

**Inflammation and Cortisol Response in
Coronary Artery Disease**

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TO
ANGI, EDDIE, VICYMIA
&
FADO

*A Good Teacher is the One who Teaches you how to Learn, thus Looking for the Truth and
Finding it is Science*
(Edward Elías)

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This thesis is based on the following papers, which will be referred to by their Roman numbers

- I. **Nijm J**, Wikby A, Tompa A, Olsson AG, Jonasson L.
Circulating levels of proinflammatory cytokines and neutrophil-platelet aggregates in patients with coronary artery disease
Am J Cardiol 2005; 95:452-456.

- II. **Nijm J**, Kristenson M, Olsson AG, Jonasson L
Impaired cortisol response to acute stressors in patients with coronary disease. Implications for inflammatory activity
Journal of Internal Medicine 2007; 262: page 375-285.

- III. **Nijm J**, Nilsson L, Jonasson L
A sustained elevation of serum matrix metalloproteinase-9 is associated with diurnal salivary cortisol in patients with acute myocardial infarction-a 3-month follow-up.
Manuscript, submitted.

- IV. Särndahl E, Bergström I, Patcha Brodin V, **Nijm J**, Lundqvist Setterud H, Jonasson L.
Neutrophil activation status in stable coronary artery disease.
PLoS ONE 2007; 2:e1056.

- V. Särndahl E, **Nijm J**, Bergström I, Forslund T, Perretti M, Jonasson L.
Enhanced neutrophil expression of annexin-1 in coronary artery disease.
Manuscript, submitted.

Abstract

Atherosclerosis is characterized by a chronic inflammation, involving autoimmune components, in the arterial wall. An increase in proinflammatory activity relative to anti-inflammatory activity is considered to cause a progression of the disease towards plaque instability and risk of atherothrombotic events, such as acute coronary syndrome (ACS). Cortisol, the end product of the hypothalamus-pituitary-adrenal (HPA) axis, is a powerful endogenous anti-inflammatory mediator. Disturbances in the HPA axis have been reported in chronic inflammatory/autoimmune diseases, like rheumatoid arthritis. The aim of this thesis was to study various markers of systemic inflammation in patients with acute and stable conditions of coronary artery disease (CAD) and relate these findings to the cortisol response.

Both patients with ACS and patients with stable CAD had high levels of C-reactive protein (CRP), interleukin (IL)-6 and IL-1 receptor antagonist, compared with healthy controls. In addition, patients with stable CAD had significantly more neutrophil-platelet aggregates than controls, as a possible indicator of neutrophil activation.

The cortisol response was determined in two different cohorts of CAD patients; one consisting of patients with a first-time myocardial infarction and one consisting of patients with long-term stable CAD. From the acute phase to 3 months, the patients with a myocardial infarction showed a higher 24-h cortisol secretion and a flattened diurnal slope caused by higher cortisol levels in the evening, as compared with healthy controls. The patients with long-term stable CAD showed similarly high levels of cortisol in the evening. The levels of evening cortisol were strongly correlated with CRP and IL-6. When exposed to acute physical or acute psychological stress at 3 months, the ACS patients showed a markedly blunted cortisol response compared with healthy controls. Following the stress tests, a significant increase in CRP was observed in the patients but not in the controls, indicating a failure of the HPA axis to compensate for stress-induced inflammation in CAD.

In the ACS patients, the time course of matrix metalloproteinases (MMPs) and their tissue inhibitor TIMP-1 was determined during the 3 months follow-up. A major finding was that the MMP-9 and TIMP-1 levels remained significantly higher in the patients at all time points compared to the controls. MMP-9 and TIMP-1, but not MMP-2, MMP-3 or MMP-7, were related to inflammatory activity, as assessed by CRP and IL-6. MMP-9 and TIMP-1 showed significant correlation with evening cortisol, even after adjustment for CRP and IL-6, lending further support for a link between 'high' flat cortisol rhythm and systemic inflammatory activity.

The activation status of neutrophils in stable CAD was further examined by measuring the expression, affinity state and signalling capacity of $\beta 2$ -integrins and the innate production of reactive oxygen species (ROS). However, the neutrophils in patients were not more activated *in vivo* than were cells in healthy controls, neither were they more prone to activation *ex vivo*. The data rather indicated an impaired function of neutrophils in stable CAD.

The neutrophils in CAD patients showed a significantly lower number of total glucocorticoid receptors (GRs) and a lower GR α :GR β ratio compared to healthy controls, indicating a chronic over activation of the HPA axis and, possibly, a state of glucocorticoid resistance. Moreover, the evening cortisol levels in patients were associated with an overexpression of annexin-1, the 'second messenger' of glucocorticoid action. In contrast to neutrophils in controls, the neutrophils in patients also showed a hyper responsiveness to exogenous annexin-1 resulting in impaired neutrophil function. To conclude, clinically stable CAD was associated with a systemic inflammatory activity, involving a high MMP-9:TIMP-1 ratio and an increased inflammatory response to acute stress but not any activation of neutrophils. This inflammatory activity was associated with a dysregulated cortisol secretion, defined by a flat diurnal rhythm and a blunted cortisol response to stress. Although the clinical relevance remains to be verified, an intriguing hypothesis is that a hyporesponsive HPA axis favours the development towards plaque instability.

Populärvetenskaplig sammanfattning

Åderförkalkning (ateroskleros) orsakas av inflammatoriska härdar (plack) i kärlväggen. Om denna inflammation tillåts bli alltför aggressiv och inte i tillräcklig grad bromsas upp av kroppens eget antiinflammatoriska system kan placken i kärlen bli sköra och ´instabila´. Detta innebär att de kan spricka och blodproppar bildas med en kärldatastrof, t.ex. hjärtinfarkt, som följd. Kortisol är en mycket viktig kroppsegen inflammationshämmande substans. Störningar i kortisolutsöndringen har påvisats vid andra kroniska inflammatoriska sjukdomar såsom ledgångsreumatism. Syftet med denna avhandling var att mäta graden av inflammation hos kranskärlssjuka patienter och samtidigt studera om och hur inflammationen påverkades av kortisolutsöndringen.

Inte bara patienter med akut hjärtinfarkt utan även patienter med kärldatakramp hade höga koncentrationer av inflammatoriska ämnen i blodet jämfört med friska personer. Dessa inflammatoriska ämnen utgjordes av bl.a. olika signalsubstanser och ämnen som bryter ner stödjevävnad i placken. Hos patienter med kärldatakramp sågs också tecken till kronisk aktivering av neutrofila celler, en särskild typ av vita blodkroppar som utgör kroppens första försvarslinje.

Både patienter med akut hjärtinfarkt och kärldatakramp uppvisade en ökad kortisolutsöndring jämfört med friska personer, och samtidigt ett förändrat dygnsmonster av kortisolhalten med förhöjda nivåer på kvällen. Ju högre kortisolhalterna var på kvällen, desto högre var nivåerna av inflammatoriska ämnen i blodet. När patienter och friska försökspersoner utsattes för akut stress i form av hård ansträngning eller psykisk stress förmådde dock inte patienterna utsöndra lika mycket kortisol som de friska. Samtidigt orsakade stressen en ökning av inflammatoriska ämnen i blodet hos patienter men inte hos friska individer.

Känsligheten för kortisol undersöktes hos patienter med stabil kärldatakramp och hos friska individer. Neutrofila celler från patienter visade tecken till att vara kroniskt överstimulerade av kortisol. När funktionen hos neutrofila celler studerades i detalj visade det sig även att celler från patienter hade en försämrad funktion jämfört med celler från friska personer.

Sammanfattningsvis kunde en ökad inflammatorisk aktivitet i blodet och ökade kortisolhalter påvisas hos patienter med kranskärlssjukdom, även hos de som var välmedicerade och utan några egentliga symtom. Det fanns ett klart samband mellan akut stress, förändrad kortisolutsöndring och inflammation. Fortfarande vet vi alltför lite om vad som startar en hjärtinfarkt men en ny förklaring kan vara att ett förändrat kortisol svar minskar kroppens förmåga att motverka inflammation och därmed gynnar en utveckling av 'instabila' plack i kärlväggen.

1 Introduction

Coronary artery disease (CAD) is the most prevalent manifestation of cardiovascular diseases (1,2). It involves two processes: a slow atherosclerotic process that causes gradual lumen narrowing and a dynamic and potentially reversible process that interrupts the slow process in a sudden and often unpredictable way, causing an acute atherothrombotic event. The asymptomatic coronary atherosclerosis develops over decades and may begin early in life (3, 4). As atheromatous plaques gradually increase in size and the coronary blood flow becomes inadequate to meet cardiac metabolism demand during exercise or stress, symptoms of angina will appear (5). If someone's angina is unchanging or progressing slowly it is described as 'stable'. For patients with stable CAD, it may be useful to classify the severity of symptoms using a grading system such as Canadian Cardiovascular Society Classification (6) (Table 1). The patient may remain asymptomatic or symptomatic with a stable course or turn into a life-threatening acute coronary syndrome (ACS), including unstable angina and myocardial infarction. In a number of cases, the ACS will even be the first manifestation of the disease. Unstable angina is characterized by a sudden worsening of angina symptoms, which become more frequent, more prolonged and more severe occurring at a lower threshold or during rest. Myocardial infarction, often characterized by prolonged angina (> 30 minutes), is defined by myocardial necrosis. The common pathological background of ACS is erosion, fissure or rupture of an atherosclerotic plaque associated with platelet aggregation, leading to different degrees of lumen-obstructing thrombi (7). A classification of myocardial infarctions is based on the electrocardiogram (ECG): ST-elevation that generally reflects a total coronary occlusion, and non-ST-elevation.

Table 1. Grading of angina pectoris by the Canadian Cardiovascular Society classification system (6).

Class I	Angina only during strenuous or prolonged physical activity
Class II	Slight limitation, with angina only during vigorous physical activity
Class III	Symptoms with everyday living activities, i.e. marked limitation
Class IV	Inability to perform any activity without angina or angina at rest, i.e. severe limitation

Lifestyle changes as well as public health and medical care advances in the prevention and treatment of CAD during previous decades have been accompanied by a marked decline in CAD mortality in Western countries. Nevertheless, CAD remains the leading cause of death in developed nations and is predicted to achieve that status worldwide within 10-15 years (8). The determinants of both the subclinical and clinical stages of the disease are numerous and varied, including risk factors for individual persons, group characteristics of entire populations, and environmental exposures. Risk factors include genetic, biomedical, behavioural and lifestyle characteristics. Those firmly established, for example, total and low density lipoprotein (LDL) cholesterol levels, blood pressure, smoking and diabetes are supported by results of numerous epidemiological and clinical studies (9,10). There is also convincing evidence to indicate that dietary factors, physical inactivity and psychosocial stress are important determinants of cardiovascular risk (11-13). A group of high-risk patients can thus be identified but many of the ACS events occur in the far more numerous individuals deemed to be at moderate or low risk by standard screening methods. Moreover, many events occur in those with known CAD who have already had the benefit of optimal current therapy including percutaneous coronary intervention and aggressive medical therapy with cholesterol-lowering and anti-platelet agents.

1.1 Atherosclerosis – an inflammatory disease.

For almost a century, lipid accumulation in the wall of medium- and large-sized arteries was considered the major initiator and maintainer of atherosclerotic disease (3, 4). However, during the last two decades, there have been tremendous advances in research and

management of atherosclerosis establishing a fundamental role of inflammation (14, 15). A model linking lipids and inflammation has thus emerged. In a simplified picture, atherosclerosis can be regarded as an inflammatory response to invading lipoproteins in the arterial wall involving both innate and adaptive immunity. Among innate immune cells, macrophages remain the most frequently studied (16). The monocytes are initially attracted to the arterial wall by cell-adhesion molecules expressed on activated endothelial cells. The differentiation into macrophages includes a substantial up-regulation of so-called scavenger receptors that normally function in the recognition and internalization of pathogens and apoptotic cells. However, scavenger receptors also recognize altered molecular patterns present on modified lipoproteins, and thus mediate the engulfment of entrapped lipids that will transform monocytes into macrophage foam cells. The death of lipid-laden foam cells ultimately leads to the formation of a necrotic, cholesterol-rich core that becomes walled off by a fibrous cap of extracellular matrix proteins. In addition to the uptake of lipoproteins, macrophages in the arterial wall secrete growth factors, cytokines and inflammatory mediators that influence the growth of the plaque. They are also likely to amplify the oxidative reactions in the plaque by the expression of enzymes like myeloperoxidase and lipoxygenases. Moreover, lipid-laden macrophages may contribute to the hyperthrombotic state of human atherosclerotic lesions by the production of tissue factor, which may activate the extrinsic coagulation pathway.

Macrophages in the lesion also participate in the acquired immune response by collaborating extensively with T cells through cell-cell and cytokine-mediated interactions. Immunohistochemical studies have shown that both CD4⁺ and CD8⁺ T cells are present in human lesions through all stages of plaque development, although CD4⁺ cells dominate in later stages of plaque development (17, 18). The number of T cells in the lesion is also related to the overall plaque morphology. In stable fibrous plaques, the number of T cells is low whereas lesions with large lipid pools contain larger proportions of T cells. The CD4⁺ T helper cells recognize processed antigens presented by MHC class II (HLA-DR) molecules on antigen-presenting cells (19). The stimulation by exposure to antigen-presenting cells induces the proliferation of T cells and the secretion pattern of cytokines that is specific for the type of T cell involved. CD4⁺ Th1 cells produce proinflammatory cytokines like interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), while CD4⁺ Th2 cells produce cytokines like interleukin (IL)-4 and IL-10 (20). Experimental animal studies have consistently shown the importance of Th1 immunity in atherogenesis (14, 15). Several reports have also confirmed

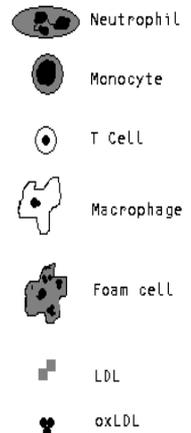
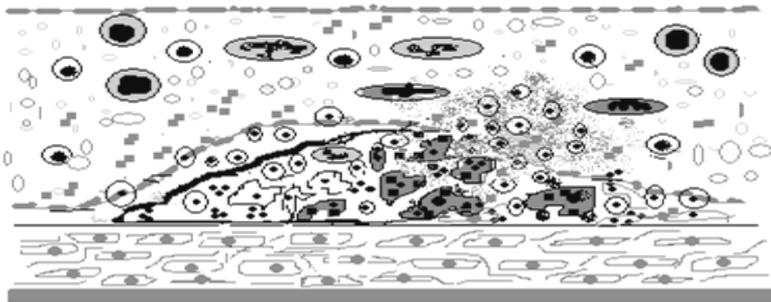
the presence of a dominant Th1 response in human plaques, as assessed by analysis of plaque-derived mRNA and functional analysis of plaque-derived T cell clones (21, 22). In addition, there is an expansion of Th1 cells (CD4+IFN- γ +) in peripheral blood of patients with CAD, with the largest numbers of Th1 cells in patients with ACS (23).

The antigen specificity of T cells in atherosclerosis has been the focus of much research and several lines of evidence support that oxidized LDL cholesterol may be a key antigen (14, 15). Although the T cells in human plaques reveal a polyclonal population, T cell clones specific for oxidized LDL have been isolated from human advanced lesions (24). Furthermore, the proliferative response of blood-derived T cells to oxidized LDL has been shown to increase significantly in patients with unstable angina compared to stable patients, suggesting an antigen-driven response (25). However, it has been argued that the high frequency of stimulated T cells in blood from patients with ACS is unlikely to represent an immune response driven by a single antigen. Instead, the possibility of several atherosclerosis-related antigens and/or even the presence of non-specific T cell stimulating factors that sustain the inflammatory activity have been discussed (26-28).

Lesions that are prone to rupture are rich in activated macrophages, have high contents of lipid and necrotic debris and thin fibrous caps. Given that the extracellular matrix components collagen and elastin are responsible for the structural integrity of the fibrous cap, a shift towards enhanced matrix degradation in the plaque may promote plaque rupture. The activation of Th1 cells and macrophages is associated with an increased release of proteases like matrix metalloproteinase (MMP)-9 (29, 30). Accordingly, coronary plaques from patients with unstable angina have shown an increased gene expression of MMP-9, accompanied by infiltrations of macrophages and T cells, compared with plaques from stable patients (31, 32).

Figure 1. Various features of an atherosclerotic plaque, a) a ruptured unstable plaque b) a stable plaque. In the unstable plaque, the majority of thrombi occur at sites of fissure or rupture of a thinned fibrous cap overlying a large lipid/necrotic core. At this stage activated immune cells are abundant. In the stable plaque, the fibrous cap is more cell rich and contains large amounts of connective tissue. Compared to the unstable plaque, the lipid core is smaller and the immune cells are fewer.

a.



b.



The inflammatory activity in atherosclerosis is not only detectable within lesions but also in peripheral blood. The systemic inflammatory state, involving increased levels of acute phase proteins, is a well-known characteristic of patients with clinically unstable CAD (33, 34). On the other hand, studies that compare systemic inflammation in patients with stable atherosclerotic disease and healthy individuals are fewer and have produced slightly different results (35-37).

1.1.1 C-reactive protein

The innate immune molecules can be generally classified into several functional groups, among them acute phase proteins. One of the major acute phase proteins in man is C-reactive protein (CRP), identified in 1930, as a precipitin of the C-polysaccharide of pneumococcus (38). As a member of the pentraxin family, CRP consists of 5 identical 22-kDa subunits (39). The synthesis of CRP in the liver is predominantly under the control of IL-6 (39, 40). However, IL-1 and TNF- α may also contribute to hepatic synthesis and secretion of CRP. After initial tissue injury, plasma levels of CRP begin to increase very early and may continue to increase several hundred-fold within 24-48 hours (39). In healthy individuals, CRP is increased following IL-6 infusion reaching a peak level 21 h after the cessation of IL-6 (41). The half-life in the circulation is estimated to be 19 hours (42). There is increasing evidence for a physiologic function of CRP as an anti-inflammatory scavenger molecule. It acts as an opsonin for bacteria, parasites, and immune complexes, activating the classical complement pathway (43). It binds to modified lipoproteins and facilitates their removal by phagocytes, thus contributing to the clearance of apoptotic and necrotic cells (44).

Numerous trials have demonstrated the ability of CRP levels to predict future cardiovascular events, including cardiovascular death, myocardial infarction, stroke, revascularization, the development of peripheral vascular disease, and sudden cardiac death (45-47). In contrast, an independent association between CRP and direct measures of atherosclerosis has not been clearly shown, as assessed by carotid intima-media thickness or coronary artery calcification (48, 49). This has led to the proposal that elevated levels of CRP may reflect the presence of vulnerable plaques that are at high risk for rupture, rather than solely reflecting the burden of atherosclerosis.

CRP is present in the atherosclerotic lesion, where it co-localizes with monocyte-derived macrophages (50). The direct contribution of CRP to atherosclerosis via direct proinflammatory effects, involving complement activation, interactions with cell surface receptors and thrombosis, has been widely discussed (51-53). However, the direct role of CRP in the arterial wall apparently remains to be clarified after it was shown that a number of the proatherogenic effects of CRP *in vitro* could be explained by contamination of the

CRP preparation (53, 54). In addition, a recently published study reported that CRP slowed atherogenesis in a CRP transgenic mouse model (55).

1.1.2 Cytokines

The cytokines are a group of low molecular weight regulatory proteins secreted by white blood cells as well as a variety of other cells in response to stimuli. They regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating the secretion of antibodies or other cytokines (56). Soluble cytokine receptors regulate the inflammatory and immune events by functioning as agonists or antagonists of cytokine signalling. As such, they act within complex receptor systems that include signalling receptors and soluble receptor antagonists (57, 58).

1.1.2.1 Interleukin-1 and interleukin-1 receptor antagonist

IL-1 is the prototypically inflammatory cytokine produced mainly by activated monocytes and macrophages but also by several other cell types. By inducing adhesion molecules, clotting factors, chemokines, and MMPs, it is a critical early mediator of inflammation. The term IL-1 is generally used to describe IL-1 α and IL-1 β , both of which exercise the same biological effects. The IL-1 receptor antagonist (IL-1Ra) is a member of the IL-1 family (58). In fact, there is evidence that IL-1Ra, like CRP, is an acute-phase protein (59). It binds to IL-1 receptors without transmitting an activation signal and represents a physiological inhibitor of preformed IL-1. Because IL-1 α and IL-1 β lack a signal peptide, they are not readily secreted from the cells into the systemic circulation. Hence, levels of IL-1 α and IL-1 β in the circulation in patients with infectious or inflammatory disease are often marginal. On the other hand, IL-1Ra has a signal peptide and is readily secreted into the blood. During experimental endotoxemia in humans, IL-1 β increases in the circulation by a factor of 2 to 2.5, whereas IL-1Ra increases by a factor of 10 to 20 (60). Therefore, measurement of IL-1Ra rather than IL-1 α and IL-1 β is considered a more reliable assessment of production of IL-1 family members.

Experimentally, IL-1 deficiency decreases the severity of atherosclerosis in apolipoprotein E-deficient mice (61). In patients with unstable angina the plasma levels of IL-1Ra were shown to be elevated and related to impaired clinical outcome (62). However, although plasma concentrations of IL-1Ra may be a reliable marker of IL-1 activity, IL-1Ra at the cellular level is thought to reflect its anti-inflammatory capacity. A possible imbalance between IL-1 and IL-1Ra in unstable conditions of CAD has been illustrated by Waehre T and coworkers (63). They showed markedly increased mRNA levels of IL-1 α and IL-1 β in peripheral blood mononuclear cells in both stable and particularly unstable angina, accompanied by only modestly increased IL-1Ra levels in the unstable patients.

1.1.2.2 Interleukin-18

IL-18 is related to the IL-1 family in terms of structure, receptor family, and function. It activates T cells promoting their expression of IFN- γ , a key mediator in plaque progression (64). Results from experimental animal studies have consistently shown a proatherosclerotic effect of IL-18 (65). IL-18 mRNA is also expressed at higher levels in unstable human carotid plaques relative to stable plaques (66). In a prospective study, IL-18 in plasma was an independent predictor of cardiovascular events in patients with CAD regardless of the clinical status at admission (67). In a few clinical studies, the circulating levels of IL-18 have been significantly increased in patients with both stable and unstable conditions of CAD compared to healthy individuals (68-70).

1.1.2.3 Interleukin-6

IL-6 is a 26 kDa cytokine, produced by many different cells, including lymphocytes, monocytes, vascular smooth muscle cells, endothelial cells and adipocytes (71). IL-6 is often induced together with the proinflammatory cytokines TNF α and IL-1 in many alarm conditions, and circulating IL-6 plays an important role in the induction of acute phase reactions. Although IL-6 is used as a proinflammatory marker, endogenous IL-6 plays a crucial anti-inflammatory role in both local and systemic acute inflammatory responses by controlling the level of proinflammatory, but not anti-inflammatory, cytokines (72). It has a rapid turnover in plasma but soluble IL-6 receptors bind IL-6 with an affinity similar to the membrane IL-6 receptor, thereby prolonging the IL-6 half-life (73). A study in men in which samples were collected every 3 h by direct venipuncture, showed a large circadian variation in

circulating IL-6. On average, values were greater than the mean throughout the night, with a peak at 01:00 PM and less than the mean throughout the day, with a nadir at 10:00 AM (74).

Patients with ACS have increased circulating levels of IL-6 compared with patients who have stable angina (34). Among patients with unstable angina, an increase in IL-6 levels that occurred 48 hours after admission, compared with the admission value, was associated with a poor prognosis (62). In the FRISC-II study including ACS patients, elevated IL-6 levels were associated with higher 6- and 12-month mortality and were additive to and independent of cardiac troponin T status (75). In addition, the benefit of an early invasive strategy was enhanced in patients with elevated IL-6 levels. These results suggested that the measurement of IL-6 may be useful to select high-risk patients for intensified therapy. However, the large circadian variations in IL-6 levels may clearly limit the applications of IL-6 as a biomarker in ACS.

1.1.2.4 Interleukin-10

IL-10 is an anti-inflammatory cytokine mainly expressed in monocytes, Th2 cells and regulatory T cells. It down-regulates the expression of Th1 cytokines, MHC class II antigens and co-stimulatory molecules on macrophages (76, 77). It also decreases the synthesis of MMP-9 and tissue factor and enhances B cell survival, proliferation and antibody production (78, 79).

The balance between proinflammatory and anti-inflammatory cytokines is thought to be important for the development of several inflammatory disorders, including atherosclerosis and ACS. Decreased levels of IL-10 have been reported in patients with unstable angina compared with clinically stable patients (80). In a prospective study performed in patients with unstable angina, elevated IL-10 levels were associated with a decreased risk of death or nonfatal myocardial infarction. Furthermore, the patients with elevated CRP and elevated IL-10 were at lower risk than were patients with elevated CRP but no elevation in IL-10, suggesting that IL-10 may be protective against proinflammatory mediators in ACS (81).

1.1.2.5 Interleukin-2

The proliferation of T helper cells is mediated by an IL-2-dependent autocrine mechanism (82). Thus, T cells stimulated by antigens or other mitogenic stimuli secrete IL-2 and express membrane receptors for IL-2. The IL-2 receptor (IL-2R) complex is a $\alpha\beta\gamma$ trimer, in which all three chains are in contact with the ligand (83). The α subunit of this complex, also known as CD25, is a 55 kilodalton transmembrane glycoprotein. A soluble form of IL-2R α appears in serum, concomitant with its increased expression on T cells (84). Increased levels of the soluble IL-2R α in biological fluids correlate with activation of T and/or B cells. Results from a number of studies suggest a correlation of levels of IL-2R α in serum with disease activity in autoimmune and infectious disorders as well as in transplantation rejection (85-87).

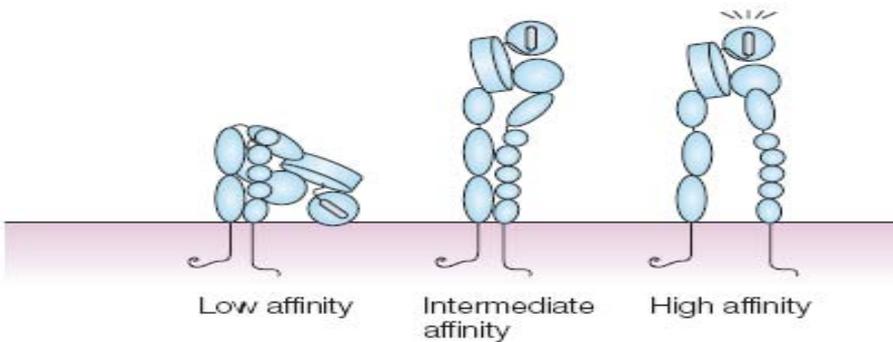
In advanced atherosclerotic lesions of apolipoprotein E knockout mice, the CD25 subunit of the IL-2R is expressed in areas rich in CD4 (88). In peripheral blood of stable CAD patients, the number of CD25+CD4+ T cells is significantly increased compared to healthy subjects (37). In accordance with these findings, elevated plasma levels of IL-2 or soluble IL-2R have been detected in patients with stable symptoms (36, 37). In patients with unstable angina, the levels of soluble IL-2R were shown to be significantly higher than in stable angina patients with a gradual decrease over 12 weeks (35).

1.1.3 Neutrophil activation

Polymorphonuclear neutrophils, often just called neutrophils, represent more than 50 % of the total circulating leukocytes and play a pivotal role in innate immunity. They develop from the same early precursors as monocytes and macrophages but in contrast to these cells, neutrophils have a very short lifetime (1-2 days) (89, 90). When primed in the circulation and at inflammatory sites, neutrophils mediate their effects through the production of proteases such as cathepsins, MMPs and elastase, cytokines like IL-1 β , TNF- α and IL-8, and the generation of reactive oxygen species (ROS) (90, 91). Neutrophil transmigration across the vascular endothelium is a highly regulated process that requires the up-regulation of neutrophil adhesion molecules (92, 93). One of the most important adhesion molecules involved in the firm adhesion, Mac-1 (CD11b/CD18), belongs to the β 2-integrin family that is expressed exclusively on leukocytes. Integrins are thought to exist in different conformations,

from a low-affinity to a high-affinity state. The latter is responsible for high-affinity ligand binding (94, 95). When neutrophils are activated, integrins switch from the low-affinity to the high-affinity state by a so-called inside-out signalling (96) (Figure 2). This activation of $\beta 2$ -integrins is highly regulated, and soluble guidance signals like chemokines and chemotactic factors (*e.g.* IL-8, leukotriene B₄ (LTB₄)) play an essential role in the process by increasing the adhesive activity of the $\beta 2$ -integrins (94, 97).

Figure 2. The low-affinity state, the intermediate affinity ('closed') state and the high-affinity ('open') state of the $\beta 2$ -integrin. The high-affinity conformation is induced and stabilized by separation of the two subunits.



In experimental studies, neutrophils are the first inflammatory cells that appear in intimal lesions in animals. The importance of neutrophil activation in vascular disease was also supported by a study in mice, which showed that blocking neutrophil-platelet interactions resulted in significantly decreased leukocyte accumulation and reduced neointima formation after arterial injury (98). In human atherosclerosis, neutrophil activation has been mainly associated with plaque rupture and ACS. Neutrophil infiltrations have been demonstrated in atherectomy specimens from unstable angina patients and in culprit lesions obtained at autopsy from patients with acute myocardial infarction (99). Moreover, neutrophil activation in peripheral blood, as assessed by CD11b up-regulation, neutrophil-platelet aggregates and elastase release, is a well-known characteristic of ACS patients (100-102). Although the numbers of circulating neutrophils in clinically stable patients correlate with angiographic stenosis complexity (103), there has been no consistent evidence for an enhanced neutrophil function in stable CAD. One early study showed increased neutrophil chemotactic activity

and LTB₄ generation in patients with stable angina (104) while others have reported that neutrophils in patients with established CAD or in individuals at high risk for vascular events possess a “primed” character, *i.e.* an increased functional potential *ex vivo* compared to neutrophils from healthy individuals (105,106). However, the neutrophil expression of β 2-integrins has been shown to be consistently similar in patients with stable CAD and in healthy controls (107). One recent study even demonstrated that neutrophils in stable CAD patients had a reduced capacity to up-regulate CD11b and to produce hydrogen peroxide following *in vitro* stimulation (108).

1.1.4 Matrix metalloproteinases

MMPs constitute a family of closely related zinc-containing proteases that together have the capacity to degrade all components of the extracellular matrix (109, 110). By now, more than 20 members have been identified in humans, as shown in Table 2 (111). On the basis of substrate specificity and primary structure, they can be divided into groups including collagenases, gelatinases (MMP-2, -9), stromelysins (e.g. MMP-3), matrilysins (e.g. MMP-7), elastase, membrane-type MMPs and “other MMPs”. Fully activated MMPs can be inhibited by interaction with naturally occurring specific tissue inhibitors (TIMPs) (112). At present, the TIMP family consists of four structurally related members, TIMP-1, -2, -3, and -4. The TIMPs bind non-covalently to active MMPs in a 1:1 molecular ratio. Among the TIMPs, TIMP-1 has been shown to potentially inhibit the activity of most MMPs. The induction of MMPs at the transcriptional level is mediated by a variety of cytokines such as IL-1, TNF- α and IL-6 (113-116). On the other hand, other cytokines like IL-4 and IL-10 inhibit the synthesis of certain MMPs (117,118). All MMPs are expressed as inactive zymogens and requires proteinases such as plasmins, to be activated. The activity is then controlled by the TIMPs. Cytokines that have been reported to be involved in the induction of TIMPs are IL-10 and transforming growth factor- β (118-120).

Table 2. MMP family members and their substrates.

Group	Name	MMP	Substrate
Collagenases	Fibroblast	MMP-1	fibrillar collagen
	Neutrophil	MMP-8	”
	Collagenase-3	MMP-13	”
	Collagenase-4	MMP-18	”
Gelatinases	Gelatinase A	MMP-2	Gelatin, collagen IV, fibronectin, elastin, laminin
	Gelatinase B	MMP-9	Gelatin, elastin, fibronectin, vitronectin
stromelysins	Stromelysin 1	MMP-3	Gelatine, fibronectin, casein, laminin, elastin, MMP-2/TIMP-2
	Stromelysin 2	MMP-10	“
	Stromelysin 3	MMP-11	Fibronectin, laminin, gelatine, aggrecan
Matrilysins	Matrilysin 1	MMP-7	Fibronectin, vitronectin, laminin, gelatine, aggrecan
	Matrilysin 2	MMP-26	Collagen IV, gelatine, fibronectin, fibrin, fibrinogen, type 1 gelatine
Elastase	Metalloelastase	MMP-12	Elastin, gelatine, collagen IV, fibronectin, laminin, vitronectin, Proteoglycan
Membrane Type	MT1- MMP	MMP-14	Pro MMP-2, procollagenase 3
	MT2- MMP	MMP-15	Pro MMP-2
	MT3- MMP	MMP-16	“
	MT4 – MMP	MMP-17	Gelatine, TNF- α precursor, fibrin
	MT5- MMP	MMP-24	-
	MT6- MMP	MMP-25	Collagen IV, gelatine, laminin
Other MMPs	Enamelysin	MMP-19	Collagen IV, gelatine, laminin, nidogen, tenacin, fibronectin, aggrecan
		MMP-20	Amelogenin
		MMP-22	Synthetic MMP substrate
		MMP-23	
		MMP-27	
		MMP-28	

In normal arterial tissue, MMP-2, TIMP-1 and TIMP-2 are expressed by smooth muscle cells while other MMPs, mainly MMP-1, MMP-3 and MMP-9, have been localised to macrophages, smooth muscle cells and endothelium in atherosclerotic lesions (31, 32,121). MMP activity in the lesion is thus believed to play a crucial role in the weakening of the fibrous cap. Accordingly, several studies have reported elevated plasma levels of MMP-9 and TIMP-1 in patients with myocardial infarction and unstable angina (122-125). Elevated levels of MMP-9 and TIMP-1, though less pronounced than in ACS patients, have also been

reported in patients with stable CAD (124, 125). Furthermore, MMP-9 and TIMP-1 have both been identified as independent predictors of future cardiovascular events, supporting their potential role in plaque vulnerability (126-128). In contrast to MMP-9 and TIMP-1, other MMPs, like MMP-2 and MMP-3, show lower levels in the acute phase of myocardial infarction than during the stable phase (124, 129). It has therefore been hypothesised that some MMPs may promote plaque rupture while others could be protective. It is also widely discussed that disruptions in the balance of TIMPs and MMPs, e.g. increased MMP-9/TIMP-1 ratio, are implicated in atherosclerosis and other chronic inflammatory disorders (130-133).

1.2 The hypothalamic-pituitary-adrenal axis and glucocorticoid action.

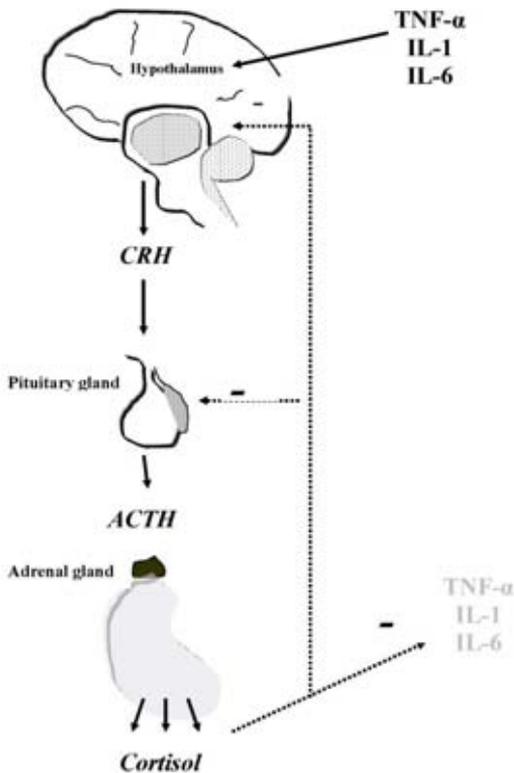
1.2.1 The hypothalamic-pituitary-adrenal axis.

The central nervous system regulates the immune system through two major mechanisms: a) the hormonal stress response and the production of glucocorticoids, and b) the autonomic nervous system with the release of noradrenalin. The central nervous system can also regulate the immune system locally via the peripheral nerves with release of neuropeptides such as substance P and locally produced corticotrophin-releasing hormone (CRH) (134, 135) The focus of this thesis will be the first mentioned mechanism regarding glucocorticoids.

The main regulator of the glucocorticoid effect on the immune system is the hypothalamic-pituitary-adrenal axis (HPA) axis (Figure 3) (136-140). It interacts with the immune system, sensing inflammatory signals and modulating the activity of this system primarily via its end product, glucocorticoids. Three cytokines – TNF- α , IL-1 and IL-6 – account for most of the HPA axis-stimulating activity in plasma. Systemic IL-6 concentrations also increase during stress unrelated to inflammation, presumably stimulated by catecholamines acting through β 2-adrenergic receptors. The first step in HPA axis activation is the release of CRH from intrahypothalamic neurons. CRH, travel from the hypothalamus via the hypophyseal-portal blood vessels to the anterior pituitary gland where it acts via specific receptors to trigger the release of the adrenocorticotrophic hormone (corticotrophin, ACTH) from specific ACTH-

producing cells into the systemic circulation. ACTH in turn acts on the adrenal cortex via melanocortin receptors to initiate the synthesis of cortisol, which is released immediately into the systemic circulation by diffusion. The magnitude of the HPA response to incoming stimuli is tempered by the glucocorticoids which act at the levels of the pituitary gland and hypothalamus to suppress the synthesis and release of ACTH and CRH. The molecular mechanisms by which the glucocorticoids exert their negative feedback effects are complex and include a) processes which lead to down regulation of the genes encoding ACTH and CRH and b) more immediate effects which suppress the release of stored hormones and thereby enable the axis to adapt rapidly to changes in circulating glucocorticoid levels.

Figure 3. Diagram showing the hypothalamo-pituitary-adrenocortical (HPA) axis and principal loci of glucocorticoid feedback control.



1.2.2 Glucocorticoids

The principal endogenous glucocorticoids are cortisol and corticosterone. Both steroids are produced by most mammalian species but the ratios in which they are secreted vary from species to species. Cortisol is the predominant glucocorticoid in man. It also constitutes the active form while cortisone is its inactive precursor. The glucocorticoids exert widespread actions in the body, which are essential for the maintenance of homeostasis and enable the organism to prepare for, respond to and cope with physical and emotional stress (141, 142). They promote the breakdown of carbohydrate and protein and exert complex effects on lipid deposition and breakdown. They are also important regulators of immune and inflammatory processes and are required for numerous processes associated with host defence. These properties underlie many of the stress-protective actions of the steroids as they quench the pathophysiological responses to tissue injury and inflammation and, thereby, prevent them proceeding to a point where they threaten the survival of the host.

Initially, glucocorticoids were thought to have mainly immunosuppressive effects. In 1948, it was shown for the first time that a synthesized version of cortisone was capable of reversing the inflammation of rheumatoid arthritis (143). However, it is important to recognize that glucocorticoids in pharmacological doses exert different effects than they do under physiological conditions (141, 144). Pharmacological doses (higher concentrations than physiological) are immunosuppressive at virtually every level of immune and inflammatory responses, whereas physiological levels of glucocorticoids are immunomodulatory rather than solely immunosuppressive. Their role in immunosuppression is mainly exerted through the suppression of nuclear factor (NF) κ B, which is a major factor involved in the regulation of cytokines and other immune responses (145). The expression of cytokines like IL-1, IL-6, IFN- γ and TNF- α , is down-regulated. The net effect of glucocorticoids is a shift of cytokine production from a primarily pro-inflammatory to an anti-inflammatory pattern, roughly corresponding to Th1 and Th2, respectively. The shift of Th1 to Th2 is considered to be due mainly to down-regulation of Th1 cytokines, thus allowing dominant expression of the Th2 cytokines (138, 139).

The transcriptional actions of glucocorticoids are mediated by supposed diffusion of the steroid hormone across the cell membrane and its binding to intracellular glucocorticoid receptors (GRs) (144, 146). The interaction of the steroid with its receptor forms a receptor-ligand complex and triggers the translocation of the receptor to the nucleus, where it binds to

a hormone response element and regulates gene transcription. Two human isoforms of the GR have been identified, termed GR- α and GR- β , which originate from the same gene by alternative splicing of the GR primary transcript (147-149). GR- α is the predominant isoform of the receptor and the one that shows steroid binding activity. In contrast, GR- β does not either bind glucocorticoids or transactivate target genes. The possible physiological role of GR- β is currently a matter for debate. In cotransfection studies, it has been shown that, when GR- β is more abundant than GR- α , GR- β acts as a dominant negative inhibitor of GR- α activity. However, other investigators found no evidence for a specific dominant negative effect of GR- β on GR- α activity. Instead, it has been argued that the ability of GR- β to regulate GR- α activity *in vivo* would depend on its expression level relative to that of GR- α . Increased expression of GR- β has been associated with glucocorticoid resistance (148,150).

Measurements of cortisol in the circulation provide a reasonable index of the activity of the HPA axis, although they poorly reflect the delivery of the steroids to receptors in their target cells. Most of the cortisol in the circulation is bound to a carrier protein and, in principle, only the free steroid has ready access to target cells. Cortisol shows a robust diurnal pattern in healthy adults with the strongest secretory activity of the adrenal cortex during the early morning hours. Peak cortisol levels are observed shortly after awakening with steadily decreasing values thereafter, except for sizable, short-term increases in response to stimuli like lunch meal, exercise or threat-provoking stressors. The nadir of cortisol secretion is reached around 2 or 3 AM with only minimal levels of the steroid detectable (134, 135, 151).

1.2.3 Annexin-1

Annexin-1 (ANXA1), previously referred to as lipocortin, is an important mediator of the anti-inflammatory actions of glucocorticoids (152). It is expressed in peripheral blood leukocytes, particularly in cells of the innate immune system such as neutrophils and monocytes. A large number of experimental studies including ANXA null mice have emphasized the role of ANXA1 as an endogenous down regulator of innate immunity (153-156). ANXA1 is mainly localised within the cytosol, but upon cell activation, it becomes rapidly mobilised to the cell surface where it acts in an autocrine/paracrine fashion by direct binding to a member of the formyl peptide receptor family, called FPRL-1 (157, 158). The

mechanisms by which ANXA1 exerts its anti-inflammatory effects are, however, complex and involve the suppression of various proinflammatory genes, e.g. IL-1 and IL-6, and the blockage of eicosanoid [157]. In macrophages, it has been shown to stimulate the release of IL-10 (159). The pharmacological effects of ANXA1 on neutrophils are probably the best characterized, including inhibition of migration, L-selectin shedding, suppression of enzyme release, and proapoptotic effects (160-162).

Exogenous glucocorticoids have been shown to induce ANXA1 production by peripheral blood mononuclear cells in vivo in man (163). In addition, a recent study demonstrated that ANXA1 expression in neutrophils was strongly correlated with the serum cortisol production, proposing a role for ANXA1 in mediating the anti-inflammatory effects of endogenous glucocorticoids (164). Based on these results, it was even suggested that ANXA1 expression in neutrophils might serve as an index of tissue sensitivity to endogenous glucocorticoids.

1.3 Disturbances in the interaction between the HPA axis and immune-mediated inflammation.

1.3.1 Defects of the HPA axis.

Chronic activation of the HPA axis or chronic inflammation results in reciprocally protective adaptations. For instance, immune suppression in Cushing's syndrome is mild, suggesting the development of tolerance to glucocorticoids. On the other hand, animals with chronic inflammatory disease have mild rather than severe hypercortisolism (165). However, disturbances at any level of the HPA axis or glucocorticoid action may lead to an imbalance of the system and enhanced susceptibility to infection and inflammatory/autoimmune diseases. Indeed, the association between a blunted HPA axis and susceptibility to autoimmune/inflammatory disease has been clearly shown in many animal models, e.g. when comparing two highly inbred rat strains, Fischer rats and Lewis rats (166-168). The Lewis rats

are highly susceptible to a wide variety of autoimmune/inflammatory diseases, while Fischer rats are resistant to these diseases. The Lewis rats exhibit a blunted HPA axis response, compared to Fischer rats with an excessive HPA response compared to outbred rats. In Lewis rats treated with low-dose dexamethasone or transplanted intracerebroventricularly with fetal hypothalamic tissue from Fischer rats, the autoimmune disease was markedly attenuated (169).

The abnormalities in Lewis rats may also have parallels in humans. A blunted HPA axis response has been shown in autoimmune diseases, in particular rheumatoid arthritis. Although basal morning cortisol levels did not differ, patients with rheumatoid arthritis showed a lower cortisol response after insulin-induced hypoglycaemia compared to healthy subjects (170). In another study, patients with rheumatoid arthritis showed a failure to increase cortisol secretion following surgery, despite high levels of IL-1 β and IL-6, compared to subjects with chronic osteomyelitis (171). Furthermore, studying the 24-h diurnal secretion of IL-6 and HPA axis hormones in early untreated rheumatoid arthritis, showed a positive temporal correlation between plasma levels of IL-6 and ACTH/cortisol (172). Based on the latter data, authors concluded that the overall activity of HPA axis remained normal and was clearly insufficient to inhibit ongoing inflammation in these patients. Interestingly, it was recently shown that an acute psychological stress test induced an increase in CRP in patients with rheumatoid arthritis, but not in patients with chronic osteomyelitis (173). A hypoactive HPA axis has also been demonstrated in patients with Sjögren's syndrome as they exhibit a blunted ACTH and cortisol response to CRH stimulation (174). In patients with atopic dermatitis and systemic lupus erythematosus, the basal morning cortisol levels are not different compared to controls. However, the ACTH and cortisol responses to acute psychological stress or insulin-induced hypoglycaemia are significantly lower in these patients compared to healthy subjects (175, 176).

1.3.2 Defects of the glucocorticoid target tissue.

Excessive immune-mediated inflammation may also arise from glucocorticoid resistance in target tissue. For instance, despite increased susceptibility to autoimmune/inflammatory

diseases, elderly people do not show an overall loss in HPA function (177, 178). However, using an in vitro assay of dexamethasone inhibition of lipopolysaccharide-induced cytokine production in whole blood, the glucocorticoid sensitivity has shown age-related changes, which may contribute to diseases in the elderly (179). In addition, by using the same assay, glucocorticoid resistance has been demonstrated in patients with autoimmune diseases (180).

The sensitivity of a cell to glucocorticoids is closely correlated with the number of GRs. A number of studies using different human cell lines have shown that cytokines like IL-1, IL-6 and TNF- α , increase the expression of GRs and thereby increase the glucocorticoid sensitivity of the respective cell. Early studies have shown that the concentration of GRs in circulating leukocytes in rheumatoid arthritis is reduced by approximately 50% (181). However, variations in the levels of GR α and GR β could also be associated with glucocorticoid resistance, both initial and acquired. Congenital forms of glucocorticoid resistance have been described. For example, a polymorphism of the human GR β gene increases the stability of GR β mRNA and is associated with rheumatoid arthritis (182). In addition, treatment with TNF- α or IL-1 in vitro has been shown to influence the balance between GR α and GR β by inducing a relative over-expression of GR β . This increase in GR β expression correlated with the development of glucocorticoid resistance (183). Lately, a number of studies have demonstrated enhanced GR β expression in leukocytes from patients with glucocorticoid-resistant rheumatoid arthritis (184), glucocorticoid-resistant asthma (185) and glucocorticoid-resistant ulcerative colitis (186) compared to patients with glucocorticoid-sensitive disease.

1.3.3 The cortisol response in coronary artery disease.

In a cross-sectional epidemiological study, six salivary cortisol samples, from awakening to bedtime, were collected from middle-aged adults on a single day. Results showed that the flatter the cortisol slopes throughout the day, the greater the likelihood of any coronary calcification (187). In another population-based study of middle-aged men, the established risk factors for cardiovascular disease were tightly associated with a pathological HPA axis, characterized by low variability, a poor lunch-induced cortisol response and a blunted dexamethasone suppression of cortisol (188). Clinical studies of cortisol levels in patients

with cardiovascular disease are, however, few and rely solely on one measure of plasma cortisol. Cortisol, determined in plasma collected between 8:00 and 9:00 PM in a fasting state, was independently related to significant coronary stenosis in middle-aged women with a prior history of ACS (189). In another clinical study, serum cortisol and IL-6 were measured in blood samples taken between 9:00 and 12:00 AM in patients with stable or unstable conditions of CAD. The cortisol levels were found to be 'inappropriately' normal in patients with high IL-6 levels and gave rise to speculations that the endogenous cortisol production was insufficient to limit inflammation in these patients (190).

2 Aims

The overall aim of this thesis was to study the systemic inflammatory activity in patients with stable and unstable conditions of CAD. A particular aim was to evaluate the cortisol secretion pattern and its relation to inflammatory activity. More specifically, the aims were:

- to study the systemic inflammatory profile in patients with stable and unstable CAD and relate the findings to peripheral immune cell populations including neutrophil-platelet aggregates.

- to study the relation between systemic inflammatory activity and diurnal cortisol secretion in stable CAD patients during basal conditions and acute stress.

- to study the MMP profile in patients with myocardial infarction, its change over 3 months and relation to diurnal cortisol secretion.

- to evaluate the neutrophil activation status in patients with stable CAD.

- to study the neutrophil expression of GRs and ANXA1 as well as the neutrophil response to exogenous ANXA1 in stable CAD patients and relate the findings to diurnal cortisol secretion.

3 Methodological considerations

3.1 Study populations

Study participants were recruited in three separate projects, termed OXIMMUN (Oxidation and Immune cells), MIMMI (Mental – Immune Interactions in Myocardial Infarction) I and MIMMI II. The three different patient and control populations are described below. Their clinical and laboratory characteristics are given in Table 3 and 4.

3.1.1 Patients

In OXIMMUN (paper I), 65 patients with angiographically verified CAD (45 with stable angina, 20 with unstable angina/non-ST-elevation myocardial infarction) were included at Höglandssjukhuset in Eksjö between November 2000 and November 2002. The diagnosis of stable angina was defined as effort-related angina of Canadian Cardiovascular Society functional class I and II without any worsening of symptoms the latest 6 months. Among the ACS patients in OXIMMUN, 13 had unstable angina and 7 had myocardial infarction.

In MIMMI I (paper II and III), 30 patients with a first-time myocardial infarction were consecutively included at Höglandssjukhuset in Eksjö between November 2000 and October 2002. Eleven had non-ST-elevation and 19 had ST-elevation myocardial infarction. All patients, in the first group, underwent revascularization therapy (two coronary artery bypass grafting) within one week, but mostly within 1-3 days. Eight patients, in the latter group, received treatment with thrombolytic therapy and 11 underwent primary percutaneous coronary intervention. All patients were assessed at day 1-3, 2 weeks and approximately 3 months after the index cardiac event. At 3 months they were all in a clinically stable metabolic condition without any evidence of infectious or inflammatory disorder. Two patients had non-insulin-treated type II diabetes.

Table 3. Clinical and laboratory characteristics of patients included in OXIMMUN, MIMMI I and MIMMI II, respectively.

VARIABLE	<u>OXIMMUN</u>		<u>MIMMI I</u>	<u>MIMMI II</u>
	Stable (n = 45)	Unstable (n = 20)	(n = 30)	(n = 30)
Age (yrs)	57 (5)	56 (7)	60 (6)	63 (5)
Male/female	45/0	20/0	25/5	25/5
Body mass index (kg/m ²)	28 (3)	26 (4)	27 (3)	27 (4)
Blood pressure (mmHg)				
Systolic	140 (18)	131 (24)	148 (20)	135 (18)
Diastolic	83 (9)	80 (13)	83 (10)	84 (10)
Smokers' n (%)	14 (31)	12 (60)	7 (24)	4 (13)
Medication n (%)				
Beta blockers	37 (82)	18 (90)	27 (90)	28 (93)
Low-dose aspirin	40 (90)	20 (100)	28 (93)	30 (100)
Statin	36 (80)	13 (65)	22 (73)	30 (100)
ACE-I/ARB ¹⁾	7 (16)	5 (25)	15 (48)	16 (52)
Clopidogrel	0	20 (100)	1 (3)	10 (33)
Lipids¹⁾, mmol/l				
Total cholesterol	5.1 (0.9)	5.4 (1.1)	4.4 (0.8)	4.5 (1.1)
LDL-cholesterol	2.9 (0.7)	3.2 (0.9)	2.4 (0.6)	2.4 (0.9)
HDL-cholesterol	1.3 (0.3)	1.1 (0.2)	1.3 (0.3)	1.4 (0.4)
Triglycerides	2.0 (1.0)	2.8 (1.6)	1.7 (0.7)	1.6 (0.7)

1) LDL, Low density lipoprotein; HDL, High density lipoprotein

Table 4. Clinical and laboratory characteristics of control subjects included in OXIMMUN, MIMMI I and MIMMI II, respectively.

<i>PROJECT</i>	<u>OXIMMUN</u>	<u>MIMMI I</u>	<u>MIMMI II</u>
<i>VARIABLE</i>	(n = 45)	(n = 30)	(n = 30)
Age (yrs)	49 (6)	61 (7)	63 (5)
Male/female	45/0	25/5	25/5
Body mass index (kg/m ²)	25 (3)	27 (3)	26 (4)
Blood pressure (mmHg)			
Systolic	125 (18)	143 (24)	143 (17)
Diastolic	80 (9)	88 (13)	85 (11)
Smokers' n (%)	7 (16)	9 (31)	4 (13)
Medication n (%)			
Beta blockers	-	5 (17)	3 (10)
Low-dose aspirin	-	0	1 (3)
Statin	-	3 (10)	3 (10)
ACE-I/ARB ¹⁾	-	3 (10)	2 (7)
Clopidogrel	-	0	1 (3)
Lipids¹⁾, mmol/l			
Total cholesterol	5.7 (0.9)	5.9 (0.9)	5.5 (0.9)
LDL-cholesterol	3.9 (2.8)	3.8 (0.9)	3.0 (0.8)
HDL-cholesterol	1.5 (0.4)	1.4 (0.3)	1.7 (0.5)
Triglycerides	1.5 (1.0)	1.7 (0.9)	1.6 (0.8)

¹⁾ LDL, Low density lipoprotein; HDL, High density lipoprotein

In MIMMI II (paper IV and V), 30 patients with angiographically verified stable CAD were recruited at the Department of Cardiology, Linköping University Hospital from November 2005 to April 2006. The patients had effort-related angina in accordance with the Canadian Cardiovascular Society functional classes I and II without any worsening of symptoms the latest 6 months. None of the patients had type II diabetes.

Exclusion criteria in OXIMMUN, MIMMI I and MIMMI II were age > 70 years, severe heart failure, chronic inflammatory/immunologic disorder including type I diabetes, neoplasm disease, evidence of acute or recent (<2 months) infection, recent major trauma, surgery or revascularization procedure, drug/alcohol abuse, poor mental function or continuous treatment with immunosuppressive/anti-inflammatory agents (except low-dose aspirin). In OXIMMUN, the exclusion criteria differed to some extent in an attempt to apply a study population as homogenous as possible. The OXIMMUN exclusion criteria included age > 65 years, female gender and both type I and II diabetes. Only middle-aged men were enrolled in order to avoid the influence of age- and gender-related immune differences.

Blood samples were always obtained by venal puncture in the morning after a 12-h fast. In patients with ACS, blood samples were always drawn before coronary intervention.

3.1.2 Controls

In OXIMMUN, 45 men were recruited from the health care staff at Högländssjukhuset, Eksjö. In MIMMI I and II, respectively, 30 men and women of equivalent age (+/- 5 years) were randomly selected from a population register representing the hospital recruitment area. The control subjects were all self-reported healthy, presently and anamnestically. In MIMMI I and II, a few controls received treatment with anti-hypertensive drugs and statins for the primary prevention of cardiovascular disease.

3.2 Serological assays

3.2.1. CRP and cytokines

Serum or plasma samples were assayed for CRP by a highly sensitive, latex-enhanced turbidimetric immunoassay with a lower detection limit of 0.03 mg/l (Roche Diagnostic GmbH, Vienna, Austria). The interassay coefficient of variation was 1.7 % for CRP. Serum samples were assayed for IL-6, IL-1Ra, and IL-10 using commercially available, high-sensitivity enzyme-linked immunosorbent assays according to the manufacturer's recommendations (Quantikine HS, R&D Systems Europe Ltd, Abingdon, Oxon, United Kingdom). Lower limits of detection for IL-6, IL-1Ra and IL-10 were 0.04 pg/ml, 22 pg/ml and 0.5 pg/ml, respectively. The interassay coefficient of variation was 8.5% for IL-6. Serum IL-2R and plasma IL-18 were measured by sandwich enzyme immunoassay (Bio Source Europe S.A., Nivelles, Belgium and Medical & Biologic Laboratories CO. Ltd., Nagoya Japan, respectively). Lower limits of detection were 10 pg/ml for IL-2R and 12.5 pg/ml for IL-18.

3.2.2. MMPs and TIMP-1

In paper III, serum samples were assayed for MMP-2, MMP-3, MMP-7, MMP-9 and TIMP-1 using a highly sensitive ELISA immunoassay (Quantikine HS, R&D Systems Europe Ltd, Abingdon, Oxon, United Kingdom). These assays measure the total levels of MMP, i.e. the proform, active form and MMP bound to TIMP. The lower limits of detection for TIMP-1, MMP-3, MMP-7, MMP-9 and MMP-2 were 0.48 ng/ml, 0.95 ng/ml, 1.06 ng/ml, 1.87 ng/ml and 6.28 ng/ml respectively. The interassay coefficients of variation for the assays were < 5 % for MMP-3, MMP-7, MMP-9, TIMP-1 and < 10 % for MMP-2. It has been argued that serum samples are probably not appropriate to assess MMP levels because release of MMPs by platelets or leukocytes may occur during the sampling process leading to artificially high levels compared with plasma. However, a recent study comparing different time intervals between blood drawing and centrifugation found no time-dependent effects on MMP-9 and MMP-2 levels, at least within a 30 min period (191). In the MIMMI I study, the time interval

between blood drawing and centrifugation never exceeded 15 minutes. The study by Gerlach RF *et al* (191) also showed that MMP-9 and MMP-2 levels were significantly correlated in serum and plasma samples. The authors therefore suggest that using serum or plasma may have no major consequences in the comparative evaluation of these MMPs in blood samples drawn during a particular study, as long as only serum or plasma is consistently used throughout the study.

3.3 Cortisol assays

A 24-hour urine test was performed in order to reflect the cortisol secretion throughout an entire day (paper II and III). The measurement of 24-hour urinary free cortisol is not influenced by serum binding protein levels and is not subject to the diurnal variation seen with serum or salivary cortisol measurements. Free cortisol in saliva has been shown to have the same diurnal rhythm as serum cortisol. Furthermore, the transfer of cortisol from blood to saliva has been shown to be rapid with a reflection in saliva of a cortisol increase in blood within 60 seconds and a state of equilibrium within five minutes (192, 193). Due to several advantages over blood cortisol analyses such as stress-free sampling and laboratory independence, the determination of cortisol in saliva has become the method of choice in basic research and clinical environments. Cortisol measures over 2 – 6 days are considered necessary to achieve reliable trait measures, since state factors may bias data from a single day (194). In order to minimise the influence of day-to-day variations, the participants of MIMMI I and MIMMI II were thus instructed to collect saliva on 3 consecutive days which were not the day after a Sunday or holiday, or the day before the weekend; i.e. sampling typically started on a Tuesday and ended on a Thursday. The first sample was taken 30 minutes after awakening and the second sample in the evening before going to bed. Careful oral and written instructions were provided to avoid misunderstanding, e.g. prior to saliva sampling, the participants were instructed to avoid physical exercise, food intake and tobacco use for at least 60 minutes. Saliva was collected with Salivette cotton swabs (Sarstedt, Nümbrecht, Germany) that were placed under the tongue for 2 minutes. The salivettes were stored at -20°C until analysis (paper II, III and V).

The free cortisol levels in urine and saliva were determined by a modified commercial radioimmunoassay (Diagnostic Products Corporation, Los Angeles, US). According to repeatedly performed quality assessments, the interassay coefficient was < 10 %.

3.4 Flow cytometry

Using flow cytometry, leukocyte cell surface molecules, as well as intracellular proteins, were detected using fluorescence-conjugated antibodies. The proteins were immunolabeled in whole blood, whereafter contaminating erythrocytes were removed by cell lysis, and the cells were finally fixed in cold paraformaldehyde (0.1-1%). The cell populations were identified using a Becton Dickinson FACSCalibur (Becton Dickinson). Data were analyzed with Cell Quest software (Becton Dickinson). Instrumental setups, adjustments, viability controls, and checking of unspecific binding were performed as described in paper I, IV and V.

Some leukocyte cell surface molecules are named systematically by assigning them a cluster of differentiation (CD) antigen number that includes any antibody having an identical and unique reactivity pattern with different leukocyte populations. The anti-CD monoclonal antibodies that were used in this thesis are specified in Table 5.

3.4.1 Mononuclear cells

In paper I, the distribution and activation status of mononuclear cells were measured by using the following combinations of monoclonal antibodies in whole blood samples: CD3+CD4+CD8⁻ (T-helper cells), CD3+CD4⁻CD8⁺ (cytotoxic T cells), CD3+CD4+CD25⁺ (activated T helper cells) and CD3+CD8+CD25⁺ (activated cytotoxic T cells), CD19⁺ (B cells) and CD14⁺ (monocytes). The antibodies were then marked with one of three fluorochromes: fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein.

Table 5. CD molecules identified by monoclonal antibodies in paper I, IV and V.

CD antigen	Alternate name	Function	Cellular expression in blood
CD3		Signalling component of T cells, part of a bigger complex which includes the T cell receptor.	All T cells
CD4		Co-receptor for MHC class II molecules.	T helper cells, monocytes (weak)
CD8		Co-receptor for MHC class I molecules.	Cytotoxic T cells, subset of NK cells
CD14		High-affinity receptor for lipopolysaccharide.	Monocytes
CD19	B cell surface antigen B4	Involved in the regulation of B cells. Forms complex with complement receptor 2.	B cells
CD25	IL-2R α -chain	In conjunction with IL-2R β - and IL-2R γ , the CD25 antigen forms the high-affinity IL-2R complex.	Activated T and B cells.
CD45	Leukocyte common antigen	Tyrosine phosphate, mediating signalling through B and T cell receptors	All differentiated haematopoietic cells except erythrocytes and plasma cells
CD62L	L-selectin, LAM1	Mediates rolling interactions between leukocytes and endothelium	B cells, T cells, monocytes, NK cells and granulocytes
CD89	Fc α R	Neutrophil Fc receptor for IgA	Neutrophils, monocytes
CD11b	Mac-1 α chain, complement receptor 3	Subunit of β 2-integrin, associated with CD18.	Granulocytes, monocytes, NK cells, subsets of T and B cells
CD11b I-domain		Conformation to the high-affinity state of β 2-integrin	As above (activated)
CD18	Mac-1 β chain	The second subunit of the β 2-integrin, associated with CD11b	Granulocytes, monocytes, NK cells, subsets of T and B cells
CD41a	Glycoprotein IIb	Part of the glycoproteinIIb/IIIa complex which is the soluble receptor for fibrinogen	Platelets

3.4.2 Neutrophil-platelet aggregates

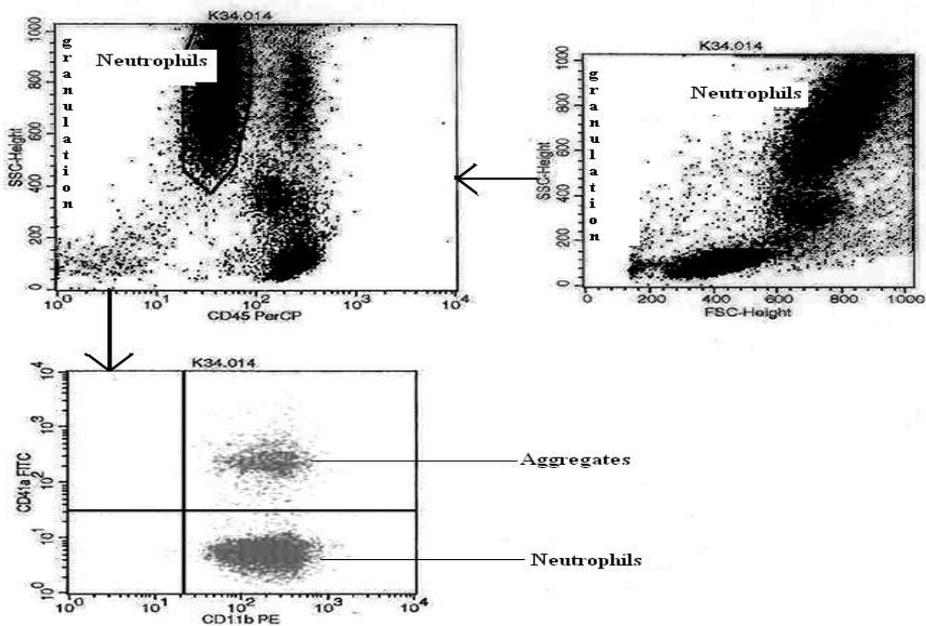
In paper I, the neutrophils were identified in whole blood as CD62L+CD89+CD14- cells. The number of neutrophil-platelet aggregates was determined by using the combination CD41a+CD11b+CD45+ (Figure 4). As a final step, the antibodies were marked with one of the three fluorochromes described above.

3.4.3 Surface-expression of proteins on neutrophils

The surface expression of the β 2-integrin, CD11b/CD18 was measured using an anti-CD11b antibody followed by a secondary -conjugated antibody (paper I) or detected by a FITC-

conjugated anti-CD18 antibodies (paper IV and V). In order to study the high affinity state of the β 2-integrins, a FITC-conjugated anti-CD11 antibody recognizing the ligand binding domain, i.e. the “I domain” (94) was used (paper V). The surface expression of the receptor for ANXA1 was determined by using a polyclonal anti-FPRL-1 antibody followed by a FITC-conjugated F(ab')₂. The antigen surface expression of the different proteins on neutrophils was quantified by measuring mean fluorescence intensity (MFI).

Figure 4. A representative FACS plot showing the definition of neutrophil-platelet aggregates.



3.4.4 Expression of intracellular proteins in neutrophils, i.e. GRs and ANXA1

Leukocytes were harvested from whole blood after red cell lysis. For the detection of GRs, the cells were fixed in cold paraformaldehyde (2%). To get access to the intracellular proteins, the cells were permeabilised with 0.02% saponin (all following steps were performed in the presence of 0.02% saponin). The cells were then incubated with antibodies directed against ANXA1 (157) or the GRs (GR- α , GR- β , GR-total) followed by incubation with a FITC-conjugated F(ab')₂.

3.5 Stimulatory assays of neutrophils

Pro-inflammatory mediators that induce receptor-mediated neutrophil activation both in vivo and in vitro include the cytokine IL-8 and the lipid-derived mediator LTB₄. The latter is formed within the neutrophils after stimulation, whereas IL-8 is mainly produced by monocytes and macrophages. In vitro studies have shown that IL-8 also activates neutrophil arachidonate-5-lipoxygenase to release LTB₄ (195). IL-8 and LTB₄ each bind and activate specific receptors belonging to a small subfamily within the 7-transmembrane spanning, G-protein-coupled receptor family. They are both characterized as intermediary chemoattractants (196), i.e. generate weaker/less potent neutrophil activation. Still, IL-8 is a highly selective neutrophil chemoattractant and activator, and LTB₄ causes e.g. adhesion and chemotactic movement of leukocytes (197).

Serum opsonized yeast particles (prepared as previously described (198) were used to test the adhesive property and/or signalling capacity of CD11b/CD18 (also known as complement receptor 3). Determining the ROS production was used as a read-out for these experiments (paper IV).

PMA (phorbol 12-myristate 13-acetate), which bypasses receptor activation and directly activates protein kinase C, was used to examine the maximal non-receptor-mediated ROS production elicited in the cells (paper IV).

Whole blood was stimulated with IL-8 (paper IV) or LTB₄ (paper IV and V) before the amount of total β 2-integrins or of β 2-integrins displaying the high-affinity epitope was evaluated by flow cytometry (section 3.4.3). The effects of IL-8 (paper IV), LTB₄ (paper IV and V), C3bi-opsonised yeast (paper IV), or PMA (paper IV) on the production of ROS was determined in isolated neutrophils using chemiluminescence technique (section 3.6).

To study the anti-inflammatory effect of ANXA1, exogenous ANXA1 (158) was added in some of the experiments prior to stimulation with the pro-inflammatory chemoattractants (paper V).

3.6 ROS production

To measure production of reactive oxygen species (ROS), neutrophils were isolated from whole blood by density gradient centrifugation (199). The ROS production was determined using luminol-amplified chemiluminescence technique (200), and the measurements were performed in a six-channel Biolumat LB9505 (Berthold Co., Wildbad, Germany) (199,201). The amplifying molecule luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) is excited when it reacts with the generated oxygen species and emits light upon relaxation to the ground state. Since luminol can cross biological membranes, both the intracellular and extracellular ROS production can be measured (202). To measure extracellularly synthesized ROS, HRP (horseradish peroxidase) is added to substitute the endogenous intracellularly located peroxidase MPO (myeloperoxidase). To measure only the intracellular production, large molecular scavengers like superoxide dismutase (SOD) and catalase are added to remove ROS that are released from the neutrophils. The extracellular ROS production can be measured exclusively if luminol is exchanged for the very similar, but membrane impermeable molecule, isoluminol (203).

Light emission, reflecting ROS interacting with luminol/isoluminol was continuously monitored. The peak value was registered for each sample, and expressed as counts per minute (cpm).

3.7 Stress tests in patients and controls

In paper II, the patients (at 3 months after the cardiac index event) and controls underwent two standardised stress tests; one physical and one psychological test. The time interval between the tests was 10-14 days. In both cases, the participants came to the hospital in a fasting state. Prescribed drugs were taken as usual. The stress tests always started at 7:30 AM. The participants first lay down on a bed reclining comfortably. Baseline blood pressure and heart rate were recorded. Saliva samples for baseline cortisol were taken before initiating

venepuncture to avoid a possible stress effect. Blood samples were always collected at baseline and after 24 hours.

3.7.1 Physical stress test

The test was performed on an electrically braked bicycle (Rodby 990, Rodby Innovation AB, Hagby, Sweden) stepwise increasing the workload by 10 watt/min. from the starting load of 30 watt. A continuous signal averaged 12-lead electrocardiogram was recorded before, during, and until 4 min. after exercise. Cuff brachial artery systolic pressure was recorded at rest, at the end of each workload and until 10 min. after exercise. Anginal chest pain, leg fatigue and feeling of general exhaustion were graded at the end of each workload applying the 10-graded Borg scale (204). Exercise-induced ST-depressions were defined as pathological indicating myocardial ischemia if they were ≥ 1 mm ST horizontal or down-sloping at a time of 60ms after the J point and were noted in at least 2 adjacent electrocardiographic leads. The exercise parameters that were measured included heart rate and systolic blood pressure at peak exercise and maximal relative predicted work capacity (watt %) = % achieved maximal work capacity (watt)/expected average maximal work capacity adjusted for gender, age and body weight. A saliva sample was obtained directly after maximal work capacity was achieved.

3.7.2 Psychological stress tests

Two kinds of psychological stressors were presented in a fixed sequence with 2 min. intervals in between, to allow time for instructions. The first stressor was the “anger recall” (205). The participant was instructed to recall an event that made him/her angry, frustrated, or upset and had 6 min. in which to relate what had happened and how he/she had felt. The second stressor was a mental arithmetic test (206). The participant was instructed to count backwards, 700 minus 7 as quickly and correctly as possible and to try to reach zero within 4 minutes. Blood pressure and heart rate were recorded at the end of each stressor and until 34 min. after the start of the first stressor. Saliva samples were obtained at 22, 26 and 34 min.

3.8 Statistics

Data were analyzed using various versions of the statistical program SPSS (version 11.5; 12.0; 15.0, SPSS, Inc, Chicago, Illinois, USA). Data are presented as mean (SD) or as median (inter-quartile range). In general, the Student's *t* test was used to compare differences in means between patients and controls (paper I, II and V). When data were skewed, the non-parametric Mann-Whitney U-test was used to evaluate continuous variables (paper III and IV). In paper I, subgroups were analyzed with one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test to evaluate the significance of any differences in means between subgroups.

Correlation analyses were performed using either Pearson's or Spearman's correlation coefficients. To assess the independent contribution and interaction of different factors, the linear multiple regressions were performed and standardized β was determined (paper II and III).

All flow cytometric data on neutrophils as well as data on neutrophil ROS production in paper IV and V were analyzed using Wilcoxon Signed rank test, thus comparing levels between a patient and his/her paired control.

Two-tailed *p* values < 0.05 were considered statistically significant.

4 Results and Discussion

4.1 Systemic inflammation in ACS patients

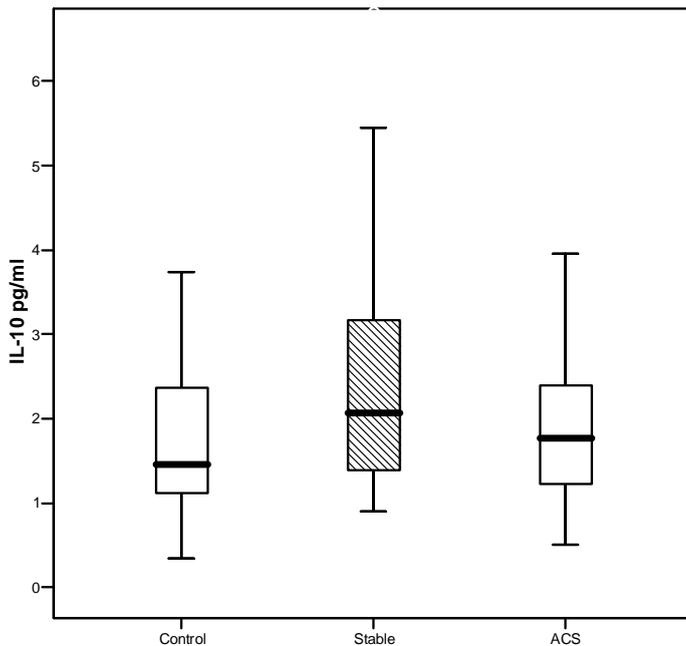
4.1.1 Inflammatory biomarkers and mononuclear cell subsets

A major, whilst not unexpected, finding associated with ACS was the marked increase in circulating markers of inflammation. As shown in paper I, the CRP levels were significantly higher in patients with ACS than in stable CAD patients and healthy controls. Similarly, the CRP levels were markedly increased in patients with a first-time myocardial infarction 1-3 days after admission compared with 2 weeks and 3 months follow-up (Paper III). The cause of CRP in patients with ACS has been a highly debated issue for several years. It has been postulated that an early CRP increase in ACS might reflect the exacerbation of inflammatory activity leading to plaque rupture or thrombus formation. Moreover, high CRP at admission increase the probability of having a new cardiac event in the follow-up, further strengthening the role of CRP as an indicator of plaque vulnerability (33, 34). However, it is also well-known that CRP undergoes a dynamic process in ACS patients and rise in parallel with markers of myocardial damage (207, 208). The strong influence of myocardial necrosis on CRP increase in ACS was also illustrated by our own results showing the highest CRP levels in unstable patients with troponin T release compared with unstable patients without troponin T release (data not shown).

Not only CRP but also IL-6 (paper I and III) and IL-1Ra (paper I) were significantly increased in ACS patients compared to stable CAD patients and controls. These data are in line with several previous studies showing increased levels of inflammatory cytokines in ACS. However, in contrast to earlier studies, we did not observe any increase in the IFN- γ - inducing cytokine IL-18 (paper I). Earlier studies have shown elevated levels of IL-18 in patients with both stable and unstable conditions of CAD (68-70). In addition, Mallat and coworkers (68) showed that IL-18 levels remained unchanged in ACS patients during 1-3 months follow-up, thus contradicting an association between circulating IL-18 levels and clinical instability.

In paper I, the IL-10 levels in ACS patients were similar when compared to healthy controls but tended to be lower when compared to patients with stable CAD patients (Figure 5). Among the cytokines, IL-10 differs from other proposed ACS biomarkers by its anti-inflammatory actions. Its anti-atherosclerotic effects have been demonstrated in experimental animal models (209, 210). Interestingly, one observational study reported that patients with unstable angina had significantly lower serum IL-10 concentrations at the time of admission to hospital than did patients with stable angina (80). A lack of IL-10 increase in ACS patients may thus reflect a failure to compensate inflammatory activity. However, this is highly speculative and single measurements of IL-10 in such a dynamic phase as ACS do probably not allow such an interpretation.

Figure 5. Serum levels of IL-10 in healthy controls, patients with stable CAD and patients with ACS. No significant differences were seen between the groups.



The total numbers of T helper cells, cytotoxic T cells, B cells or monocytes did not differ significantly in ACS patients compared to stable CAD patients or controls. The numbers of CD4+ cells expressing the IL-2R were significantly higher in ACS patients compared to healthy controls but did not differ from stable CAD patients. CD4+CD25+ T cells display a range of intensities of CD25 expression from intermediate to high levels. A small subpopulation of CD4+ cells (less than 5 %) exhibiting high CD25 intensity have been proposed to represent regulatory T cells, so-called T_{regs}.(211). T_{regs} protect the host from autoimmune disease by suppressing self-reactive cells and reduced numbers of T_{regs} have been shown in a variety of autoimmune diseases (212). Recently, it was demonstrated that patients with ACS exhibited significantly reduced numbers of peripheral T_{regs} as compared with patients with stable angina (213). However, in the OXIMMUN study, we did not make any attempt to differentiate between different CD4+CD25+ cell subsets but used CD25 as a general marker of recent activation. In addition, the soluble form of IL-2R in serum tended to be higher in the ACS patients. The data thus indicated the presence of systemic T cell activation in ACS, as has been previously shown by several others (23, 35, 37,214).

4.1.2 Neutrophils

The serum levels of CRP, IL-6 and IL-1Ra in ACS patients did not correlate to T cell activation markers but, instead, showed a strong correlation to neutrophil counts ($r = 0.64$, $p < 0.01$ between IL-6 and neutrophils). The number of neutrophils was also significantly higher in the ACS patients compared to patients with stable disease. However, an unexpected finding was that the number of circulating neutrophil-platelet aggregates tended to be lower in ACS patients compared to stable CAD patients. Even more, the neutrophil expression of CD41a was significantly lower in the ACS group compared to the stable group. The findings are in contrast to several previous findings of increased numbers of leukocyte-platelet aggregates, including monocyte-platelet and neutrophil-platelet aggregates, in ACS (100-102, 107). However, one potential explanation is the more extensive anti-thrombotic treatment of all ACS patients in the OXIMMUN study. All ACS patients received a combination with low-dose aspirin and clopidogrel. Clopidogrel, alone or in combination with aspirin, has been shown to be a potent inhibitor of neutrophil-platelet aggregate formation while monotherapy with aspirin is not (215, 216).

4.1.3 MMPs and TIMP-1

In the MIMMI I study (paper III), the course of MMP and TIMP-1 serum levels over time was studied in patients with a first-time myocardial infarction. In the acute phase of MI, the serum levels of MMP-9 and TIMP-1 were significantly increased compared to those 3 months later. Increased levels of MMP-9 and TIMP-1 have been associated with ACS in a number of previous studies (122-124) although a direct relationship between MMPs and actual plaque rupture has been less established. Much of the existing data implicating MMPs in plaque rupture has been obtained from patients undergoing carotid endarterectomy or coronary endarterectomy (31, 32, 113). Plaques, classified as symptomatic from clinical evidence, show a significantly increased expression of MMP-9 compared to asymptomatic plaques (217,218). In a recent study, the relation between plaque morphology as assessed by intravascular ultrasound before percutaneous coronary intervention and serum MMP levels was evaluated. Interestingly, MMP-9 was found to be an independent predictor of plaque rupture in ACS patients (125).

In contrast to MMP-9 and TIMP-1, the levels of MMP-2 and MMP-3 were significantly reduced in the acute phase compared to follow-ups at 2 weeks and 3 months. The expression of MMP-2 and MMP-3 has been found to be increased in atherosclerotic lesions (31, 219) while serological studies have yielded more conflicting results. One previous study showed significantly lower levels of MMP-2 in ACS patients (124) while others have shown increased levels of MMP-2 in ACS compared to stable disease (122, 220). On the other hand, serological data on MMP-3 are more consistent. The MMP-3 levels are decreased in patients with ACS compared to stable CAD patients (124,129) but increase during the recovery phase (129). Moreover, patients with premature CAD have decreased plasma levels of MMP-3 (221). Altogether, it may be speculated that decreased plasma levels of MMP-3, if accurately reflecting MMP-3 action in the arterial wall, indicate increased matrix degradation and enhanced risk of plaque rupture promoted by other MMPs.

There was no significant difference in MMP-7 levels between the acute phase and 3 months later (4.0 (3.1-4.7) vs. 3.9 (3.3-4.4) ng/ml). The MMP-7 levels in patients were slightly higher than in healthy controls (3.2 (2.9-4.2) ng/ml) although the difference did not reach statistical significance. In atherosclerotic plaques, MMP-7 has been shown to be prominently expressed

(222). Only one previous study has measured circulating levels of MMP-7 in CAD patients demonstrating that MMP-7 was significantly elevated in patients with both stable and unstable conditions compared to healthy subjects, thus suggesting MMP-7 as a marker of existing atherosclerotic disease (124).

MMP-9 and TIMP-1 were both strongly associated with systemic inflammation, as assessed by CRP and IL-6, whereas MMP-2, MMP-3 and MMP-7 did not show any correlation with these markers. This highlights the complexity of MMPs, indicating that their function and regulation are not obligatory linked to the inflammatory response.

4.2 Systemic inflammation in patients with stable CAD

4.2.1 Inflammatory biomarkers and mononuclear cell subsets

The studies of systemic inflammatory activity in CAD have mostly focused on the acute symptomatic coronary event while cross-sectional studies comparing stable and unstable CAD with healthy controls are few and have yielded inconsistent results. In the OXIMMUN study (paper I), patients with stable CAD had lower levels of CRP, IL-6 and IL-1Ra compared to ACS patients but their levels were significantly higher than in healthy controls. Based on these results, we hypothesised that a low-grade systemic inflammation was chronically present in patients with clinically stable disease. It is known that plaque rupture may be clinically silent (223,224). In a histopathological study of carotid plaques, unstable plaques with a high content of macrophages and T cells were associated with increased serum levels of CRP (225). Recently published studies have also shown that CRP and other serological markers of inflammation are correlated with angiographic characteristics of plaque instability (226) and positive coronary artery remodelling, estimated by intravascular ultrasound (227) in patients with stable symptoms. The source of CRP and cytokines may be the inflammatory sites of atherosclerotic lesions (228,229) although other origins like adipose tissue (230) and infectious/inflammatory sites in other organs such as joints and lungs must be taken into account.

However, in the following studies (MIMMI I and II), the CRP and IL-6 levels in stable CAD patients did not any longer differ from controls. Several randomised placebo-controlled trials have consistently shown that a rapid reduction of inflammatory markers, predominantly CRP, is achieved by administration of a statin (231-233). Earlier cross-sectional studies of CAD patients, subdividing the patients *a posteriori* with regard to statin treatment, have further illustrated the potent influence of statins on systemic inflammatory markers (37, 234). In vitro, statins have been shown to exert a direct inhibitory effect on IL-6-induced expression of CRP in human hepatocytes (235, 236). Other experimental studies have shown that statins suppress the production of inflammatory cytokines in various cell types, e.g. through the inhibition of NFκB (237, 238). In the OXIMMUN study, 80% of the patients received long-term treatment with statin compared to 96 % of the patients in MIMMI I and all patients in MIMMI II. Moreover, the statin doses administered in OXIMMUN were generally lower according to earlier guidelines. The patients in OXIMMUN were also less likely to be treated with angiotensin converting enzyme (ACE)-inhibitors than were patients in MIMMI I and II (16%, 48% and 52%, respectively). Angiotensin II has proinflammatory properties and a number of studies have shown that ACE-inhibitors and angiotensin II receptor blockers reduce CRP up to 30 %. However, there are also reports that claim no effect of certain ACE-inhibitors including enalapril, on CRP levels [reviewed in 239].

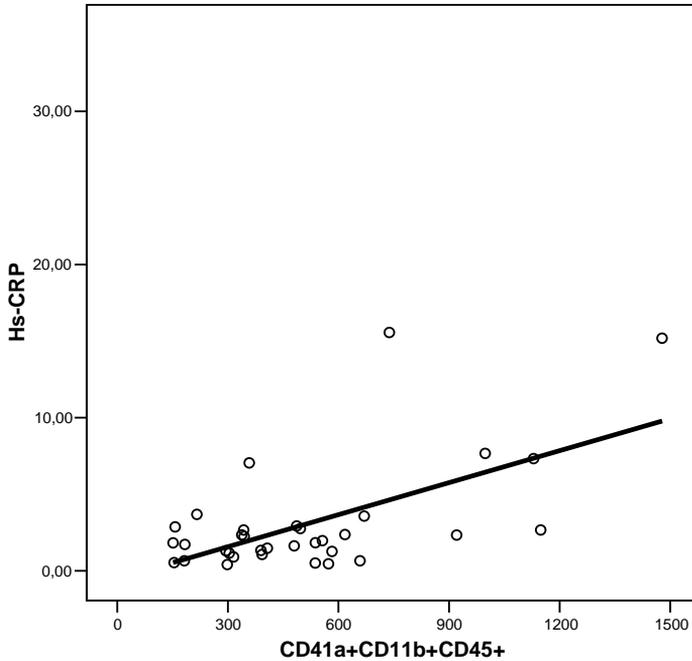
As shown in Figure 5, the median levels of IL-10 tended to be increased in stable CAD patients compared to both healthy controls and ACS patients. In view of the anti-inflammatory effects of IL-10, it may be interesting to speculate that patients with higher IL-10 values represent patients with a better prognosis.

The numbers of all T cells, B cells and monocytes did not differ between stable CAD patients and control subjects while the numbers of CD4+CD25+ T cells were found to be modestly increased, indicating the presence of chronic low-active T cell activation. In a previous study, a markedly increased systemic T cell activation was detected in stable CAD patients (37). In that study, a post-hoc analysis also showed that the T cell response was much more pronounced in patients without statin therapy compared to patients with long-term statin therapy.

4.2.2 Neutrophils

The patients (OXIMMUNE study) with stable CAD had significantly more neutrophils and neutrophil-platelet aggregates than healthy controls (paper I). Circulating neutrophil-platelet aggregates are sensitive markers of in vivo platelet activation but are also considered to be markers for neutrophil activation since neutrophil activation is associated with enhanced adhesion to fibrinogen and platelets (100). The correlation between CRP and neutrophil-platelet aggregates was even stronger in patients with stable CAD than in ACS patients ($r = 0.65$, $p < 0.001$, see Figure 6). However, the neutrophil expression of CD11b (paper I) or CD18 (paper IV) did not differ between stable CAD patients and controls contradicting an ongoing neutrophil activation in the patient group. Although an enhanced expression of CD11b/CD18 is a well-established activation marker of neutrophils, it has to be considered that the absolute numbers of $\beta 2$ -integrins does not necessarily reflect the neutrophil adhesive capacity. In MIMMI II (paper IV), we therefore compared the high-affinity state of neutrophil $\beta 2$ -integrins in patients with stable CAD and healthy control subjects. However, the neutrophil expression of high-affinity CD11b did not differ between patients and controls during basal conditions, neither did it differ after stimulation with IL-8 or LTB₄. A 'primed' state of circulating neutrophils has been suggested in patients with stable CAD (105,108). Our data did not verify that the neutrophils in stable CAD patients were more prone to activation than were cells in control subjects. In contrast, we obtained evidence for an impaired activation status in the patients, as assessed by a decreased ROS production in response to complement receptor-mediated phagocytosis and a comparably decreased ROS production in response to a non-receptor-mediated stimulus.

Figure 6. The correlation between levels of CRP and numbers of neutrophil-platelet aggregates in patients with stable CAD ($r = 0.65$, $p < 0.001$).



4.2.3 MMPs and TIMP-1

In the MIMMI I project we determined the serum levels of MMPs and TIMP-1 in ACS patients from the acute event to 3 months (paper III). At 3 months, the patients had been clinically stable for at least 2 months and circulating levels of CRP and IL-6 had declined to 'control' levels. In spite of this the serum levels of MMP-9 and TIMP-1 was still significantly elevated at 3 months compared to healthy controls. The findings of high MMP-9 levels in stable CAD are in agreement with a number of previous studies (124, 240, 241). On the other hand, TIMP-1 in the circulation has been less studied giving contradictory results. Nilsson L *et al* (124) found increased levels of TIMP-1 in stable CAD patients whereas Tayebjee M *et al* (241) did not. In the present study, the ratio between serum MMP-9 and TIMP-1 concentrations was significantly higher in the ACS patients at 3 months follow-up compared to control subjects. TIMP-1 is the most specific inhibitor of MMP-9 and the MMP-9/TIMP-1

ratio has been suggested to indirectly define the serum MMP-9 activity in vivo. The MMP-9/TIMP-1 ratio has also been proposed as a marker of disease activity in chronic inflammatory diseases, like multiple sclerosis (130, 131, 133). However, much remains unclear regarding the origin as well as the functional role of MMP-9 and TIMP-1 in the circulation of CAD patients. One intriguing possibility is that the increased serum levels reflect an increased expression in atherosclerotic tissue and, thus, may be used as a direct noninvasive assessment of extracellular matrix turnover and propensity to plaque rupture. However, the systemic levels of MMP-9 and TIMP-1 may also reflect the production by activated leukocytes and platelets in the circulation and, as such, be implicated in leukocyte adhesion, leukocyte migration and platelet aggregation. Interestingly, one study has shown increased neutrophil linked MMP-9 in patients with stable CAD (242). Similar to the acute phase, MMP-9 and TIMP-1 levels during recovery were both associated with systemic inflammation.

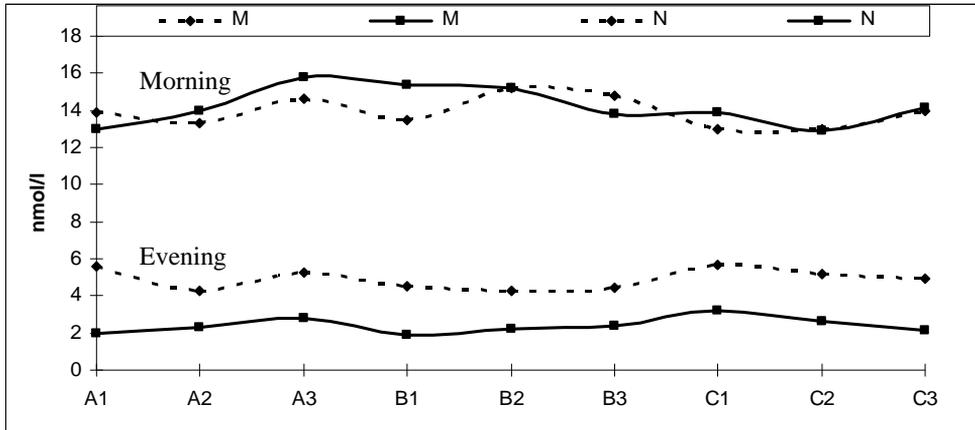
Several experimental studies have shown that lipophilic statins, e.g. simvastatin, reduce MMP-9 secretion by human macrophages, smooth muscle cells and endothelial cells in culture (243, 244). However, statin-mediated lowering of MMP-9 levels has not been consistently verified in clinical trials (245-247). In this regard, our present data do not support that statin therapy in ACS patients resulted in any potent suppression of serum MMP-9.

At 3 months follow-up, the levels of MMP-2, MMP-3 and MMP-7 in the ACS patients did not significantly differ from healthy controls. In a previous study (124), the levels of MMP-2 in stable CAD patients did not differ from healthy controls whereas MMP-3 showed significantly lower and MMP-7 significantly higher concentrations in the patients. In vitro, the macrophage expression of MMP-3 and MMP-7 decrease with statin treatment (222, 248,249). In the present study, it cannot be excluded that the extensive statin use has influenced the circulating levels of MMP-3 and MMP-7. As during the acute phase, the MMP-2, MMP-3 and MMP-7 levels at 3 months follow-up did not show any correlation with markers of inflammation.

4.3 The cortisol secretion pattern in CAD

In the MIMMI I study, the measurement of free cortisol in 24 h urine collection was used to obtain an index of the total diurnal production of cortisol. The highest values of total cortisol output were seen during the first 3 days of myocardial infarction but at 3 months follow-up, the values were still significantly higher in the patients than in control subjects (paper II and III). In order to evaluate the diurnal variation in cortisol secretion, salivary cortisol levels were measured repeatedly over 3 days, 30 min after awakening and in the evening. In both the MIMMI I and MIMMI I studies, the data were based on mean cortisol levels from these 3 days. The average intraindividual variation in salivary cortisol levels was less than 15 %. The mean salivary cortisol levels in patient and control groups at different time points are given in Figure 7. The morning cortisol levels did not differ between the ACS patients (at any time point) and controls (MIMMI I, paper II and III), neither between the patients with long-term stable CAD and controls (MIMMI II, paper V). On the other hand, both studies showed that the evening cortisol levels were significantly higher in patients compared to controls, indicating a flatter cortisol profile over the day in CAD patients independent of clinical stage. Although the determinants or consequences of having a flat cortisol rhythm are not clear, it is generally considered a result of long-term HPA over-stimulation. A flat rhythm has also been proposed to constitute a marker of disease progression. Abnormal circadian rhythms have been observed in patients with cancer and as an example; patients with metastatic breast cancer whose diurnal cortisol rhythms are flattened have earlier mortality (250). In a 5-year follow-up study of middle-aged men, an abnormal cortisol secretion pattern, including low variability, was associated with an increased incidence of cardiovascular-related events and type 2 diabetes (251). Moreover, in a cross-sectional population study, a flat diurnal cortisol pattern was associated with the risk of coronary calcification (187).

Figure 7. The mean values of morning and evening cortisol at different timepoints in patient (broken lines) and control subjects (continuous lines) of the MIMMI I study. A, B and C represent different 3 day's series of saliva collection.



4.3.1 The relation between diurnal salivary cortisol and systemic inflammation

During the acute event of ACS and at 2 weeks, correlations were seen between the serum levels of inflammatory markers (CRP and IL-6) and 24-h urinary cortisol (e.g. between IL-6 and urinary cortisol $r = 0.49$, $p < 0.001$). At all time points (1-2 days, 2 weeks and 3 months), strong correlations were seen between the inflammatory markers and evening salivary cortisol (paper II and III). Similarly, there was a strong correlation between the levels of CRP and evening cortisol in the group of long-term stable CAD patients in MIMMI II (paper V). Cytokines such as IL-1 and IL-6 can increase glucocorticoid secretion by enhancing the synthesis and release of CRH and ACTH. Cortisol, in its turn, inhibits the production of TNF- α , IL-1 and IL-6, thus decreasing the serum levels of cytokines that activate the HPA axis [reviewed in 140]. Our findings indicate that a low-grade systemic inflammation is associated with a continuous HPA stimulation in stable CAD patients, resulting in a 'high' flat cortisol rhythm. Suggestions have been made that patients with rheumatoid arthritis and CAD that exhibit high levels of IL-6 and, at the same time, 'inappropriately' normal morning cortisol levels, have insufficient capacity to limit inflammation (172, 190). However, the correlations between cortisol and inflammatory markers do not prove a cause-and-effect relationship and

there are inherent difficulties in evaluating the appropriateness of a given level of cortisol for a particular level of ongoing inflammation in individuals. In addition, the possible effects of medication on cortisol levels should be considered. However, data from previous studies investigating the effects of beta-blockers and statins have been consistent, giving no evidence for alterations in basal cortisol levels of ACTH-induced cortisol response (252-256).

The serum levels of MMP-9 and TIMP-1 were both related to systemic inflammation. As shown in Paper III, MMP-9 and TIMP-1 were also significantly correlated to evening cortisol levels and remained so after adjustment for CRP and IL-6. The MMP-9/TIMP-1 ratio also showed correlation to evening cortisol ($r = 0.39$, $p < 0.05$). On the other hand, cortisol was not related to either MMP-2 or MMP-3, and there was only a weak correlation between MMP-7 and 24-h urinary cortisol during the acute phase. As far as is known, no other studies have evaluated the relationship between serum levels of MMP and endogenous cortisol. However, several in vitro studies have shown that glucocorticoids are potent regulators of MMP-9 and TIMP-1, by inhibiting MMP-9 and enhancing TIMP-1 production (257-259). In vivo, one hydrocortisone dose given intravenously rapidly reduced the plasma concentration of MMP-9 (260). Our findings support the hypothesis that a dysfunction of the HPA axis in stable CAD patients might contribute to a systemic inflammatory state, characterized by a persistently high MMP-9/TIMP-1 ratio.

4.3.2 The response to acute stress

4.3.2.1 The cortisol response to stress

The activity of the HPA axis is often evaluated by measuring the stress-induced cortisol response. In the MIMMI I project, the ACS patients underwent both physical and psychological stress testing at 3 months follow-up, and so did the control group. During the exhaustive bicycle exercise test, the maximal relative predicted work capacity was significantly higher in the control subjects though the capacity of patients were well within normal range, 106% vs. 99%. The cardiovascular reactivity during the physical stress test, as assessed by blood pressure and heart rate, was similar in patients and controls. During the psychological stress test, the cardiovascular reactivity in general was less pronounced than

during the bicycle test. There were also larger differences between patients and controls during psychological stress, i.e. higher peak values of systolic blood pressure and lower heart rate values in the patient group. The cortisol response to physical stress was significantly attenuated in patients when compared to control subjects (median 31 % vs. 52% increase, $p < 0.01$), thus indicating a hypoactive HPA axis in CAD. Similarly, the cortisol response to psychological stress was markedly attenuated in patients compared to controls (median 11 % vs. 30 % increase, $p < 0.01$). The differences between patients and controls remained significant even after adjustment for possible confounding factors such as smoking, beta-blocker therapy and statin therapy. The relationship between a blunted HPA axis and susceptibility to inflammatory/autoimmune disease is well-known from animal studies [as reviewed in 165]. Blunted ACTH and cortisol responses to various stress stimuli have also been shown in a number of chronic inflammatory/autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus (172-176). The MIMMI I study is the first to demonstrate a blunted cortisol response in patients with atherosclerotic disease. However, the findings are in line with previous findings from the population-based LiVicordia study (Linköping-Vilnius Coronary Risk Assessment Study) (261). Lithuanian middle-aged men have a fourfold risk for CAD mortality compared with Swedish men. The aim of the LiVicordia study was, therefore, to compare both traditional and new possible risk factors for CAD in 50-year-old men from each of the cities Vilnius, Lithuania and Linköping, Sweden. Compared with Swedish men, Lithuanian men exhibited a significantly lower cortisol response to a standardised laboratory stress test but no difference in cardiovascular reactivity. In addition, the LiVicordia study showed a higher prevalence of early peripheral atherosclerosis in the Lithuanian men, as assessed by ultrasound measurements of intima-media thickness (262).

4.3.2.2 The inflammatory response to stress

The serum levels of CRP and IL-6 were determined before and 24 h after the stress tests. None of the inflammatory markers differed between patients and controls at baseline. Neither were there any differences in IL-6 levels between patients and controls after 24 h (1.8 (1.3 – 2.5) vs. 1.7 (1.2 – 2.4) pg/ml) probably reflecting that IL-6 levels had returned to baseline levels. On the other hand, physical stress induced a significant increase in CRP in the patients compared to the controls after 24 h (median 21% vs. 6% increase, $p < 0.01$). Similarly, psychological stress induced a significant increase in CRP in patients but not in controls

(median 18% vs. 5% increase, $p < 0.01$). The findings suggest the presence of hypofunction of the HPA axis in CAD patients. This may, in its turn, contribute to an inefficient suppression of stress-induced IL-6 release and, thereby, cause an increase in CRP in CAD patients. In a previous study, the effects of acute psychological stress were assessed in patients with rheumatoid arthritis in comparison with patients with osteoarthritis, the latter a joint disease not associated with systemic inflammation (173). A stress-induced increase in CRP was specific to patients with rheumatoid arthritis and the authors discussed whether the inflammatory reaction to stress could underlie the increased risk for myocardial infarction in this patient group.

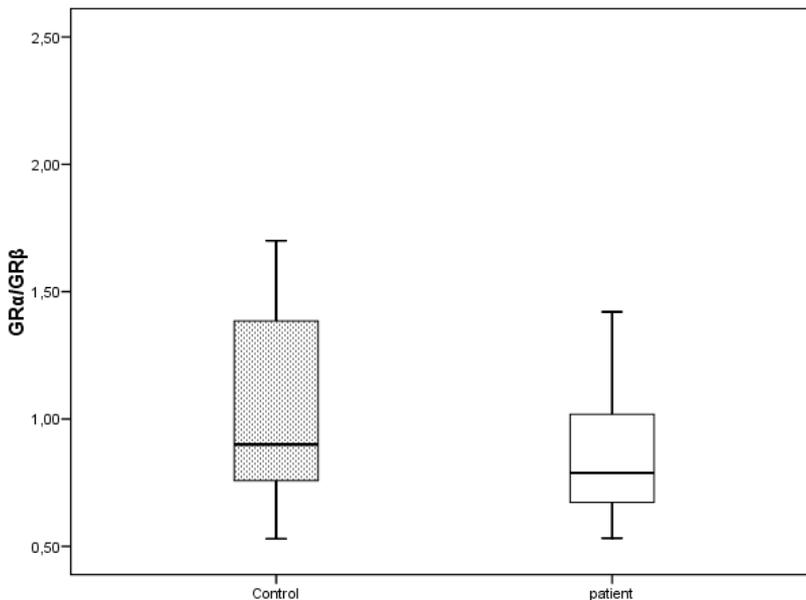
There is a strong association between external triggers and onset of myocardial infarction and sudden cardiac death beyond that expected by chance alone. Several studies have shown that both physical exertion and acute emotional stress such as burst of anger are potent triggers of myocardial infarction (263, 264). In one multicenter study, possible triggers were identified by 48.5% of the population; the most common were emotional upset (18%) and moderate physical activity (14%) (265). Lately, a case-control study with 11,119 patients with a first myocardial infarction and 13,648 age- and sex-matched controls from 52 countries (the INTERHEART study) investigated the relation of psychosocial factors to the risk of myocardial infarction. People with myocardial infarction reported a significantly higher prevalence of stress factors including stress at work and at home, financial stress and major life events in the past year (11). The mechanism by which stress increase the risk of myocardial infarction is not clarified but the interplay between stress and inflammation could play an important role. Thus, one intriguing possibility is that individuals with a blunted cortisol response will be more susceptible to atherosclerosis progression and plaque instability due to an 'abnormally' high inflammatory response to stress.

4.3.3 Is CAD associated with glucocorticoid resistance?

Ablated anti-inflammatory effects of cortisol may be associated with glucocorticoid resistance and alteration in the levels of GRs. In MIMMI II (paper V), it was investigated whether the levels of GR $_{\alpha}$ and GR $_{\beta}$ in neutrophils differed in patients with stable CAD compared with levels in healthy subjects. The expression of GR $_{total}$ and GR $_{\alpha}$ was significantly decreased in

patients compared to controls while the GR_{β} expression tended to be higher in the patient group. The net effect was a significantly lower ratio of GR_{α}/GR_{β} expression in the patients compared to controls (Figure 8). A reduction of GR_{total} has previously been demonstrated in patients with rheumatoid arthritis (181). Moreover, a down-regulation of GR_{α} at both the mRNA and protein levels occurs following chronic exposure to glucocorticoids (266, 267). There is also increasing evidence that an increase in the expression of GR_{β} relative to GR_{α} is associated with glucocorticoid insensitivity in a number of inflammatory disorders, including rheumatoid arthritis (268). The GR findings in MIMMI II confirm a state of HPA axis overstimulation in long-term stable CAD patients but may also indicate the presence of glucocorticoid resistance.

Figure 8. The ratio of GR_{α}/GR_{β} expression in patients with stable CAD and healthy controls (paper V).



On the other hand, the expression of ANXA1 in neutrophils was found to be significantly enhanced in the CAD patients compared to the controls, (74 (12-83) vs. 63 (10-66), $p < 0.01$),

as might be expected in a state of hypercortisolism. In consequence, the ANXA1 expression was positively correlated to the levels of evening salivary cortisol and negatively correlated to the GR_{total} expression. Glucocorticoids induce the synthesis and secretion of the anti-inflammatory protein ANXA1 and the neutrophil expression of ANXA1 were recently proposed as an indicator of glucocorticoid sensitivity (164). Taken together, the data in MIMMI II may seem inconsistent not fully supporting the theory of glucocorticoid resistance. However, glucocorticoid resistance is not an 'all-or-nothing' state but can be generalised or tissue-specific, transient or permanent, partial or complete, and compensated or non-compensated (269, 270). Hypothetically, a relative glucocorticoid resistance in CAD patients can be compensated for by increased cortisol secretion during basal conditions but upon stress stimuli, this compensation may probably become less efficient.

Although the number of ANXA1 receptors did not differ between patients and controls, the *ex vivo* results suggested an enhanced signalling capacity of ANXA1 receptors and/or a hyperresponsiveness to ANXA1 in the CAD patients. Exogenous ANXA1 suppressed LTB₄-mediated production of ROS in neutrophils from patients but not in cells from controls. To sum up, the original hypothesis about more inflammatory neutrophils in stable CAD was not proven in the MIMMI II study (paper IV and V). In contrast, the increased neutrophil expression of ANXA1 may indicate that circulating neutrophils in stable CAD are cells with enhanced anti-inflammatory properties and as such, take part in the down-regulation of inflammatory responses. However, the impaired neutrophil function in CAD may also be the result of a prolonged and/or overcompensated glucocorticoid-induced inhibition and in the long run, have negative consequences for the innate immune system.

5 Concluding remarks

- Serum levels of CRP, IL-6 and IL-1Ra in patients with stable as well as unstable conditions of CAD were associated with numbers of circulating neutrophil-platelet aggregates, suggesting a state of neutrophil activation (paper I).
- Patients with both stable and unstable conditions of CAD exhibited an altered cortisol pattern compared with healthy controls, involving hypercortisolism and a flat diurnal slope due to high evening cortisol values. The evening cortisol correlated positively with CRP and IL-6 levels. In addition, when exposed to acute physical or psychological acute stress, the patients showed a blunted cortisol response. Following the stress tasks, a significant increase in CRP was observed in the patients but not in the controls indicating a failure of the HPA axis to contain inflammatory activity (papers II, III and V).
- The serum levels of MMP-9 and the MMP-9/TIMP-1 ratio were high at the acute onset of myocardial infarction and remained significantly elevated compared to controls during a 3 months follow-up. MMP-9 in serum and the MMP-9/TIMP-1 ratio correlated with evening cortisol levels further supporting a link between a flat cortisol rhythm and systemic inflammation (paper III).
- The neutrophils in patients with stable CAD were not more activated *in vivo* compared to neutrophils in healthy controls, neither were they more prone to activation *ex vivo* (paper IV).
- The high evening cortisol levels in CAD patients correlated negatively with GR_{total} expression and positively with ANXA1 expression on neutrophils. In contrast to controls, the neutrophils from patients were highly responsive to exogenous ANXA1 possibly reflecting a chronic overactivation of the HPA axis (paper V)

To summarize, systemic inflammation in stable CAD was characterized by a high MMP9/TIMP-1 ratio, an increased inflammatory response to stress and an impaired neutrophil function. This inflammatory activity was associated with a dysregulated cortisol secretion involving a flat diurnal rhythm and a blunted cortisol response to stress. An intriguing hypothesis is that a hyporesponsive HPA axis may favour the development towards plaque instability.

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