Inflammation, platelet aggregation and prognosis in acute myocardial infarction

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ABSTRACT

The incidence of stroke and re-infarction is noticeably high in the first few days following acute myocardial infarction. This finding has raised questions whether the systemic inflammatory reaction secondary to myocardial necrosis is involved. The inflammation might affect the activation of platelets leading to insufficient effect of the antiplatelet treatment given. Furthermore, the importance of platelet reactivity and inflammation in terms of long-term prognosis is not fully understood. The prognostic importance of C-reactive protein (CRP) in relation to clinical variables also needs to be clarified.

The present studies are aimed at describing the dynamics of platelet function during the first days of an acute myocardial infarction, in relation to diabetes and inflammation. We also investigated whether increased platelet reactivity or the increased concentration of CRP in blood were related to a worse outcome. Finally, we examined if CRP levels contributed to a predictive model using clinical variables known to affect outcome in patients with AMI.

We used two novel platelet function tests to measure platelet reactivity; the PA-200 (a laser light aggregometer) and the PFA-100 (measures primary haemostasis in whole blood).

Platelet aggregation increased during the initial course of an acute myocardial infarction. The increase in platelet aggregation was most pronounced in diabetics and in patients showing higher systemic inflammatory reaction, assessed by measuring the concentration of CRP in blood. The pronounced platelet aggregation occurred despite ongoing antiplatelet and antithrombotic treatment.

There was a significant association between the levels of CRP and the degree of platelet reactivity. However, while the CRP levels were associated with a worse outcome (AMI, stroke and death), the results of the platelet function tests were not. The importance of CRP in predicting prognosis depended on which adjustments were made for confounding factors.

CRP and prognostic variables in a statistical model predicting death, however, showed that CRP was excluded. Thus CRP did not predict outcome beyond clinical prognostic variables.

The results of these studies reinforce the importance of clinical variables such as heart failure, age, atrial fibrillation, smoking status, diabetes and impaired kidney function - all of which were associated with worse prognosis in multivariable analysis.
ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to by their respective Roman numerals I-IV:


II. Karlsson F, Modica A, Mooe T. Dynamics of platelet activation in diabetic and non-diabetic subjects during the course of an acute myocardial infarction. Thrombosis Research 2007 May 31


IV. Modica A, Karlsson F, Mooe T. Prognosis after acute myocardial infarction as predicted by CRP and clinical variables. Submitted

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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<tr>
<td>ARB</td>
<td>Angiotensin receptor blockers</td>
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<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AMI</td>
<td>Acute myocardial infarction</td>
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<tr>
<td>CABG</td>
<td>Coronary by-pass grafting</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>COX-1</td>
<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Closure time</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>Non-ST elevation myocardial infarction</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
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<tr>
<td>SPA</td>
<td>Small platelet aggregates</td>
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<tr>
<td>STEMI</td>
<td>ST-elevation myocardial infarction</td>
</tr>
<tr>
<td>TIA</td>
<td>Transitoric ischemic attack</td>
</tr>
<tr>
<td>TnT</td>
<td>Troponin T</td>
</tr>
<tr>
<td>TxA</td>
<td>Thromboxane A_2</td>
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<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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INTRODUCTION

Although the incidence of new cardiovascular events after an acute myocardial infarction (AMI) seems to be declining, recurrent events are still a major issue (1-5). In fact, patients suffering an acute coronary event have a 2 to 6-fold higher incidence of death and non-fatal myocardial infarction as compared to patients with stable coronary disease (6). The highest incidence of recurrent ischemic events is seen the first month after the incident AMI and a large number of the new events are not related to the initial culprit lesion, but arise from new plaque ruptures in other segments of the coronary tree (5, 7). Studies of the coronary arteries in AMI have shown that the unstable plaque formation is not just a local inflammatory phenomenon, but reflects the pan-coronary process of vulnerable plaque formation (8, 9). In addition, these vulnerable vascular features are also more common in the carotid artery among patients with unstable angina as compared to patients with stable angina (10), which corresponds to a substantial increased risk of stroke early in the AMI period (11-13).

Furthermore, epidemiologic studies have shown that the risk of AMI and stroke increases during the first days after a pneumonia or upper-tract respiratory infection (14-16). The mechanisms connecting inflammation or infection with AMI are probably multi-factorial. Besides endothelial dysfunction, platelets ought to have a part in the mechanisms since platelets play a central role in the development of thrombosis and in new ischemic events happening in the early phase after AMI (17, 18).

Diabetes mellitus is associated with increased risk for recurrent cardiovascular events, including ischemic stroke after an AMI (11-13). The frequency of cardiovascular deaths among diabetics is about 2 to 3-fold as compared to patients not having diabetes, irrespective of sex (19). The reason for this is not fully understood, but since diabetes is associated with endothelial dysfunction, chronic inflammation, accelerated atherosclerosis and a state of hypercoagulability, hyper-reactive platelets could be one of the reasons (20). Enhanced platelet activation among diabetics is previously documented (20), but the dynamics of platelet function during ongoing AMI has never been described. In addition to diabetes mellitus, there is a known set of conventional risk factors explaining the majority of cardiovascular events (21). However, the timing of stroke in relation to AMI suggests that conventional risk factors do not explain the marked increase in the risk for ischemic stroke during the AMI process. Interestingly, ST-elevation myocardial infarction and more extensive myocardial necrosis in AMI have been shown to be related to early occurrence of stroke (12, 22).

The tissue damage in myocardial infarction induces an inflammatory response with release of pro-inflammatory cytokines, C-reactive protein (CRP) and several other molecules that are involved in a complex interplay between the endothelial, immune and haemostatic systems (23-26). The inflammatory reaction might strengthen the activation of platelets, leading to an insufficient effect of the antiplatelet treatment given.
Several studies have shown that the prognosis for the patients with AMI improves by inhibiting platelets early during the acute myocardial infarction process. This was first shown for acetylsalicylic acid (aspirin) (27) and later for clopidogrel (18, 28-30). By combining these two antiplatelet drugs there is a synergic effect (31). Unfortunately, far from all patients seem to respond to this treatment since new events occur and different laboratory tests show insufficiently inhibited platelets (32, 33). Many drugs used in medical treatment are given in relation to the weight of the patient, but this is not the case for antiplatelet pills, all patients are given a standardized dose. Different studies have not been able to show benefits of higher doses of ASA, although, some dose-response effects have been shown for clopidogrel (34). New and more effective ADP-inhibitors have recently been studied in clinical trials. They offer better antiplatelet effects and fewer cardiovascular events, but at the expense of more bleeding complications (35-37).

In summary, the prognosis after an AMI has improved substantially by introducing more effective antiplatelet and antithrombotic therapies. The therapies, however, do not appear to be satisfactory since the risk for new early thrombotic events remains. A better understanding of the mechanisms involved in thrombosis during acute myocardial infarction would open for further therapeutic improvements and more individualized treatment regimens.

Respiratory infection and myocardial infarction or stroke

Epidemiologic studies have shown a strong association between recent respiratory infection and subsequent AMI or stroke. These studies also showed that the risk was greatest for the first few days following infection and then fell off, so that there was no observed excess risk after about two weeks.

Meier et al reported in 1998 a case-control study from the General Practice Research Database, and found a significant association between respiratory infection within the previous month and AMI (14). From the same database, a larger case-series study, conducted by Smeeth et al., confirmed the finding on AMI and also reported an association with stroke (15). They also showed an increased risk of AMI and stroke after a urinary tract infection. Recently, a large study using a different general practice database, the IMS Disease Analyzer Mediplus database (IMS), definitely confirmed the evidence of strong association between recent respiratory infection and AMI or stroke (16). This case-control study showed that the odds ratios for infection three days prior to the index date were 3.8 for MI and 4.1 for stroke. There was a strong association of an increased risk of an AMI immediately following infection, which decreased over time (trend test p<0.001). The adjusted odds ratio of AMI within seven days of an infection was 2.1 (95% CI 1.4-3.2), whereas the odds ratios for one to three months and three months to one year were not significant. They failed to show any association between urinary tract infection and AMI. However, the adjusted odds ratio for stroke within a month following a urinary tract infection was 2.7 (95% CI 1.3-5.5).
The risk of stroke following acute myocardial infarction

The risk of stroke is remarkably high during the first few days after an AMI and then declines rapidly.

Mooe et al. showed that the daily rate of stroke during the first days after AMI is approximately eight events per 10,000, as compared to 0.14 events per 10,000 in an age-adjusted reference population (13). The risk of stroke was highest during the first few days after AMI and then declined rapidly later on.

In a cohort study, the risk of stroke following AMI was markedly high, particularly early after onset of AMI. The increased risk was 44-fold during the first month after AMI (11).

The timing of stroke in relation to MI suggests that the inflammatory response secondary to myocardial necrosis may have a role in the marked increased risk of stroke during the early period after AMI. Conventional risk factors such as age, atrial fibrillation, diabetes, heart failure, hypertension, prior stroke, prior MI and anterior location of index MI have been shown in different studies, yet not consistently, to be associated with an increased risk of ischemic stroke after AMI (22, 38). However, conventional risk factors do not explain the temporal incidence pattern with the highest risk initially and with the rapidly diminishing incidence the following month (11, 13).

These studies have also shown that stroke after an AMI is associated with more extensive disability and increased risk of death, as compared to stroke not preceded by AMI. This constitutes an important public health matter for persons with coronary disease, since improved survival after AMI leads to an increased number of people at risk.

Inflammation, endothelial dysfunction and platelet aggregation

In healthy volunteers, a very brief exposure to endotoxin or certain cytokines has been shown to impair endothelium-dependent relaxation for many days. The effect has been termed endothelial stunning (39). The mechanisms underlying the temporary endothelial dysfunction produced by inflammation are not yet known, but the consequences are that the normal properties of the endothelium, antiplatelet, anticoagulant and fibrinolytic properties are attenuated. The endothelial dysfunction following acute infection or inflammation may thus provide an additional transient risk factor for acute myocardial infarction and stroke (40). In addition, infection of endothelial cells (41) or exposure to certain pro-inflammatory cytokines induces the production of tissue factor, cell surface adhesion molecules and induction of procoagulant activity (40). Interestingly, CRP has been proposed to be involved in atherothrombosis by different mechanisms leading to endothelial dysfunction and a more procoagulant environment (42).

Activated platelets are seen in chronic pathological inflammatory states such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, cystic fibrosis, in the progression and metastatic spread of malignancies and in the acute immune response to bacterial challenge (43). Platelets upon activation stimulate the trans-
membrane migration of inflammatory cells in vulnerable endothelium. Activated platelets adhere to inflamed endothelium, secret pro-inflammatory mediators activating leukocytes and endothelium, promoting further atherosclerotic progression and a local prothrombotic environment (43).

In a setting of acute systemic inflammatory reaction such as in AMI, the levels of monocyte-platelet aggregates are increased (44). A relatively recent study showed that CRP promoted monocyte-platelet aggregation ex vivo and in vivo and the authors suggested an inflammatory-thrombotic link that is regulated by high levels of CRP (45). However, although these aggregates appear in AMI, their pathobiological significance is unclear.

C-reactive protein and cardiovascular events
The association between CRP and increased risk for cardiovascular events is well established in the cardiovascular research field. This association is seen both in healthy persons with moderately increased concentrations of CRP (46-48) as well as in AMI patients with the highest concentrations of serum CRP (26, 49-57).

According to the review article by Casas, over 40 population-based prospective studies have reported on the association of “baseline” CRP levels with cardiovascular disease outcomes. The adjusted odds ratio for cardiovascular events with increased CRP levels was 1.5 and 2.0 in two different meta-analysis (58).

Suleiman et al. showed that CRP was an independent predictor of heart failure and mortality in 1044 survivors of AMI. Adjusted HR for the combined endpoint, for patients in the fourth quartile as compared to patients in the first, was 2.5 (95% CI, 1.5 to 4.1; p <0.0001) (54). A large prospective study with a population of 1773 consecutive patients with unstable angina or ACS showed an almost two-fold risk, in the 30-day combined endpoint of death or AMI, having a CRP on admission > 10 mg/L (56). There are several other studies confirming this association. Unfortunately, however, most of these studies have several limitations. The lack of adjustments for clinical variables is most important (49, 51, 55, 57). Some studies are retrospective and contain selected cohorts of sub-studies from clinical pharmaceutical studies (51, 57, 59, 60).

The pathophysiology of acute coronary syndrome
Atherosclerosis
The atherosclerotic process (fatty streak), which starts already in the second decade of life, is considered a natural process in aging. These early atherosclerotic lesions are benign in nature, i.e. they will not cause any manifested disease (preclinical coronary artery disease). As the aging process continues and other stimuli for atherosclerosis come in to play, such as factors in the environment and genetic constitution, the atherosclerotic process may accelerate and become more malignant. The factors contributing to atherosclerosis can be organized within three classes: genetic factors that have been difficult to identify given the multigenic complexity; factors related to
the environment, such as cigarette smoking, a sedentary lifestyle or hypercholesterolemia; and a stochastic component (by chance). The accelerating atherosclerotic process includes smooth muscle cell migration and proliferation, and cell death.

**Plaque rupture**

Due to complex mechanisms involving the endothelium, the inflammatory cell system and platelets, the atherosclerotic plaques grow and may either develop into stable significant plaques causing stable coronary symptoms or into unstable plaques. Among factors contributing to the transformation of atherosclerotic stable plaque into unstable and plaque rupture are acute systemic infections such as influenza or pneumonia. In a couple of days, an acute infection may trigger severe and abrupt inflammation in a high-risk atherosclerotic plaque (61). The apoptotic death of smooth muscle cells in the atheroma leads to a weaker fibrous cap, since the production of collagen needed to repair and maintain the matrix of the fibrous cap is diminished. Macrophages in the atherosclerotic lesions over-express matrix metalloproteinases and elastolytic cathepsins break down the collagen and elastin of the arterial extracellular matrix. This leads to degradation of the fibrous cap. The mechanisms of reduced collagen synthesis and increased degradation lead eventually to vulnerable and unstable plaques, which rupture and expose the sub-endothelial thrombotic surface, leading to adherence of platelets and subsequent thrombosis, impeded blood flow and myocardial infarction. Coronary spasm may also contribute to the impaired blood flow.

**Platelet activation**

Upon exposure of the sub-endothelial surface due to plaque rupture, the platelets adhere by different receptor systems and become activated. The platelets secrete their granules into the surrounding environment, activating more platelets and the immune system. The activation of platelets leads to platelet aggregation in the core of the ruptured plaque and gradually to formation of a thrombus. The main inducers of platelet activation are collagen and the soluble agonists such as adenosine diphosphate (ADP), epinephrine, platelet activating factor, thrombin and thromboxane A\(_2\) (TxA\(_2\)). Thrombin is considered to be the most potent physiological activator (62). The exposure of the sub-endothelial surface also activates the haemostatic system. This is basically a natural physiological function of the coagulation system securing haemostasis after vascular injury.

**The haemostatic system**

Side by side with the inflammatory process and endothelial dysfunction, when platelets adhere and aggregate on the subendothelial structures, the cascade of the coagulation system activates. The activation of the haemostatic system and the extrinsic pathway (via tissue factor) of the coagulation cascade promote local thrombin generation, which bind to adherent platelets and amplify the platelet activating process. Thrombin induces cleavage of fibrinogen to fibrin polymers, which can then be cross-linked to form a stable clot with platelets (secondary haemostasis). The
coagulation cascade can be partly inhibited by anticoagulants such as low molecular weight heparin (LMWH).

The inflammatory system
Leukocytes (mainly monocytes) adhere to diseased endothelium by different leukocyte adhesions molecules. Upon stimulation by chemo-attractant cytokines (released by activated platelets and endothelium), the leukocytes start to move between endothelial cell junctions to enter the intima (monocytes are now called macrophages), where they begin to accumulate lipids and transform into foam cells. These primary lesions are called fatty streaks and are considered to be reversible to some extent. As the infiltration of macrophages, T-cells and mast-cells continue, the atheroma grows. At the site of the ruptured plaque, activated immune cells are abundant and produce inflammatory mediators. The activation of T-cells leads to a cascade of cytokine release and in the end substantial amounts of interleukin–6, which stimulates the release of acute-phase reactants such as serum amyloid A and CRP from the liver (63). Blood concentrations of interleukin–6 and CRP are raised in patients with ACS and high concentrations are related to worse prognosis (26, 64). This acute-phase reaction in AMI is secondary to the myocardial necrosis and not to the plaque rupture (23). However, even moderately elevated CRP concentrations among apparent healthy persons are associated with cardiovascular disease (46). This raised CRP concentration, in contrast to the situation in AMI, probably mirrors the local cellular inflammation going on in the atherosclerotic vessels. Not only interleukin 6 and CRP of the acute-phase reactants are related to cardiovascular disease, but also many other inflammatory markers such as erythrocyte sedimentation rate, levels of fibrinogen and circulating soluble adhesion molecules such as soluble intercellular adhesion molecule 1, soluble VCAM-1, and soluble P-selectin, which are shed by activated cells (65, 66).

The endothelial system
Hypercholesterolemia causes focal activation of endothelium in large and medium-sized arteries. An inflammatory reaction in the arterial endothelial wall is triggered by the infiltration of LDL particles. The oxidation of LDL leads to released phospholipids which can activate endothelial cells. Platelets, immune cells and endothelial cells interact in an intricate way to maintain the atherosclerotic process.

The antiplatelet and antithrombotic drugs
Aspirin
Aspirin irreversibly inhibits, by acetylation, the cyclooxygenase-1 dependent (COX-1) synthesis of TxA₂, during the entire life span of the platelet. TxA₂ is a potent vasoconstrictor and a necessary agonist for the full aggregation response of platelets. The antiplatelet property of aspirin varies between individuals, not only because of different degrees of COX inhibition, but presumably because of the many different biochemical pathways activating platelets by by-passing COX-1 (33). Among patients
with acute or previous cardiovascular or cerebrovascular events, treatment with aspirin has been shown to effectively reduce the relative risk of new cardiovascular thrombotic events by 25% (27). The ISIS-2 study showed the benefits of aspirin as a complement to treatment with thrombolysis. In patients with ST-elevation myocardial infarction, the treatment with aspirin reduces mortality to an extent similar to that of the thrombolytic agent streptokinase (STEMI) (17). Most patients are given the same low-dose regime (75-160 mg) and as of present, there are no bed-side tests in use to evaluate if the antiplatelet effect is satisfactory.

**Thienopyridines**

The thienopyridines irreversibly block the adenosine diphosphate (ADP) receptor P2Y12 on the platelet surface. There are two ADP-receptor inhibitors available: clopidogrel and ticlopidine. The latter is not frequently used because of the risk of neutropenia. Both are pro-drugs which need to be metabolized in the liver to active metabolites.

Dual antiplatelet therapy with aspirin and clopidogrel in ACS has convincingly shown an additive benefit over aspirin monotherapy in various clinical trials (18, 28-30, 67). For instance, during a mean follow-up period of nine months, the risk for cardiovascular events (AMI, stroke and cardiovascular death) was reduced significantly by 20 percent by adding clopidogrel (300 mg loading dose followed by 75 mg daily) to aspirin 75 to 325 mg (RR 0.80; 95% CI, 0.72 to 0.90; P<0.001) (28).

**Anticoagulation**

Low molecular weight heparins (LMWH) are well studied in AMI. These fractionated heparins inhibit the formation of thrombin by inhibiting factor Xa in the coagulation cascade. There are two different LMWH (dalteparin, enoxaparin) used and both have shown good clinical effect in reducing cardiovascular events. Enoxaparin has been shown to reduce cardiovascular events by 15-20 percent as compared to unfractionated heparin (68). The pentasaccharide fondaparinux is also an inhibitor of factor Xa in the coagulation cascade. Treatment with fondaparinux has been shown to be as effective as enoxaparin in the Oasis-5 study but caused less major bleedings.
AIMS OF THE STUDY

The aims were:

- To study relationships between the inflammatory response, infection and platelet reactivity (I, III).
- To study the dynamics of platelet aggregation in patients with diabetes mellitus and acute myocardial function (II).
- To evaluate the prognostic importance of the inflammatory response and of platelet function in acute myocardial infarction (III).
- To assess the clinical importance of CRP and clinical variables in terms of prognosis (IV).
MATERIAL AND METHODS

Study population

The studies were conducted in Östersund Hospital, which is the primary referral hospital for the County of Jämtland, Sweden. The catchment area has about 128,000 inhabitants. The aim was to include all consecutive patients with chest pain admitted to the intensive coronary care unit in 2002 and 2003, but the inclusion of patients during the weekends, holidays and vacation-periods was sparse.

One thousand and twenty-eight (1028) patients were finally included in the database, of which different numbers of patients were included in the separate papers according to the sub-paper inclusion criteria (Fig. 1). Among those patients eligible during the inclusion period, 78 patients with ACS or AMI were not included due to different reasons: 24 patients were not included because of time-delay; seven patients did not want to participate; three patients suffered impaired cognitive functions; six patients died before inclusion in the study; one patients was from abroad; six patients were difficult to do venous puncture on and 31 patients were not included for unknown reasons.

Acute myocardial infarction was diagnosed according to the guidelines of the European Society of Cardiology (69). Acute coronary syndrome was defined as either unstable angina or myocardial infarction with typical chest pain and objective evidence of cardiac ischemia (ECG changes and/or elevated cardiac markers). Five-hundred and ninety (590) patients had ACS of whom 534 patients had AMI (troponin T positive).

Patients receiving any treatment for diabetes mellitus were classified as diabetics. No glucose tolerance tests were performed. Patients were classified as hypertensive if they had a diagnosis of hypertension. Any histories of atrial fibrillation or atrial fibrillation during current hospitalization were classified as atrial fibrillation. Patients with signs of heart failure during hospitalization were classified as Killip class > 1. Conventional antiplatelet and antithrombotic treatments were used as clinically indicated. The routinely used aspirin dose was 75 mg per day. Informed consent was obtained from all subjects. The characteristics of the patients included are summarized in Table 1.

All studies comply with the Declaration of Helsinki and were approved by the Regional Ethical Review Board in Umeå.

Paper I

Three hundred and fifty-eight (358) patients with ACS were included, of whom 66 patients had an infection during their hospital stay. An in-hospital stay of at least three days and aspirin treatment on discharge were required for inclusion. An infection was arbitrarily defined as: (i) the occurrence of fever of >38.0 C for >2 days; (ii) urinary tract infection (UTI); or (iii) pneumonia and other significant clinical infections. We used these simple clinical conditions since they are common in the
clinical situation. The diagnosis of infection was made by the physician on duty, who did not participate in the study.

**Paper II**

One hundred and ninety-five (195) non-diabetics and 48 diabetics, all with AMI, were included. They all had complete PA-200 data until the fifth day of the hospital stay. An in-hospital stay of at least five days was required in order to assess the dynamics of platelet activation by repeated measurements. The treatment for diabetes was diet in 6, oral medication in 17, insulin in 18 and both oral treatment and insulin in 7 subjects. Another 170 patients with AMI were admitted during the inclusion period but were not included because of death or discharge before the required five in-hospital days (n=131) or incomplete aggregation data.

**Paper III**

All patients with a diagnosis of AMI during the inclusion period were considered for inclusion. An in-hospital stays of at least three days, aspirin treatment on discharge and complete PFA-100 and measured peak CRP data were required for inclusion. Three hundred and thirty-four (334) patients with AMI were included in the study. Another 163 patients with AMI were admitted to the hospital during the inclusion period but were not included because of death or discharge before the required three in-hospital days (n=90), not being on aspirin treatment on discharge (n=29) or incomplete laboratory data (PFA-100, CRP) (n=64).

**Paper IV**

All patients with a diagnosis of AMI were considered for inclusion. A blood sample of CRP on admission was required for inclusion, which three patients did not fulfil. Thus five hundred and thirty-one (531) patients were included.

**Study design**

Papers I-II are cross-sectional observational studies. Paper III is both a cross-sectional observation study and a cohort study. Paper IV is a cohort study.

**Statistics**

**General**

Statistical analysis was performed by using the software SPSS, release 13 (Chicago, IL, USA). Group data are expressed as means (SD) for continuous variables and as rates for variables on a nominal scale. Median values and interquartile range were used when the distribution of data made it appropriate. Correlation between two samples was analyzed using Spearman rank correlation coefficient, $r_s$. Differences between two groups were assessed using the t test for independent data or Mann–Whitney U test when appropriate. Differences between multiple independent groups were analyzed using Kruskal-Wallis test. Differences between proportions were analyzed using the chi-square test. The null hypothesis was rejected for $p < 0.05$. When a direction of
the difference between two samples could be predicted as to their means, i.e. the mean of a sample 1 was “greater than” or “less than” that of sample 2, the test was one-tailed. Otherwise, if the focus of the test was on differences between samples, the test was two-tailed.

**Papers I and III**
Troponin T and CRP values were divided into quartiles in the analyses. The measured peak values of PA-200 and CRP were used in paper III. Repeated measurements of platelet activation during hospitalization were analyzed with the non-parametric Friedman test. Independent variables associated with platelet aggregation measured by the PA-200 were identified using a linear regression model. Age, CRP, troponin T, the use of LMWH, and the use of clopidogrel were considered to be of potential importance and were included in the models. An approximate normal distribution with improved skewness and kurtosis was achieved using the square root transformation of the PA-200 results. Independent predictors of high residual platelet reactivity, assessed by PFA-100, were identified using a logistic regression model. The variables of age, CRP, troponin T, platelet count, von Willebrand factor, treatment with thrombolysis, the use of LMWH and the use of clopidogrel were included in the model. The relation between measured peak CRP concentration and closure times was investigated using Spearman rank correlation. In paper III we used Cox-regression analysis to calculate the unadjusted and adjusted relative hazard ratios and 95 percent confidence intervals for stroke, myocardial infarction and death of any cause in relation to measured peak CRP, platelet aggregation and the state of high residual platelet reactivity. The variables of age, sex, smoking status, diabetes, atrial fibrillation, Killip class > 1, intervention with CABG or PCI, baseline glomerular filtration rate (GFR) and troponin T were included in the adjusted model. The assumptions for Cox-regression analysis were evaluated by Kaplan-Meier curves for all variables included.

**Paper II**
Correlation was assessed by Spearman rank correlation method. Repeated measurements of platelet activation during hospitalisation were analyzed using Friedman test.

**Paper IV**
CRP values were divided into quartiles. In the logistic regression analysis we included variables that were associated with death in univariate analysis, theoretically had a potential to affect outcome or were of importance based on previous experience. To assess whether CRP contributes to a model predicting death at two years after AMI, logistic regression analysis was performed. The variables were analyzed using stepwise forward (log-likelihood ratio). Tests were made for confounding and interactions in the model. Tests on interaction by age and by Killip class > 1 produced a significant interaction term, age*Killip class > 1. The default significance
level for entry was <0.05 while that for removal was 0.10. The order of entry of the variables gives their relative importance to the separation of the patient groups examined (Table 4).

The univariate absolute risk of death (number and percentage of deaths) was calculated for statistically significant variables in the logistic regression analysis. Calculations were made for single variables as well as for any combination of two variables in order to illustrate the potential clinical usefulness. The variable age was dichotomized to age above or below the median value (75 years) and renal function to GFR above or below 60 ml/(min*1.73m²).

Control group and reference values concerning PFA-100
In paper I we used previous established cut-off value for normal closure time (CT), according to previously published studies. Since it is questionable to use other laboratories’ historical reference values, we used a control group of 278 volunteers, randomly selected from the population registry, to establish in-house reference ranges. The volunteers (mean age 58 years, range 30 to 93), had no known cardiovascular disease and no previous history or laboratory results indicative of platelet dysfunction, meaning no anaemia, no extraordinary bleeding tendency and normal platelet count. The use of non-steroidal anti-inflammatory drugs was not allowed. Since it is not unusual to find persons with mild von Willebrand disease in a selected volunteer population, and because individuals might be unaware that they have actually taken aspirin-like substances, we discarded all CT-values above the 95th percentile before defining the normal range. The reference range (90-197 sec) was then determined based on a 90% central interval of the results. The coefficient of variation (CVs) was calculated at 15.3% for the collagen and epinephrine cartridge (CEPI). A CV at 15.3 % for the PFA-100 is higher in comparison to previously reported studies. Higher CVs has been reported among patients taking aspirin (70), as such it can not be excluded that among our control group were persons on aspirin.
The population of the county of Jämtland
128 000

Excluded for various reasons
78

The study population
Re-admittance not included
1028 patients

Unspecific chest pain
Other cardiac and other medical conditions
438

Acute coronary syndrome
AMI TnT pos 534
ACS TnT neg 56

ACS with a hospital stay of at least 3 days
PAPER III
358

AMI
Follow-up study
PAPER IV
531

ACS with a hospital stay of at least 3 days
PAPER I
331

AMI with a hospital stay of at least 5 days
PAPER II
243

Figure 1. Selection of Study Cohorts
Table 1. Clinical Characteristics of 590 patients with Acute coronary syndrome.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean, SD)</td>
<td>72 ± 12</td>
</tr>
<tr>
<td>Male</td>
<td>378 (64%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>118 (20%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>232 (39%)</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>193 (33%)</td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>164 (28%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>120 (20%)</td>
</tr>
<tr>
<td>History of heart failure</td>
<td>93 (16%)</td>
</tr>
<tr>
<td>Previous stroke or TIA</td>
<td>101 (17%)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>51 (9%)</td>
</tr>
</tbody>
</table>

Blood tests

Blood samples were obtained via venipuncture and collected in tubes containing sodium citrate (0.129 M) on admission and on days two, three and five in hospital for the PA-200 analysis and on admission and the third day for the PFA-100 analysis. A blood sample collected in serum tubes for CRP analysis was obtained on days one, two, three and five after admission. CRP was measured with C-reactive protein (latex) high sensitive immunoturbidimetric assay on Modular Analytics System (Roche). The coefficient of variation for the CRP analysis was 4.0% and 1.3% at the CRP-concentration level 0.94 mg/L and 17.1 mg/L respectively. The peak value of consecutive troponin T analyses was recorded. A blood sample for troponin T analysis was taken routinely every 8 hours. Troponin T was measured by an electrochemiluminescence immunoassay (e170 Modular). GFR calculations were based on measurements of cystatin C levels for each patient. Plasma cystatin C measurements were performed by means of a particle enhanced immunoturbidimetric method (Modular P). LDL was calculated from the serum concentrations of cholesterol and fasting triglycerides using the Friedewald’s formula. Von Willebrand factor was measured with ELISA using reagents from DAKO (Copenhagen, Denmark). Analyses with the platelet function tests were conducted within 180 min after blood sampling.

The platelet function tests

General

To evaluate the platelet aggregation and reactivity we used two different kinds of tests: the PA-200 aggregometer (Kowa Inc., Tokyo, Japan) and the PFA-100 (Platelet Function Analyzer, Dade Behring, Germany).

Historically, platelet function tests have been developed to identify disorders of bleeding and thrombosis. The original aggregometers were developed in 1970s. Since
then, the aggregometers have developed to an instrument designed specifically for measuring platelet aggregation that is basically a spectrophotometer attached to a chart recorder or computer. Earlier platelet function tests were expensive, hard to handle, time consuming and therefore confined to specialized haemostatic laboratories (43). As the methods have developed and the platelet function tests have become more easy to use, the interest for measuring platelet function has spread to other areas of medicine. In cardiology there is a need, in a point-of-care manner, to evaluate the platelet response to antiplatelet drugs in order to assess the risk for new thrombotic events. Thereby the antiplatelet therapy could become more individualized.

At present there are no standardized methods to objectively measure platelet activity in vivo. This is not surprising since the platelet is a complex organism and the primary haemostatic process is complicated, with adhesion, activation and aggregation stages. Different laboratory tests measure different mechanisms in the platelet function. Therefore it is not surprising that the correlation among the separate platelet function tests is poor (71).

The term Aspirin resistance, which has been used in many medical articles for some years, is controversial because its definition, diagnosis, prevalence, causes and clinical consequences are still uncertain (72). The dependence on the TxA₂ pathway in platelet activation varies between different individuals and with the type of platelet function test used. The term “aspirin resistance” should therefore be confined to the failure of aspirin to have an impact on its pharmacological target, documented by specific tests.

**PA-200 system**

The PA-200 is an aggregometer which differs from traditional light transmission aggregometers since it uses laser-light scattering technique. It is therefore more sensitive in detecting the formation of small platelet aggregates, which conventional aggregometers underestimate. The PA-200 also allows evaluation of the size of platelet aggregates formed upon activation, that is; small platelet aggregates (SPA), medium platelet aggregates and large platelet aggregates (LPA). The conventional light transmission aggregometer offers just one parameter of platelet aggregation, i.e. a change in light transmission of platelet suspensions while the PA-200, on the other hand, provides two parameters such as size and number of platelet aggregates. It can also be used as a conventional light transmission aggregometer.

An optical dense aggregometer or a light transmission aggregometer uses light passing through a medium and the more transparent the medium is, the more light is allowed to pass. As a reference, it is standard to use each subject’s autologous platelet-poor plasma (PPP) as the maximum transparent situation and the platelet-rich plasma (PRP) as the most opaque setting. As platelets aggregate in PRP, the plasma becomes more transparent and the increase in light transmission through the sample is recorded. In the platelet aggregation test, it is necessary to use an agonist to initiate the aggregation. There are a variety of agonists with advantages and disadvantages. In studies with aspirin, it is common to use epinephrine as an agonist.
The typical response to epinephrine in optical density curves is a two phase-wave: a small wave followed by a full scale secondary response. The second response, when present, is inhibited by aspirin. This second wave corresponds to the transition of small-sized platelet aggregates to medium-sized platelet aggregates and then to large-sized platelet aggregates (Fig. 2).

![Figure 2](image)

**Figure 2.** The output of PA-200 is illustrated where the separate platelet aggregation waves and the optical density curve are shown.

In the separate studies in the present dissertation, an epinephrine concentration of 0.12 µM was used. The optimal concentration was set after a pilot study where aggregation to different concentrations of epinephrine was initiated and corresponded well to concentrations earlier used in published papers with the PA-200 (73). This concentration thus represents a well-balanced aggregation level and was below the common used concentration of 1 to 10 µM (43).

**PFA-100 system**

The PFA-100 is an instrument and test cartridge system in which primary haemostasis is simulated in vitro, that is, the adhesion and aggregation of platelets to a stimulus. The coagulation system does not affect the results of PFA-100. PFA-100 tries to mimic the high shear stress conditions that platelets are exposed to in a capillary. In the test cartridge, anticoagulated whole blood is aspirated from a sample reservoir through a capillary and an aperture in a membrane, exposing platelets to high shear flow conditions. The membrane is either coated with collagen and epinephrine (CEPI) or collagen and ADP (CAPD), which trigger the platelets to adhere and activate. CEPI,
which was originally thought to be sensitive to the aspirin-induced inhibition of platelet function, was used in papers I and III. The time (s) from the start of the test until the platelet plug occludes the aperture is the closure time (CT). The maximum time recorded is 300 s and, if no occlusion is detected at that time, the measurement is ended. Recordings of 300 s or above were arbitrarily assigned 300 s for statistical calculations. This ceiling effect of the PFA-100 affects the CVs estimated as we are unable to use the exact value. In paper I we used previous established cut-off value to define aspirin non-responsiveness, <193 s. In paper III we used a volunteer population to establish the in-house reference range, <197 s (see earlier heading, Control group and reference values concerning PFA-100).

Recently, the capability of PFA-100 to measure the inhibition of platelets by aspirin has been questioned, since PFA-100 does not measure the pharmacological target of aspirin, i.e. COX-1 activity (see section The antiplatelet and antithrombotic drugs). Furthermore, PFA-100 measurement is sensitive to many other variables, including platelet function, platelet count and plasma von Willebrand factor (vWF). Therefore, even if aspirin optimally inhibits TxA_2 formation, which would per se prolong the CT, this inhibition may be outweighed by other primary haemostasis stimulating factors which are not affected by aspirin. Watala et al. demonstrated that platelet aggregation mediated by interactions between vWF and the membrane bound receptors GPIb and GPIIb-IIIa are the most important determinants for PFA-100 results, whereas other platelet receptors and triggering mechanisms for platelet adhesion or aggregation have little impact (74). Furthermore, one study showed that induced systemic inflammation among healthy volunteers had a major impact on the results obtained by PFA-100, and the closure times were inversely correlated to increased levels of vWF (75). Chakroun et al. confirmed this finding by showing that plasma vWF levels are the main determinant of short CT among aspirin-treated patients with cardiovascular disease (76). Another study has demonstrated that aspirin treatment diminished TxB_2 to the same extent in patients with short closure time and long closure time (77). As a consequence, it has been suggested that normal CT values despite antiplatelet treatment should be interpreted as a state of high residual platelet reactivity (72). We therefore used this term in paper III as compared to paper I where we used the old and misleading term aspirin non-responsiveness. Lately, studies with PFA-100 have reported higher CVs for patients on aspirin treatment (70). The limited precision may thus erroneously classify patients as aspirin responders or non-responders.
RESULTS

Platelet aggregation during the course of an acute myocardial infarction

Papers II and III, irrespective of patient population, showed that platelet aggregation increased during the course of an AMI and peaked on day three. The increase in platelet aggregation was most pronounced among the diabetics (paper II) and occurred despite ongoing antiplatelet and antithrombotic treatment.

Infection or systemic inflammatory response and platelet reactivity

There was an association between infectious/inflammatory state, CRP and platelet reactivity, as described in papers I and III. In paper I, platelet aggregation was most pronounced among those patients with pneumonia and the proportion of aspirin non-responsiveness was twice compared to those not having any infections (Table 2). The numbers of small platelet aggregates were highest among the patients with the highest CRP concentration and the CT values were lower (Table 3). Patients with UTI or fever also had a higher occurrence of platelet aggregates as compared to patients not having any signs of infection. These results are not adjusted for differences in patient characteristics. However, the concentration of CRP was associated with platelet aggregation ($r_s = 0.31; p < 0.001$). There was a small negative correlation between measured peak CRP and closure time, $r_s = -0.17; p = 0.002$ (paper III). There was also an association between the concentration of CRP and aspirin non-responsiveness adjusted for age, troponin T, and use of clopidogrel and LMWH. ($P < 0.001$, OR = 1.54; 95% CI: 1.23–1.96).

Platelet function and outcome

Pronounced platelet aggregation during AMI was associated with poorer outcome in a univariate Cox-regression model but not in a multivariable model including clinical relevant variables (Fig. 3) (paper III). Median follow-up time was 44 months (inter-quartile range 35–55 months). During that period of time there were 141 primary endpoint events (ischemic cerebral event, myocardial infarction, or death of any cause). The results of PFA-100 were not significantly associated with poorer outcome.

Concentration of C-reactive protein and outcome

Concentrations of serum CRP were associated with poorer outcome as is shown in paper III. A CRP concentration more than about 65 mg/L, during the course of an AMI, corresponded to adjusted two-fold odds in subsequent occurrence of AMI, stroke and death of any cause during a mean follow up-time of 44 months (HR 2.0, 95% CI 1.1-3.7) (Fig. 4). The rates for the combined endpoint of stroke, AMI and death in the different quartiles of measured peak CRP were 24 (28.2%), 31 (37.8%), 34 (40.5%) and 52 (62.7%) in the first, second, third and fourth quartile, respectively ($p<0.0005$) (paper III). During the entire follow-up period, a total of 28 cases of ischemic cerebral events, 65 myocardial infarctions and 88 deaths occurred.
However, as shown in paper IV, the association between baseline CRP and death in patients with acute myocardial infarction was absent when including the significant interaction term *age by Killip class > 1*.

**Clinical characteristics and outcome**

*Age, atrial fibrillation, Killip class >1* (heart failure), *smoking status* and *diabetes* predicted a worse prognosis, as is shown in paper III. *Killip class > 1* had the strongest association with poorer outcome (AMI, stroke and death of any cause) with a hazard ratio of 1.8 (95% CI 1.2-2.6). In paper IV over half of the patients with *Killip class > 1* were deceased after two years following AMI. The odds of being deceased in two years was three-fold as compared to those not having heart failure during hospitalization. *Intervention* with either CABG or PCI was independently associated with better outcome. However, patients who did not undergo intervention were older, had worse kidney function and probably more co-morbidity, which might have influenced the difference in outcome. The variables *age by Killip class > 1, GFR, intervention* and *atrial fibrillation* were retained in the logistic regression model and predicted death or not in 82% of the patients. See Table 4. Elevated CRP is associated with death in patients with acute myocardial infarction, but the association is absent in the multivariable model when the interaction term *age by Killip class > 1* is included. CRP has no value beyond clinical variables to predict death after an AMI (paper IV).

Table 2. Aspirin non-responsiveness among patients diagnosed with and without pneumonia in acute coronary syndrome.

<table>
<thead>
<tr>
<th></th>
<th>Aspirin non-responsiveness</th>
<th>Aspirin responsiveness</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No infection, day 1</td>
<td>110 (42%)</td>
<td>155 (58%)</td>
<td>265</td>
</tr>
<tr>
<td>Pneumonia, day 1</td>
<td>4 (44%)</td>
<td>5 (56%)</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>114</td>
<td>160</td>
<td>274</td>
</tr>
<tr>
<td>No infection, day 3</td>
<td>128 (46%)</td>
<td>149 (54%)</td>
<td>277</td>
</tr>
<tr>
<td>Pneumonia, day 3</td>
<td>9 (90%)</td>
<td>1 (10%)</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>137</td>
<td>150</td>
<td>287</td>
</tr>
</tbody>
</table>

Aspirin non-responsiveness defined as CT ≤ 193 sec. For day 3 data, p = 0.006, using the χ²-test.
**Table 3.** Comparison of platelet aggregation, aspirin non-responsiveness, closure times (CT) and troponin T (TnT) levels according to quartiles of peak C-reactive protein (mg/L).

<table>
<thead>
<tr>
<th>Quartiles of C-reactive protein</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; (n=85) (0.05-5.3)</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; (n=82) (5.4-19.6)</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; (n=84) (19.7-64.5)</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; (n=83) (64.6-304)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak SPA, (Count, x10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>71 (22-91)</td>
<td>82 (30-120)</td>
<td>104 (45-150)</td>
<td>117 (70-160)</td>
</tr>
<tr>
<td>Aspirin non-responsiveness (%)</td>
<td>45</td>
<td>44</td>
<td>51</td>
<td>64</td>
</tr>
<tr>
<td>CT (sec)</td>
<td>259 (150-300)</td>
<td>231 (143-300)</td>
<td>184 (137-300)</td>
<td>162 (124-300)</td>
</tr>
<tr>
<td>TnT (µg/L)</td>
<td>0.49 (0.21-1.26)</td>
<td>1.09 (0.33-2.64)</td>
<td>1.93 (0.92-5.18)</td>
<td>3.99 (2.02-7.36)</td>
</tr>
</tbody>
</table>

Peak SPA=The highest measured count of small platelet aggregates during hospitalization. Aspirin non-responsiveness defined as closure time < 197 sec. Values are expressed as medians with the interquartile range in parentheses. Calculations using Kruskall-Wallis test.

**Figure 3.** Cumulative event-free survival (AMI, stroke, all death) in relation to peak platelet aggregation quartiles. Multivariable Cox-regression analysis.

![Graph of cumulative event-free survival](image-url)
Table 4. Final logistic regression model to predict death after two years of follow-up after an acute myocardial infarction.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*Killip class &gt; 1</td>
<td>1.03</td>
<td>1.02-1.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GFR</td>
<td>0.97</td>
<td>0.96-0.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intervention</td>
<td>0.27</td>
<td>0.12-0.61</td>
<td>0.003</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>1.79</td>
<td>1.04-3.09</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Logistic regression using forward stepwise (Likelihood ratio) method. OR=Odds ratio. CI=Confidence interval. GFR= Glomerular filtration rate (ml/min*1.73m²), as continuous variable. The initial multivariable model included CRP in quartiles, sex, smoking, hypertension, diabetes mellitus, previous AMI, previous stroke, chronic obstructive pulmonary disease, troponin T and low cholesterol lipoprotein.
**DISCUSSION**

Platelet function and biology is complex. It is difficult to comprehend all aspects of interaction of different mechanisms involving the platelets way of contributing to a thrombus formation. However, during the past few years, the literature expressing this field has literally exploded, and it has become clear that there is an intricate interplay between the endothelial system, inflammatory system and platelet-coagulation system. In the future, it is likely that we will have to control each single system in order to prevent or treat cardiovascular diseases optimally.

One of our hypotheses concerns how the inflammatory reaction secondary to myocardial muscle necrosis affects the platelets to become more active. It is earlier described that CRP peaks on day three during the course of AMI (23, 49, 53), which is in accordance to our experience. The platelet aggregation increased during the first days and peaked on day three, following AMI (paper II). Paper I showed a significant association between the concentration of serum CRP and platelet aggregation on day three. The platelet aggregation was most prominent among patients with pneumonia, UTI and fever (paper I).

Interestingly, the risk of thrombotic events is also highest the first days after a systemic inflammatory reaction. According to a sub-analysis of two clinical trials, analyzing mode and timing of recurrent ischemic events after ACS, the peak incidence of re-infarction also occurred on day three (5). Furthermore, as described in the introduction section, the risk of stroke is markedly high early following AMI and seems to be related to the size of the myocardial infarction. Finally, the risk for both AMI or stroke is highest in the three days following respiratory infection (16).

The link between the inflammatory acute response and the risk for new cardiovascular events could be either via the endothelial system or the activation of platelets. One might have expected a stronger relation between CRP and platelet aggregation. However, the platelets were influenced by the antiplatelet treatment given, probably biasing the results of the platelet function tests. The association between CRP and platelet aggregation might thereby have been attenuated. Furthermore, when considering the dynamics of CRP with rather abrupt rise to stimulus and a relatively short half-time, it is obvious that it is difficult to capture the true peak of the CRP concentration in blood, which might have given a stronger association. Besides CRP, there could be other cytokines or markers of inflammation, known or unknown, that have a stronger effect on platelet function. Nevertheless, it is likely that proinflammatory/thrombogenic cytokines are involved in causing platelets to be more reactive and more insensitive to antiplatelet treatment. This is indicated in paper I as aspirin non-responsiveness was twice more common among patients with pneumonia. An example of a possible inflammatory-thrombotic link has been suggested to be the formation of monocyte-platelet aggregates (MPAs). CRP has been shown to induce monocyte-platelet aggregation (45). The formation of MPAs in the blood is associated with acute cardiovascular disease.
Inflammation or infection also alters the normal antithrombotic function of endothelial cells, which might contribute to a more thrombotic environment (78). In analogy with respiratory infections increasing the risk for acute cardiovascular events by causing abrupt inflammatory changes in susceptible atherosclerotic lesions, it is not excluded that similar inflammatory mechanism might be responsible for the increased risk for vascular events after myocardial necrosis, induced by an infarction. The consequence is that the acute inflammatory response triggers further endothelial dysfunction and pronounced platelet reactivity, altogether acting in synergy in creating a more thrombotic environment. The mechanisms described are likely to be active not only in coronary arteries but at multiple sites, such as carotid and cerebral arteries in susceptible patients (8, 9).

Paper II shows that platelet aggregation is a dynamic process in AMI and tends to decrease after a few days although a longer follow-up with repeated measurements is necessary to confirm the decreasing trend. Our data in paper III does not support that new events in the long term are linked to more pronounced platelet aggregation during the acute course of a myocardial infarction. However, there is no doubt that the increased platelet reactivity in the early phase of AMI has an impact on the risk of new events, since further inhibition of the platelets early in the myocardial infarction process leads to less thrombotic events. The COMMIT trial (18) supports this by showing that by adding clopidogrel to aspirin in AMI, during a mean period of only 15 days, new cardiovascular events (AMI, stroke and death combined) decreased, OR 0.91 (95% CI 0.86-0.97). Despite the large number of participants enrolled in the study (45,852 patients), treatment with clopidogrel did not significantly reduce the risk of ischemic stroke (OR 0.84 CI 0.68-1.03). However, the frequency of ischemic stroke was lesser in the active treatment group, both in the COMMIT trial (18) and in the CLARITY-TIMI 28 trial (29). The CLARITY-TIMI 28 trial investigated early treatment with clopidogrel against placebo in 3491 patients with STEMI (29). This trial showed a 46% risk reduction of stroke in favour of treatment with clopidogrel (p=0.05). The explanation for absence of significant difference in these two trials could be explained by the lack of bolus dose of clopidogrel in the COMMIT trial and the lack of power in the CLARITY-TIMI 28 trial. A novel thienopyridene prasugrel, with a faster onset of action and more potent antiplatelet effect than clopidogrel, reduces the risk of the same composite endpoint further, as compared to clopidogrel, in patients with ACS (35). The benefit was seen as early as at day three. There was no significant difference between the two treatment groups in the rate of stroke. The greatest benefit appeared to be in patients with diabetes mellitus. This greater efficacy in antiplatelet activity of prasugrel is, however, accompanied with increased risk of bleeding (79). Another novel thienopyridene, ticagrelor was also more effective in preventing the composite endpoint than clopidogrel in the PLATO-study, but as in the prasugrel study, no difference was seen in the frequency of stroke between clopidogrel and ticagrelor (37).

In paper I we concluded that infection during the course of an AMI is related to more pronounced platelet reactivity. Those patients with an infection had higher
CRP concentrations as compared to patients not having infection. The concentration of CRP was also related to more pronounced platelet aggregation. Despite an association with inflammation neither more pronounced platelet aggregation nor did PFA-100 closure times independently predict new cardiovascular events in the long term follow-up. However, it is difficult to rule out a problem with insufficient power concerning the analysis of PA-200 and PFA-100 results as predictors of prognosis in the present study. To adequately explore the relationships in the short term, a larger study is needed since shorter follow-up time offers fewer events.

The poor prediction ability of platelet function tests is not surprising considering the complex mechanisms involved in the pathophysiology of thrombus formation. Various platelet tests do only measure a part of the platelet activation pathways and most of all they do not consider the two additional systems involved, namely, the immune system and the endothelial system, which the platelet function tests do not quantify. Moreover, the results of the PA-200 and PFA-100 have relatively marked variances and thus lack of power to discriminate outcome.

After an AMI, diabetics are at increased risk for adverse events at short term as well as long-term. Paper II confirms previous findings that reactive platelets are more frequent among diabetics as compared to non-diabetics. Despite antiplatelet and anticoagulant treatment, the platelet aggregation increases the first days among all patients with AMI, but significantly more among the patients with diabetes. Fibrinogen levels were higher in diabetic subjects which may have contributed to the enhanced platelet aggregation. Several other explanations for increased platelet reactivity in diabetics have been reported, such as increased reactivity to ADP, increased platelet turnover, oxidative stress resulting in aspirin insensitive thromboxane biosynthesis and impaired clopidogrel pro-drug formation to active metabolite. The result may impair the antiplatelet effect (20, 80).

There are conflicting reports whether CRP predicts cardiovascular recurrent events after an AMI. Most of these studies are small, sub-studies to clinical trials, retrospective and therefore biased by confounding factors and by publication bias, i.e., positive results are reported while negative results are not. Nevertheless, in studies showing an association between CRP and prognosis in AMI, the events are mainly death and not recurrent AMI (54, 56, 59, 81). However, the strength of the associations is weak and depends on the presence of confounding variables in the model-building stage. CRP does not contribute to the model when adding the significant interaction term, age by Killip class > 1, as shown in paper IV. The finding confirms earlier studies showing that CRP is not independently associated with death after AMI (56, 82). For that reason, the concentration of CRP, during AMI, has no value of clinical importance beyond clinical prognostic variables to predict death.

However, in pathophysiological perspectives, CRP in AMI is still of interest, since CRP might contribute to worse remodelling via complement activation (58). It has been shown in animal models that injecting authentic pure human CRP into rats after they have undergone coronary artery ligation or ischemia-reperfusion injury causes significant worsening of cardiac function and increased mortality. This adverse
effect of human CRP in rats is completely removed by administration of a specific CRP inhibitor drug, 1,6 (bis)-phosphocoline hexane. In contrary, genetically elevated CRP in humans does not increase all cause mortality (83, 84), which contradicts the support for CRP per se, to cause cardiovascular disease.

It still remains to clarify if the relatively high short-term risk of recurrent AMI and stroke following AMI and the high risk of first ever AMI and stroke in acute respiratory infection is caused by an inflammatory systemic reaction. The lack of association between CRP concentration in AMI and risk of subsequent AMI could theoretically be due to obscuration by the relation of CRP and death, since elevated CRP levels highly correlate with low left ventricular ejection fraction. Patients with the highest CRP levels also have the highest concentration of TnT, reflecting the degree of myonecrosis. TnT concentrations up to 0.63 µg/L are shown to be related to an increased risk of myocardial reinfarction. Above this level of TnT concentration the risk of infarction decreases but the risk for death increases. Such a relation has been explained by the fact that patients with the highest TnT concentrations have the least amount of functioning coronary tree left and sizable amounts of myocardium at risk (85).

The result in papers III and IV reinforces the importance of clinical variables such as heart failure, age, smoking status, atrial fibrillation, diabetes and impaired kidney function, all of which were associated with worse prognosis in multivariable analysis.

Limitations
There are limitations in this dissertation that must be clarified. The most important limitation concerns the use of new medical equipment not standardized to daily medical practice. The use of platelet aggregometers also requires well-standardized methods from the technique of taking blood samples, handling the test tube on its way to the laboratory, and preparing the samples finally to be analyzed in the aggregometer. Furthermore, it is also questionable if in vitro platelet aggregation in PRP, preceded by all the preparations including adding an agonist, mirrors the complex pathophysiologic circumstances occurring in vivo.

Soon after the platelet function test PFA-100 was developed, it was thought to be excellent for monitoring the effect of aspirin in platelets and to use as a bedside test because of its simplicity in daily practice. Over the course of time it has become evident that the PFA-100 is not specific for, nor predictive of any particular disorder (86). The results of PFA-100 seem to be influenced by several factors such as age, gender, smoking and various blood parameters. The International Society on Thrombosis and Haemostasis Scientific and Standardization Subcommittee (ISTH SSC) concluded in 2006 that use of the PFA-100 in therapeutic monitoring of platelet function is currently best restricted to research studies and prospective clinical trials (87).

The diagnosis of infectious states such as pneumonia and UTI was made by the physician on duty, not participating in the study. Since the clinical diagnosis was not standardized, it is possible that some patients were misclassified. For instance it is not always obvious, in a clinical situation, if the patient is suffering of pneumonia or
heart failure. Furthermore, the frequency of aspirin non-responsiveness was twice as common in the group with pneumonia, but since the sample size is limited, the finding is questionable.

In the present thesis, the central statistical models used were based on regression models such as linear regression, logistic regression and Cox regression. The difficulty of the models may be controlling the presence of potential confounding factors and/or interactions in a model. Furthermore, the assumptions of a statistical model should not be violated and the criteria required for the sample size, concerning both population and the frequency of events, should be met. A well known rule of thumb is the 1 in 10 rule: for linear models, this means that one predictor can be used for every 10 patients; for logistic or Cox regression one predictor can be studied for every 10 events (88, 89). Another rule of thumb is the 1 in 50 rule. When this rule is satisfied you can safely use the stepwise selection with the default p-value of 5%, since the power for selection of true predictors is large and the risk for biased stepwise selection is limited (89). The rules are rules of thumb, based on limited research. However, in our statistical models in the present thesis we have carefully selected co-variables which are known to contribute to the model. The variables are also known to affect the event studied. This approach was uncomplicated in paper IV, since outcome was diseased or not. Moreover the variables are well-known prognostic co-variables. In contrary, the variables contributing to the models showing associations with platelet reactivity were more uncertain since the knowledge of co-variables affecting platelet reactivity is limited. In paper IV the 1 in 50 rule was not met. But since the co-variables retained were well-known risk factors affecting prognosis in AMI, we concluded that no major violation had occurred. Moreover, the continuous variables age, CRP and GFR were checked for linearity in the logit. The procedure is exemplified by using the variable age and is as follows: the first step was to split the data of the variable age into quartiles. The grouping was thus based on the 25th, 50th and 75th percentiles resulting in about equal number of values in each group. The midpoints of the four groups were then calculated. Finally, univariate logistic regression was performed for each group. The values of coefficients and constants were obtained and thus the logit for each group by

\[
\text{Logit} = \text{Midpoint} \times \text{Coefficient} + \text{Constant}
\]

Fig. 5 shows the graph of the logit obtained from the univariate analysis of each subgroup versus the midpoint of subgroups of the variable age.
Figure 5. Logit versus midpoint of subgroups for age. Age is reasonably linear in the logit.

The other continuous variables GFR as well as CRP were also reasonably linear. Logit versus midpoints of CRP is shown in Fig. 6.

Figure 6. Logit versus midpoint of subgroups for CRP. CRP is reasonably linear in the logit.
An obstacle in observation studies is how to deal with exclusions and missing data. In paper II, there was no major difference between patients who were included and those who were not, except for values of maximum TnT and a limited use of ACE-inhibitors or ARBs. In paper I and II participants were excluded due to missing data of the main analyses i.e. the results of platelet function tests, which may affect the conclusions. In addition, the consequence was a limited study group with less power.
IMPLICATION AND FUTURE RESEARCH

To conduct high quality studies concerning platelet function and the risk of future cardiovascular events, we need better validated, accurate, standardized platelet function tests. The need is crucial for better understanding of the mechanisms involved in platelet thrombus formation in acute myocardial infarction. Point of care platelet function tests would facilitate the application.

In the present studies, platelet function was measured in patients on antiplatelet and anticoagulant treatment. It would be interesting to assess platelet reactivity in patients with acute systemic inflammatory conditions such as acute influenza or pneumonia but without cardiovascular disease and antiplatelet treatment. In such patients it would be of interest to assess the strength of any association between CRP or other inflammatory markers and platelet function.

Future prospective studies should include baseline and follow-up measurements of platelet function in patients with accurately monitored compliance of long-term antiplatelet use. Studies with platelet function tests before and after antiplatelet treatment should also be performed, and in patients with high residual platelet reactivity it should be tested if tailoring the antiplatelet treatment would improve the outcome.

Future studies should also address in what manner inflammation affects the platelets. It should be explored if the mechanisms involved are COX-1 dependent or not. If not, which alternative pathway is activated? Clinical studies on inhibiting the acute inflammatory responses during AMI are warranted.

Since inflammation plays a central role in atherosclerosis and in the development of ACS, future studies testing medical treatments that affect inflammatory mechanisms are needed. For example, elevated plasma levels of Lp-PLA$_2$ are associated with an increased risk of cardiovascular events. Lp-PLA$_2$ is a product of inflammatory cells and the enzymatic activation of Lp-PLA$_2$ produces pro-inflammatory and pro-apoptotic mediators. Darapladib and varespladib, Lp-PLA$_2$ inhibitors, have shown dose-dependent inhibition of plasma and intra-plaque Lp-PLA$_2$ activity (90). The results of ongoing event-driven outcome trials will show if the Lp-PLA$_2$ inhibitors are of clinical value.

Finally, by preventing influenza and pneumonia by vaccination we may reduce the risk of recurrent myocardial infarction, sudden cardiac death and stroke (61, 91, 92).
CONCLUSIONS

The following conclusions were drawn from the studies:

- Platelet aggregation is related to the inflammatory response in acute myocardial infarction (I, III).

- Pronounced platelet aggregation is seen among patients with infection during the course of acute myocardial infarction, as compared to patients not having infection (I).

- The platelet aggregation increases the first days during the course of AMI and is more pronounced among patients with diabetes mellitus (II).

- After AMI the concentration of CRP is associated with a worse prognosis concerning AMI, stroke and death, but the strength of the association depends on which adjustments for confounding factors that are made (III).

- The results of the platelet function tests, PA-200 and PFA-100, show no independent association with a worse prognosis after an acute myocardial infarction (III).

- The concentration of CRP has no value of importance beyond clinical variables to predict death after AMI (IV).
Det är allmänt accepterat att inflammatoriska processer bidrar till utveckling av åderförkalkning i hjärtats kransväg. Studierna i denna avhandling visar att den akuta inflammatoriska reaktionen vid akut hjärtinfarkt bidrar till aktivare blodplättar. Att blodplättarna aktiveras ytterligare kan vara en bidragande orsak till uppkomst av ny hjärtinfarkt, slaganfall och död.

Tidigare forskning har visat att risken för slaganfall under dagarna efter akut hjärtinfarkt är anmärkningsvärt hög. Det har också visats att risken för ny hjärtinfarkt är högst under de första dagarna. Risken för nytt slaganfall efter hjärtinfarkt avtar därefter snabbt med tiden. Det har gjort att man spekulerat i huruvida den inflammatoriska reaktionen under akut hjärtinfarkt kan vara orsaken.

Epidemiologisk forskning har dessutom visat att risken för hjärtinfarkt eller slaganfall är hög de närmaste dagarna efter insjuknande i influensa eller lunginflammation. Dessa tillstånd orsakar en akut inflammatorisk reaktion. Huvudsyftet med studierna i denna avhandling är att undersöka huruvida den inflammatoriska reaktionen påverkar blodplättarna till att bli mera aktiva, dvs., att ”klumpa ihop” sig, och om detta innebär sämre prognos för patienter med uppmätt förhöjt inflammatorisk reaktion.

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