with LPS for 19 h reduced secretion of MMP-9, while TIMP-1 secretion was increased and TIMP-2 secretion remained at a low level. However, as we show in paper I, the levels of MMP-9 and TIMPs in PBMC supernatants were negligible after 4 h of culture, independent of PMA stimulation. The discrepancy between our ex vivo studies may be due to the duration of cell culture but also, at least partly, to a less sensitive assay of MMP-9 and TIMPs in study I.
Table 5. Protein secretion of MMP-9, TIMP-1, and TIMP-2 in PBMCs cultured 19 h with or without LPS (55 patients, 30 controls). Values, expressed as ng/ml for protein secretion, are given as median (inter-quartile range).

<table>
<thead>
<tr>
<th></th>
<th>PBMC stimulation</th>
<th>PATIENTS</th>
<th>CONTROLS</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 Protein</td>
<td>Paper II</td>
<td>19-h culture, medium only</td>
<td>8.3 (5.3-14)</td>
<td>8 (4.6-11)</td>
</tr>
<tr>
<td></td>
<td>Paper II</td>
<td>19-h culture, LPS</td>
<td>4.6 (3.6-5.5)**</td>
<td>4.8 (3.4-4.6)*</td>
</tr>
<tr>
<td>TIMP-1 Protein</td>
<td>Paper II</td>
<td>19-h culture, medium only</td>
<td>12 (8.7-18)</td>
<td>14 (5.9-22)</td>
</tr>
<tr>
<td></td>
<td>Paper II</td>
<td>19-h culture, LPS</td>
<td>23 (17-33)**</td>
<td>22 (15-33)**</td>
</tr>
<tr>
<td>TIMP-2 Protein</td>
<td>Paper II</td>
<td>19-h culture, medium only</td>
<td>2.2 (1.7-2.6)</td>
<td>2.8 (1.5-3.3)</td>
</tr>
<tr>
<td></td>
<td>Paper II</td>
<td>19-h culture, LPS</td>
<td>2.3 (1.7-3.2)</td>
<td>2.5 (1.7-3.2)</td>
</tr>
</tbody>
</table>

*= P < 0.05, vs 19-h culture, medium only

*** = P < 0.001, vs 19-h culture, medium only

Brunner et al. (147) showed that an increased ratio of MMP-9/TIMP-1, both in serum and monocytic mRNA, was associated with unstable conditions of CAD. The ratio between MMP-9 and TIMP-1 levels in serum has also been proposed as a marker of disease activity in autoimmune diseases, such as multiple sclerosis (155). Our results did not find any differences in MMP-9/TIMP-1 ratios between patients and controls, neither in mRNA expression nor in protein levels. The physiological relevance of using ratios between MMP-9 and TIMPs has been criticized. Nevertheless, we can speculate that increased TIMP mRNA levels in PBMCs from stable CAD patients reflect an adequate counterregulatory response to the increase in MMP-9 expression. Hypothetically, a failure to counterregulate MMP-9 contributes to destabilization of the plaque. It is possible that differences in MMP-9/TIMP ratios might have been observed in patients with ACS. However, we believe that the choice of time point for measurements in ACS patients may have a large influence, the risk being obvious that blood samples are taken too late in the process to allow conclusions about what has preceded the event.

The mRNA expression of MMPs in human mononuclear cell subsets has been systematically mapped by Bar-Or et al. (62), who showed a predominance of MMP-9 in monocytes compared to T and B cells (62). However, it has been reported that Th1 cells upregulate MMP-9 in multiple sclerosis (156). Since T cells and monocytes are the main infiltrating cells in atherosclerosis, we further evaluated their expression of MMP-9 and TIMP-1 proteins using flow cytometry intracellular staining. The proportions of cells positively stained for MMP-9 and TIMP-1 as well as the amount of intracellular protein, assessed by median fluorescence intensity (MFI), were determined. As expected, MMP-9 and TIMP-
proteins were more abundantly expressed in monocytes than in T cells, but without any differences between patients and controls.

Serum and plasma levels of MMPs and TIMPs

Plasma and serum levels of MMP-9 and MMP-8 did not differ between patients and controls (Table 6). This is in agreement with several studies reporting similar levels of MMP-9 in stable CAD patients and controls (50, 51, 60, 147), although others have reported higher circulating levels in patients with stable symptoms (48, 49, 120). A few earlier studies have also shown increased levels of circulating MMP-8 in stable CAD compared to healthy controls (66, 157). One explanation behind the contradictory findings in stable CAD patients may be statin treatment, although this is far from clarified. Reduced plasma levels of MMP-9 in statin-treated CAD patients have been reported (158) while a study of healthy individuals with hypercholesterolemia did not find any effects of simvastatin on plasma or serum levels of MMP-9 or TIMP-1 (159).

Serum and plasma levels of MMP-9 and MMP-8 were strongly intercorrelated (r = 0.80, p < 0.001 and r = 0.87, p < 0.001, respectively). However, as expected, serum levels were greatly increased compared to plasma levels, most likely reflecting neutrophil degranulation during the clotting process in the serum tube. Accordingly, a time-dependent release of other neutrophil mediators such as leukotriene B₄ has been seen during serum preparation (160).

The levels of serum and plasma TIMP-1 did not differ between patients and controls, while plasma TIMP-2 was shown to be significantly lower in patients in paper I, but not in paper II. Decreased levels of TIMP-2 in CAD patients have been reported in some studies (48, 50), while others (60) have demonstrated increased levels. We observed significantly higher TIMP-1 levels in serum compared to plasma, probably due to activation of platelets in the clotting process. It has been reported that platelets are rich producers of TIMPs, in particular TIMP-1 (161).

The elevated mRNA levels of MMP-9, TIMP-1 and TIMP-2 in PBMCs were not reflected in the circulating protein levels. This was supported by Lichtinghagen et al. (162) who compared the mRNA expression of MMP-9, TIMP-1 and TIMP-2 in PBMCs with circulating protein levels in patients with chronic active
hepatitis C and healthy controls without finding any association. We believe that a major part of MMP-9 and MMP-8 in the circulation is originating from neutrophils. Platelets have been proposed as a source of circulating MMP-9, however, they appear to be negligible contributors, as previously shown by us (163). A leakage of MMPs from other tissues such as atherosclerotic plaques cannot be excluded but is very difficult to verify in humans. The sources of TIMP-1 and TIMP-2 may be several, including platelets, smooth muscle cells and fibroblasts (161, 164, 165). Our present findings do not provide any evidence that leukocytes are major producers of TIMPs.

**Table 6.** MMP-9, MMP-8, TIMP-1 and TIMP-2 levels in plasma and serum. Values, expressed as ng/ml, are given as median (inter-quartile range).

<table>
<thead>
<tr>
<th></th>
<th>N (PAT/CONT)</th>
<th>Patients</th>
<th>Controls</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-8</strong></td>
<td>Serum Paper I</td>
<td>10/10</td>
<td>12.4 (5.2-39)</td>
<td>8.1 (5.7-19)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper I</td>
<td>44/47</td>
<td>3.1 (1.5-3.5)</td>
<td>3.1 (2.2-5.7)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper II</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td>Serum Paper I</td>
<td>10/10</td>
<td>438 (275-1049)</td>
<td>388 (215-608)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper I</td>
<td>44/47</td>
<td>67 (38-107)</td>
<td>53 (43-85)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper II</td>
<td>57/41</td>
<td>50 (38-67)</td>
<td>49 (41-81)</td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
<td>Serum Paper I</td>
<td>10/10</td>
<td>193 (168-213)</td>
<td>180 (151-191)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper I</td>
<td>44/47</td>
<td>87 (78-95)</td>
<td>92 (80-103)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper II</td>
<td>57/41</td>
<td>89 (72-97)</td>
<td>92 (80-106)</td>
</tr>
<tr>
<td><strong>TIMP-2</strong></td>
<td>Serum Paper I</td>
<td>10/10</td>
<td>77 (72-86)</td>
<td>85 (80-87)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper I</td>
<td>44/47</td>
<td>70 (65-77)</td>
<td>79 (72-83)</td>
</tr>
<tr>
<td></td>
<td>Plasma Paper II</td>
<td>57/41</td>
<td>84 (71-92)</td>
<td>86 (78-92)</td>
</tr>
</tbody>
</table>
Exploring the potential associations between PBMC-derived levels of MMP-9, TIMP-1 and TIMP-2 and psychological factors

Depressive symptoms, assessed by CES-D, were significantly increased in post-ACS patients in comparison with healthy controls (paper II). Also, the levels of CES-D scores in patients remained unchanged up to 18 months after ACS thus speaking against that depressive mood was merely a transient phenomenon induced by the cardiac event. In agreement with our findings, previous longitudinal studies have shown persistent elevation of depressive symptoms up to 12 months after ACS (166-168). As expected, we found that depressive symptoms correlated positively with cynical hostility ($r = 0.267$, $p < 0.01$) and also, negatively with mastery ($r = -0.594$, $p < 0.001$). Cynical hostility is a strong predictor of depressive mood (169) and like depression, it has been associated with an increased risk of cardiovascular events (170, 171). Conversely, mastery has been found to independently protect against CAD (172).

We divided post-ACS patients according to sustained high or low CES-D scores. Psychological, clinical and laboratory measurements of the two groups are depicted in table 7. MMP-9 and its inhibitors, assessed in plasma or in PBMCs, were not related to depressive symptoms, cynical hostility or mastery. Neither did IL-6 in plasma show any correlations with psychological factors. In contrast to these findings, a population-based study of 402 middle-aged individuals (from the same region of Sweden as our cohort) found a significant association between plasma MMP-9 and CES-D scores, and also between plasma MMP-9 and cynical hostility, independent of traditional cardiovascular risk factors (100). Moreover, in a large case-control study using a proteomic approach, MMP-9 was one of the strongest markers associated with major depression (101). Another study showed that patients with depression had increased serum MMP-9 levels both in the acute phase and during remission (173). However, as mentioned above, the circulating levels of MMP-9 may not reflect what is going on at the cellular level (162). Interestingly, previous studies have shown that an upregulation of proinflammatory cytokines and chemokines in monocytes is associated with depressive symptoms in apparently healthy men and women (174, 175). Furthermore, it has been reported in a cohort of 56 ovarian cancer patients that elevated depressive symptoms, chronic stress and low social support were
associated with elevated levels of MMP-9 protein in tumor-associated macrophages (102). As described above, we studied MMP-9 and its inhibitors extensively by determining mRNA expression, protein release and intracellular protein levels in PBMCs without finding any associations with depressive symptoms or other psychological factors. Among our study participants, 19% of the post-ACS patients and 11% of the controls had a CES-D score above 16, which is the proposed cut-off for clinical depression (136) and in line with earlier findings that 15 to 20% of MI patients meet the criteria of clinical depression (176). Thus, it may be argued that the individuals with clinical depression in our cohort were too few to detect an association between psychological stress and MMP-9 levels. However, in population-based studies, not only clinical depression but also depressive symptoms in the subclinical range, have been associated with inflammatory markers, such as MMP-9, CRP and IL-6 in a dose-dependent manner (99, 100).
Table 7. Characteristics of post-MI patients with sustained low CES-D scores (\( \leq 6 \) on at least 2 occasions) or high CES-D scores (\( \geq 7 \) on at least 2 occasions). Values are given as median (inter-quartile range) or n (%).

<table>
<thead>
<tr>
<th></th>
<th>Sustained low CES-D scores (n = 24)</th>
<th>Sustained high CES-D scores (n = 27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychological measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CES-D*</td>
<td>3.2 (1.6-4.3)</td>
<td>13 (10-19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cynical hostility*</td>
<td>25 (21-31)</td>
<td>32 (26-36)</td>
<td>0.004</td>
</tr>
<tr>
<td>Coping*</td>
<td>25 (23-26)</td>
<td>20 (18-26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clinical measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>67 (64-71)</td>
<td>65 (56-69)</td>
<td>0.22</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>18/6</td>
<td>21/5</td>
<td>0.72</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27 (24-30)</td>
<td>28 (25-31)</td>
<td>0.33</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>2 (8)</td>
<td>3 (12)</td>
<td>0.67</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>4 (17)</td>
<td>5 (19)</td>
<td>0.73</td>
</tr>
<tr>
<td>Anti-hypertensive treatment, n (%)</td>
<td>11 (46)</td>
<td>12 (46)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Coronary angiography, 1/2/3-vessel disease, n</td>
<td>10/7/7</td>
<td>8/10/9</td>
<td>0.37</td>
</tr>
<tr>
<td>Laboratory measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9, plasma ng/ml</td>
<td>41 (30-75)</td>
<td>52 (44-67)</td>
<td>0.20</td>
</tr>
<tr>
<td>TIMP-1, plasma ng/ml</td>
<td>93 (78-100)</td>
<td>84 (70-95)</td>
<td>0.29</td>
</tr>
<tr>
<td>TIMP-2, plasma ng/ml</td>
<td>86 (77-91)</td>
<td>84 (67-91)</td>
<td>0.27</td>
</tr>
<tr>
<td>MMP-9, mRNA†</td>
<td>290 (150-520)</td>
<td>220 (120-610)</td>
<td>0.98</td>
</tr>
<tr>
<td>TIMP-1, mRNA†</td>
<td>16 (12-25)</td>
<td>12 (8-18)</td>
<td>0.11</td>
</tr>
<tr>
<td>TIMP-2, mRNA†</td>
<td>530 (330-880)</td>
<td>450 (390-560)</td>
<td>0.36</td>
</tr>
<tr>
<td>IL-6, plasma pg/ml</td>
<td>1.9 (1.4-3.3)</td>
<td>2.1 (1.6-2.8)</td>
<td>0.82</td>
</tr>
</tbody>
</table>

CES-D, The Center for Epidemiological Studies - Depression scale; MMP-9, matrix metalloproteinase-9; TIMP-1 and -2, tissue inhibitor of matrix metalloproteinase-1 and -2
* = scores in patients represent mean values derived from 2 or 3 occasions
† = mRNA values in freshly isolated PBMCs, expressed as arbitrary units


To conclude, the findings did not support our original hypothesis of higher MMP-9 levels in patients with depressive symptoms. The theory of inflammation as a link between psychological stress and CAD has become a rather well-established fact over the years (177, 178), even if this theory has been questioned lately. Some large prospective studies showing that psychological distress, such as depressivity and negative affect, predict risk of future CAD events, show that the risk remains after adjustment for the inflammatory markers CRP and IL-6 (98, 179, 180). Similarly, the inflammatory markers predict the incidence of CAD, but independently of the psychological factors. Other studies have shown that the association between inflammatory markers and psychological stress disappear.
after adjustment for health behaviors associated with depression, like physical
inactivity, smoking and high body mass index (181, 182). It is still possible that
we could have seen an association between MMP-9 and psychological factors in
a larger study population. Also, a larger sample size would have permitted us to
draw conclusions about specific subgroups, such as smokers, obese individuals
or patients with diabetes.

PBMC-derived levels of MMP-9, TIMP-1 and TIMP-2
and glucocorticoid sensitivity

The mRNA levels of GR-α and GR-β mRNA were compared in freshly isolated
PBMCs from post-ACS patients and controls (Figure 4; paper III). GR-α mRNA
was found to be markedly increased in patients while GR-β mRNA did not differ
significantly between the two groups. The levels of GR-β mRNA were
remarkably low compared to the levels of GR-α mRNA. This is in line with other
findings demonstrating that the expression of GR-β is 1000-fold less than GR-α
mRNA (183, 184). However, when we used the semi-quantitative method of flow
cytometry intracellular staining, no significant differences in GR-α and GR-β
protein levels could be detected. GR-α is known as the predominant GR isoform
with biologically functional activity while GR-β, unable to bind cortisol, has been
proposed to be a dominant negative inhibitor of GR-α-mediated effects (70). An
increase in GR-β mRNA expression or a lowered GR-α/GR-β mRNA ratio has
been associated with a state of glucocorticoid resistance in various cell lines (88,
185). Similarly, increased expression of GR-β mRNA and protein in PBMCs has
been reported in patients with glucocorticoid-resistant diseases such as asthma,
rheumatoid arthritis and inflammatory bowel disease (89-92). However, the
proposed importance of GR-β-mediated repressive effects on GR-α has been
questioned. Similar to our results, a number of previous clinical studies has not
been able to find elevated levels of GR-β mRNA expression in diseases, such as
asthma, Crohn’s disease and inflammatory bowel disease (93-95). It has been
proposed that GR-β mRNA expression might have limited relevance in
glucocorticoid resistance due to its remarkably low levels in PBMCs (70, 183).
On the other hand, our data indicated that PBMCs had similar protein expression
of GR-α and GR-β, which may contradict the former theory.
Figure 4. Expression of GR-α and GR-β mRNA levels in freshly isolated PBMCs from post-ACS patients and controls. GR-α mRNA were increased in post-ACS patients (p < .001), while the difference in GR-β mRNA expression did not reach statistical significance (p = .10). Values are expressed as arbitrary units and given as median (i-q range).

To further evaluate the sensitivity to GC in patients and controls, we performed ex vivo assays to study the dexamethasone-induced suppression of MMP-9 and TIMPs in PBMCs. After 19 h culture, the mRNA expression and protein secretion of MMP-9 was efficiently suppressed by dexamethasone in both patients and controls, as shown in Table 8. The findings are in agreement with several previous studies reporting dexamethasone-induced inhibition of MMP-9 in a variety of cell types, including human PBMCs (82, 84, 186-188). Also, in clinical settings, glucocorticoid treatment has been shown to induce a rapid decrease in MMP-9, both in circulation and in cerebrospinal fluid (189, 190).
Table 8. Suppression of MMP-9, TIMP-1 and TIMP-2 mRNA levels and protein secretion in PBMCs cultured for 19 h with dexamethasone at 10^{-7}M or 10^{-8} M. Values are given as median (i-q range) and expressed as percentage reduction of mRNA expression or percentage reduction of protein levels in supernatants of PBMCs cultured without dexamethasone for 19 h.

\[
\begin{array}{|c|c|c|c|c|}
\hline
 & Controls (n = 30) & Patients (n = 55) & P \\
\hline
 & Relative suppression & & & \\
\hline
MMP-9 mRNA & Dex 10^{-7} M & 97 (96-98) & 97 (95-98) & .19 \\
 & Dex 10^{-8} M & 76 (62-88) & 79 (71-87) & .58 \\
Secretion & Dex 10^{-7} M & 91 (81-95) & 88 (71-97) & .87 \\
 & Dex 10^{-8} M & 74 (62-79) & 72 (59-80) & .60 \\
TIMP-1 mRNA & Dex 10^{-7} M & 61 (53-75) & 69 (56-76) & .42 \\
 & Dex 10^{-8} M & 34 (7-54) & 50 (27-59) & .026 \\
Secretion & Dex 10^{-7} M & 53 (29-67) & 65 (59-72) & .002 \\
 & Dex 10^{-8} M & 36 (18-49) & 44 (31-51) & .13 \\
TIMP-2 mRNA & Dex 10^{-7} M & 30 (3-52) & 37 (16-57) & .21 \\
 & Dex 10^{-8} M & -12 (-37-25) & 19 (-1-41) & .002 \\
Secretion & Dex 10^{-7} M & 13 (-1-34) & 24 (13-37) & .19 \\
 & Dex 10^{-8} M & 13 (2-31) & 13 (-1-24) & .64 \\
\hline
\end{array}
\]

\* = p < 0.05, \text{b} = p <0.01, \text{c} = p < 0.001 compared to untreated cells within groups.

In contrast to MMP-9, the dexamethasone-induced suppression of TIMPs has been less studied and results are contradictory. Prior studies have reported that dexamethasone suppressed the secretion of TIMP-1 in PBMCs and fibroblasts (83, 187), while another study showed increased release in alveolar macrophages (84). We found that mRNA expression as well as secretion of TIMP-1 and TIMP-2 was suppressed by dexamethasone and moreover, data indicated an increased sensitivity to GC in post-ACS patients. At physiological concentrations of dexamethasone (10^{-8} M), the suppression of both TIMP-1 and TIMP-2 mRNA was significantly greater in patients than in controls (Table 8). When using pharmacological concentrations of dexamethasone (10^{-7} M), the suppression of TIMP-1 mRNA was still greater in patients.

Interestingly, the glucocorticoid sensitivity of PBMCs ex vivo, especially regarding the suppression of TIMP-1 and TIMP-2, correlated with the mRNA expression of GR-α in peripheral blood. This is in line with earlier studies showing that the total expression of GRs in a cell is closely associated with sensitivity to GC (86, 87). However, the GR-β mRNA expression, which was found to strongly correlate with the GR-α mRNA expression, also showed an
association with dexamethasone-induced suppression of TIMP-1 and TIMP-2, although it was weaker than for GR-α. Hence, our results suggest that GR-β is co-regulated with GR-α and do not support the theory that GR-β mRNA expression could function as a marker of reduced glucocorticoid sensitivity.

Upon exposure to dexamethasone, the mRNA levels of GR-α and GR-β were downregulated in both patients and controls (Table 9). Similarly, low GR mRNA expression has been associated with overexposure to dexamethasone in HeLa S3 cells (191). The other way around, in patients with hypocortisolism, the levels of GR mRNA in PBMCs are increased in order to compensate for the scarcity of cortisol (184). It can be speculated that the increased levels of GR-α mRNA in PBMCs reflect a state of relative hypocortisolism in post-ACS patients. The presence of pre-activated monocytes may thus be the result of insufficient suppression by glucocorticoids. This could also explain why the PBMCs from patients showed signs of increased GC sensitivity ex vivo. A previous study by Nijm et al. (117) showed that post-ACS patients exhibited a blunted cortisol response to both physical and psychological stressors. A life with daily stressful events, each followed by an inappropriate glucocorticoid response, may thus contribute to a state of relative hypocortisolism. However, a decreased secretory rate of GC is not the only potential explanation behind increased numbers of GRs. The bioavailability of GCs may also be reduced due to alterations in cortisol-binding globulin or the activity of 11β-hydroxysteroid dehydrogenase types 1 and 2 (70).

**Table 9.** Suppression of GR-α and GR-β mRNA levels in PBMCs cultured for 19 h with dexamethasone. Values are expressed as percentage suppression of mRNA expression in untreated and given as median (i-q range).

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 30)</th>
<th>Patients (n = 55)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative suppression %</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GR-α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex 10⁻⁷ M</td>
<td>42 (31-58)²</td>
<td>39 (22-46)⁴</td>
<td>.20</td>
</tr>
<tr>
<td>Dex 10⁻⁸ M</td>
<td>32 (17-45)³</td>
<td>34 (8-47)³</td>
<td>.85</td>
</tr>
<tr>
<td><strong>GR-β</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex 10⁻⁷ M</td>
<td>82 (65-92)²</td>
<td>81 (48-94)⁴</td>
<td>.67</td>
</tr>
<tr>
<td>Dex 10⁻⁸ M</td>
<td>56 (9-79)</td>
<td>74 (41-91)¹</td>
<td>.027</td>
</tr>
</tbody>
</table>

² = p <0.01, ³ = p < 0.001 compared to untreated cells within groups.
Release of MMP-9 and cortisol in response to acute mental stress

Acute mental stress is known to upregulate circulating inflammatory mediators. In healthy subjects, increased NF-κB DNA binding activity and cytokine gene expression are short-term effects (i.e. after 30 min) in response to stress, while increased levels of cytokines in the circulation are detected later, usually after 1-2 h (108, 114). However, the rapid release of neutrophil granules containing MMP-9, MMP-8 and MPO is likely to be detected within minutes, long before the secretion from mononuclear cells (29). Acute stress induces an early increase/mobilization of neutrophils into the blood (192). Previous studies have also shown that neutrophils are transiently activated in response to mental stress, by assessing oxidative capacity to reduce nitro-blue tetrazolium or levels of lactoferrin-bearing cells (112, 193). Here, we assessed the response to acute mental stress by measuring circulating levels of MMP-9, MMP-8, TIMP-1, TIMP-2 and MPO at baseline and 20 min after stress in patients with stable CAD (paper IV). A remarkable variation in the stress-induced release of MMPs and MPO was seen within the patient group. In line with this, previous studies have shown a significant variation in stress-induced release of cytokines, such as IL-6, in healthy subjects (108, 114). Furthermore, the differential response to mental stress was elegantly demonstrated in post-ACS patients by Strike et al (194). They divided patients into two groups: one emotional trigger group (n = 14) where the cardiac event had been preceded by negative emotional stress and one group (n = 20) without an emotional trigger. The emotional trigger group showed a greater increase in platelet activation, including neutrophil-platelet aggregates, in response to mental stress. These results highlighted the susceptibility to stress-induced neutrophil activation in the emotional trigger group.

We also measured cortisol in salivary samples before and 20 min after stress. Similar to the inflammatory response, the cortisol response varied substantially. Such a variation has also been reported in healthy subjects (114, 115) although a peak in cortisol response to mental stress is generally expected to occur 20-25 min after a stress test, with a subsequent rapid decline to pre-test levels (115, 195). However, among our CAD patients, 2/3 demonstrated lower levels of cortisol after stress than before stress. In a previous study of 13 healthy volunteers, Steptoe et al. (196) performed repeated measurements of cortisol up
to 45 min after a mental stress test and was able to show a progressive reduction from baseline, reaching lower levels of cortisol already after 20 min. Moreover, Kunz-Ebrecht et al. (115), studying 109 individuals in the Whitehall II cohort, reported that individuals who exhibited the lowest stress-induced cortisol response, so-called cortisol non-responders, had lower cortisol levels 20 min after stress compared to pre-test levels. These authors also demonstrated a weak but significant inverse association between the stress-induced change in cortisol and the stress-induced change in IL-6 (cytokines measured 45 min after stress). In agreement with this, we found a negative correlation between the change in cortisol levels and the change in serum MMP-9 in all 64 patients.

Due to the large variation, we divided the patients into tertiles depending on the stress-induced change in serum MMP-9. The relative increases in serum MMP-9, MMP-8 and MPO in the lower tertile (T1) and upper tertile (T3), respectively, are shown in figure 5. The serum levels of MMP-9, MMP-8 and MPO were strongly intercorrelated. The patients in T3 had significantly reduced levels of cortisol 20 min after stress compared to pre-test levels, whereas no significant change was observed in the lower tertile T1. (Figure 6). Unfortunately, we only measured cortisol 20 min after stress. Earlier measurement of cortisol, e.g. directly after stress, might have provided a better picture of the cortisol response. However, it can be speculate that the patients in T3 were cortisol non-responders incapable of sufficiently regulating a stress-induced neutrophil response. It is known that glucocorticoids modulate delayed genomic effects but also, they exert rapid non-genomic effects (197-199). Their direct effects on neutrophils are, however, not well known but in vivo therapy with prednisolone has rapid effects on the migration of neutrophils into inflamed joints in rheumatoid arthritis, in both humans and mice (200, 201). Moreover, in an in vitro study, both methylprednisolone and hydrocortisone were found to rapidly reduce neutrophil degranulation (202). However, high doses of $10^{-5}$ M were needed to achieve the effect and it still remains unclarified if physiological concentrations of cortisol can affect neutrophil degranulation in vivo.
Figure 5. Serum MMP-9, MMP-8 and MPO in patients divided into tertiles depending on their percentage change in serum MMP-9, tertile 1 (green) and tertile 3 (blue). Values are presented as median (inter-quartile range).

Activation of the sympathetic nervous system may be essential for the induction of inflammatory response to stress (203). In their study of healthy volunteer, Steptoe et al (196) showed that the stress-induced cytokine response was associated with sympathetic reactivity, rather than with cortisol reactivity. Kunz-Ebrecht et al (115) also showed lower heart rate variability in cortisol non-responders. In our population of CAD patients, there was no association between the cardiovascular response and inflammatory response. The blood pressure and heart rate response patterns were similar in T1 and T3 during stress. However, since the majority of patients were treated with beta-blockers, no conclusions about the impact of sympathetic reactivity can be made.

The perceived stress during the test may be important for the stress response. We did not measure the levels of perceived stress in the patients, which is a limitation. However, the similar cardiovascular stress response in T1 and T3 indicates an enhanced psychological arousal in both groups.
Figure 6. The cortisol response to stress, expressed as percentage change from baseline to 20 min after stress, in patients divided into tertiles depending on their percentage change in serum MMP-9, (lower tertile, T1 and upper tertile, T3).

We measured the basal cortisol levels 30 min after awakening and just before bedtime, i.e. at time points representing peak and nadir levels, but found no association with the stress-induced cortisol response. In line with our results, a recent study including 466 healthy older adults showed no relationship between the cortisol stress response and the cortisol slope during the day (204). Neither did we find any association between the psychological background factors including depressive symptoms (CES-D), cynical hostility and mastery, and the stress response. It has been shown that individuals with these psychological factors, particularly depression, are more prone to develop an inflammatory response to emotional stress (107). Furthermore, in men with clinical depression, stress-induced levels of IL-6 and NF-κB correlate with the severity of depression (205). Recently, Fagundes et al. (109) studied 136 healthy individuals with depressive symptoms and showed that those with elevated CES-D scores released significantly more IL-6 in response to mental stress than those with low scores. As already discussed above, it is possible that the patients with elevated CES-D scores in our study were too few to reveal a difference in stress-induced neutrophil activation.
The susceptibility to stress-induced release of MMP-9 and its association with leukocyte TL and carotid atherosclerotic burden

We found that the leukocyte TL was significantly shorter in T3 compared to T1 (Figure 7; paper IV) and also, there was a weak but significant inverse correlation between the stress-induced change in serum MMP-9 and leukocyte TL in all 64 patients ($r = -.29, p = .023$). Telomere shortening, a hallmark of cellular ageing, is known to be an independent predictor of cardiovascular events (129). Telomere attrition is also associated with chronic psychological stress but interestingly, it has been proposed that it is the physiological response to stress, rather than the stressful event itself, that is crucial for telomere shortening (206, 207). In a recent study, Zalli et al (131) demonstrated an interesting link between leukocyte TL and biological response to mental stress. The authors divided 333 healthy older individuals into subgroups depending on TL and telomerase activity in PBMCs. No differences were found in demographic/biological variables, including CRP and IL-6 levels, between the groups. However, when assessing the response to mental stress, the group with short TL/high telomerase activity showed several signs of allostatic load, e.g. a blunted cortisol response. With regard to inflammatory parameters, there were no differences in IL-6 response but the short TL/high telomerase activity group showed an increase in monocyte chemoattractant protein levels 45 min after stress.
Figure 7. Leukocyte telomere length in patients divided into tertiles depending on their percentage change in serum MMP-9, T1 and T3.

In the present study, we determined TL in leukocytes, a cell population that is composed of 50-60 % neutrophils. It is possible that the shorter TL in T3 reflects a higher activity of hematopoietic stem cells in the bone marrow, which might have been induced by an increased neutrophil activation in the periphery (208). As recently shown by Heidt et al. (209), mental stress in wild-type mice led to increased proliferation of hematopoietic stem cells in the bone marrow, resulting in a higher output of neutrophils in the circulation. Furthermore, when atherosclerosis-prone mice were subjected to mental stress, they exhibited a more inflammatory plaque activity involving increased numbers of neutrophils and increased levels of MPO. Unfortunately, we did not determine the neutrophil counts after stress. So far, it can only be speculated that the T3 group also exhibited an increased output of neutrophils as a response to the stress.

The ultrasound assessments of carotid arteries indicated a correlation between atherosclerotic burden and stress-induced increase in serum MMP-9. While all patients in T3 exhibited plaques in their right carotid artery, only 67 % of the patients in T1 did so. The results should be interpreted with caution, since the sample size was small and we did not assess atherosclerosis in other arteries. The coronary angiography findings did not provide any evidence for more severe CAD in T3. However, coronary angiography is not an appropriate method for assessing coronary atherosclerosis. We believe that our carotid ultrasound
findings may provide an indication that frequent activation of neutrophils is associated with a progress of atherosclerosis. There is also emerging evidence that neutrophils play a role in the onset and development of disease (36, 210). Interestingly, a study including 355 human carotid plaques showed that a high number of neutrophils in the plaque was associated both with high levels of MMP-9 and MMP-8 in the plaque and histopathologic features of rupture-prone lesions (32). Another interesting finding was reported by Burke et al (211). In an autopsy survey of men with CAD who died suddenly, plaque rupture was more frequent in those who died during strenuous activity or emotional stress.
Concluding remarks

When we measured the basal circulating levels of MMPs and TIMPs in CAD patients, their levels were similar to those observed in healthy controls. On the other hand, analyses of MMP-9, TIMP-1 and TIMP-2 in leukocytes revealed significant differences between patient and controls. Neutrophils in patients released significantly more MMP-9 upon mild physiological stimulation ex vivo, thus proposing a preactivated state of neutrophils. Furthermore, PBMCs in patients expressed significantly higher levels of MMP-9, TIMP-1 and TIMP-2 mRNA, suggesting that these cells also exhibited a preactivated phenotype.

An association between inflammatory activity, e.g. increased MMP-9 levels, and depressive symptoms has been proposed. We found persistently elevated depressive symptoms in CAD patients compared to controls. However, depressive symptomatology did not show any correlations with the leukocyte-derived expression of MMP-9, TIMP-1 and TIMP-2 mRNA, nor with circulating levels. Thus, our findings suggest that the preactivated phenotype of mononuclear cells and the elevated depressive symptoms are two parallel phenomena in CAD patients.

Reduced GC sensitivity may be a characteristic of chronic inflammatory disorders. However, we found no evidence for reduced GC sensitivity in patients, rather the opposite. The expression of GR-α mRNA in PBMCs was significantly increased in patients and moreover, the PBMCs from patients exhibited a normal or even increased sensitivity to GC-mediated suppression of MMP-9 and TIMPs ex vivo. The results suggest a state of relative hypocortisolism which may contribute to an impaired regulation of leukocyte-derived MMP-9 and TIMPs.

Finally, we investigated the biological response to mental stress in CAD patients, especially focusing on the rapid release of MMP-9 and cortisol. A stress-induced increase in serum MMP-9 was clearly associated with altered cortisol reactivity. It was also associated with telomere shortening in leukocytes and carotid plaque burden, as a possible consequence of a long-lasting impairment of the ability to handle stressful events in daily life. We believe that it may be important to identify patients who are susceptible to stress-induced neutrophil activation and to develop preventive and therapeutic strategies for them.
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There is a long list of persons that have contributed to this thesis in different ways and it would not have been possible to complete this work without their support. I would like to give special thanks to:

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Appendix (papers I-IV)
Papers

The articles associated with this thesis have been removed for copyright reasons. For more details about these see:
http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-114328