Inflammatory Markers and Prognosis in Acute Coronary Syndromes

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Stockholm 2010
“I hate writing. I love having written”  Mark Twain

To my parents and teachers
Abstract

**Background:** Inflammation both accelerates atherosclerosis and contributes to the activation and rupture of the atherosclerotic plaque. Several markers of inflammation, such as C-reactive protein (CRP), have shown prognostic merit in patients with acute coronary syndromes (ACS).

**Aims:** To investigate the association between circulating levels of some markers with relation to inflammation – osteoprotegerin (OPG), Chemokine (C-X-C motif) ligand 16 (CXCL16) and chromogranin A (CgA) – and prognosis in a population of patients with ACS.

**Material and methods:** Patients aged 18-79 years who were admitted to the coronary care unit at a university hospital with an ACS had blood drawn within 24 hours and after 3 months; OPG, CXCL16 and CgA concentrations were determined by an enzyme immunoassay using commercially available antibodies. Echocardiography with determination of the left ventricular ejection fraction (LVEF) was performed within 5 days of admission. Mortality data were obtained from the Swedish National Population Registry and morbidity data from the Swedish Hospital Discharge Registry. The length of follow-up was a median of 81-92 months.

**Results:** Higher OPG levels were associated with an increased likelihood of ST-elevation myocardial infarction (MI), markers of myocardial damage and indices of cardiac dysfunction such as LVEF and B-type natriuretic peptide (BNP). The patients with the higher levels were also more likely to have a history of heart failure (HF) and to be hypotensive on arrival. The circulating OPG levels were predictive of long-term mortality and the incidence of rehospitalization due to HF, a relationship that remained significant after adjustment for clinical risk factors and, in a subgroup where such data were available, after further adjustment for LVEF, CRP, BNP and troponin. The C-statistics of the prognostic information offered by OPG were significantly better than CRP and troponin and similar to BNP and LVEF. For CXCL16, as for OPG, higher levels were associated with higher age and ST-elevation MI. CXCL16 predicted long-term mortality, future hospitalizations for HF and new MI, also after adjustment for clinical risk factors. After further adjustments for LVEF, CRP, proBNP and troponin, only the combination of OPG and CXCL16 serum levels predicted cardiovascular (CV) and all-cause mortality, as well as HF rehospitalizations. This was true for both the long term and short term, even after adjustment for the Global Registry of Acute
Coronary Events (GRACE) score. Serum levels of OPG and CXCL16 at day 1 and 3 months after ACS were similarly associated with outcome.

CgA was also an independent predictor of mortality and HF after adjustments for conventional risk factors, including troponin, and was still significantly associated with mortality after further adjustment for LVEF and proBNP.

**Conclusions:** Even after adjustment for conventional risk markers, serum levels of OPG, CXCL16 and CgA were predictive of long-term mortality and rehospitalizations due to HF in patients with ACS. We also found that a combination of OPG and CXCL16 serum levels was predictive of mortality and HF hospitalizations - and gave more information than either marker alone - in both the long term and the short term, even after adjustment for the GRACE score. Inflammatory markers appear to add prognostic value above and beyond clinical information.

Key words: acute coronary syndrome, prognosis, atherosclerosis
Svensk sammanfattning


I Sverige är hjärt-kärlsjukdom den bakomliggande dödsorsaken hos 42 % av kvinnorna och 41 % av männen som dör. 2007 inträffade 665 fall av akut hjärtinfarkt per 100 000 män och 467 fall per 100 000 kvinnor ≥20 år. Dödligheten minskar – bland annat pga att kriterierna vidgats och mindre hjärtskador än tidigare ger diagnosen hjärtinfarkt, men också för att flera behandlingar tillkommit som förlänger livet hos personer med hjärt-kärlsjukdom och för att förebyggande åtgärder börjat få effekt- men fortfarande dör drygt 15 % av dem som vårdas på sjukhus för en hjärtinfarkt inom 28 dagar enligt Socialstyrelsens siffor.

Målsättningen med avhandlingen var att undersöka om några ämnen som stiger vid inflammation och därfor kan ses som markörer för farlig ateroskleros skulle kunna användas för att bättre gruppera patienter med ett akut koronarsyndrom utifrån deras risk att dö eller få en ny hjärtinfarkt alternativt utveckla hjärtsvikt.

I arbetena som ingår i denna avhandling undersökte vi relationen mellan koncentrationen av de här inflammationsmarkörerna i blodprover tagna det första vårdtyget hos patienter med akuta koronara syndrom och dödlighet samt hjärtrelaterad sjuklighet under en uppföljningstid av c:a 7 år.

Bland de patienter vi undersökte var högre nivåer av OPG, CXCL16, en kombination av dessa två eller CgA förenat med en ökad dödlighet, även efter att vi korrigerat för kliniska riskfaktorer. Likaledes var höga värden av OPG, CgA och CXCL16 eller OPG/CXCL16-kombinationen förknippade med en ökad risk för att behöva sjukhusvårdas pga hjärtsvikt, även efter korrigerande för kliniska riskfaktorer. CXCL16 var också förknippat med en ökad risk för att komma att sjukhusvårdas för hjärtinfarkt. Resultaten var oberoende av vilken diagnos patienten fick vid det första vårdtillfället, och de kan bidra till utformningen av en optimerad riskstratifiering och förebyggande behandling.
List of publications


II. Jansson AM, Aukrust P, Ueland T, Smith C, Omland T, Hartford M, Caidahl C.


IV. Jansson AM, Røsjø H, Omland T, Karlsson T, Hartford M, Flyvbjerg A, Caidahl K.
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## Abbreviations

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<td>ACE-I</td>
<td>Angiotensin-converting enzyme inhibitor</td>
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<td>ACS</td>
<td>Acute coronary syndrome</td>
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<td>ADAM</td>
<td>A disintegrin and metalloproteinase domain</td>
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<td>ARB</td>
<td>Angiotensin receptor blocker</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BNP</td>
<td>B-type natriuretic peptide</td>
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<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
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<td>CAD</td>
<td>Coronary artery disease</td>
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<td>CCU</td>
<td>Coronary care unit</td>
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<tr>
<td>CgA</td>
<td>Chromogranin A</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CK-MB</td>
<td>Creatinine kinase MB fraction</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CV</td>
<td>Cardiovascular</td>
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<tr>
<td>CXCL16</td>
<td>Chemokine (C-X-C motif) ligand 16</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ESC</td>
<td>European Society of Cardiology</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>HF</td>
<td>Heart failure</td>
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<td>HR</td>
<td>Hazard ratio</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>NSTE-MI</td>
<td>Non-ST-elevation myocardial infarction</td>
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NF-κB  Nuclear factor-kappa-B
OPG  Osteoprotegerin
PCI  Percutaneous coronary intervention
PRACSIS  Prognosis and risk in acute coronary syndrome in Sweden
RANK  Receptor activator of nuclear factor-kappa-B
RANKL  Receptor activator of nuclear factor-kappa-B
SD  Standard deviation
SMC  Smooth muscle cell
SR  Scavenger receptor
SR-PSONX  Scavenger receptor for phosphatidylserine and oxidized LDL
STEMI  ST-elevation myocardial infarction
TGF  Transforming growth factor
TNF  Tumor necrosis factor
TnI  Troponin I
TnT  Troponin T
TRAIL  Tumor necrosis factor-related apoptosis-inducing ligand
UAP  Unstable angina pectoris
VCAM-1  Vascular cell adhesion molecule 1
Introduction

In this thesis, the usefulness of a few markers of inflammation is examined with regard to their ability to predict morbidity and mortality in a population of patients experiencing an ACS.

As the majority of research articles on the subject tend to begin, cardiovascular (CV) disease is the leading cause of death in the western world. In fact, searching for the phrase “CV disease is the leading cause of death in the western world” on the search engine Google.com gives about 55,600 replies. In 2001, ischemic heart disease was the leading cause of death not only in high-income countries but also in low- and-middle-income nations.

Acute coronary syndromes

ACS is a term used for MI with and without ST elevation (STEMI and NSTEMI respectively) and unstable angina pectoris (UAP). It is most commonly caused by the rupture of an atherosclerotic plaque with subsequent thrombus formation, followed by ischemia and often the necrosis of dependent heart muscle.

MI is the most common cause of death in middle-aged men and women in Sweden. In 2007, there were 665 acute MIs per 100,000 men and 462 per 100,000 women aged ≥ 20 years. In 1995, 41% of men who had heart attacks died within 28 days, as did 45% of women. In 2001, the diagnostic criteria for acute MI were changed, resulting in more patients, with less serious disease, being diagnosed with MI. Because of this, the mortality from MI in Sweden appeared to decrease even more rapidly than was actually the case. In 2007, the lethality within 28 days of MI was down to 28% for men, and 32% for women. The main reason for the decrease in mortality, however, is the reduction of risk factors in the Swedish population, and the second reason is improved treatment.

In Sweden in 2008, CV disease was the underlying cause of death in 42% of women and 41% of men. Both the incidence and mortality of MI are strongly related to both age and gender; after adjustment for age, men are almost twice as likely to die from MI as women.

Myocardial infarction

An acute MI is defined as myocardial cell death due to prolonged myocardial ischemia. It is the necrosis of cardiac myocytes in response to ischemia due to insufficient blood supply, usually secondary to arterial occlusion, commonly manifested as retrosternal chest
pain/pressure, frequently accompanied by ECG changes. The present definition of MI was introduced in 2007 by representatives of the American College of Cardiology, the American Heart Association, the European Society of Cardiology (ESC) and the World Heart Foundation, making troponins the preferred biomarkers. An increase in the cardiac troponin concentration (I or T) greater than the 99th percentile of a reference population together with evidence of ischemia with at least one of the following:

- Symptoms of ischemia
- ECG changes of new ischemia (new ST-T changes or new left bundle branch block)
- Development of pathological Q waves on the ECG
- Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality

are considered to be diagnostic of MI.\(^5\)

**Unstable angina pectoris**

UAP can be new-onset angina or a sudden change in the pattern of previously stable angina, including pain during rest and accelerating angina, which may signal an impending heart attack. The line between what is UAP and what is MI has been moved with the new definition of MI, resulting in more patients who would previously have been given the diagnosis of UAP now receiving a diagnosis of NSTEMI.

**Endpoints**

**Mortality**

CV mortality dominates in patients who have had an ACS and is caused either by a new MI/related sudden death or by HF/lethal arrhythmia. In a previous study on the PRACSIS cohort, 5-year mortality was higher in NSTEMI patients before, but not after adjustment for risk markers.\(^6\) In a recent study on the UK and Belgian part of the GRACE, 5-year morbidity and mortality were as high in NSTEMI and UA patients as in those with STEMI.\(^7\)

**Ischemic heart failure**

HF is a common disease. In Sweden, an estimated 200,000 have HF that gives symptoms and at least as many have latent HF. The prevalence increases with age; about 1% of 50-year-olds have HF, as compared to 10% of those aged 70 and over. In 2007 in Sweden, 22,000 patients
were hospitalized for HF and 3,800 died.\textsuperscript{5} Mortality is high in subjects with HF; in a recent article, HF was associated with an unadjusted case-fatality rate of 59\% within 5 years in Sweden.\textsuperscript{9} Early diagnosis and treatment improve both the prognosis and quality of life.\textsuperscript{10-13} HF results from a variety of underlying conditions, such as ischemic heart disease, hypertension, valvular disease, cardiomyopathies, myocarditis and diabetes. Several definitions of HF have been suggested over the years. The ESC states that HF is a syndrome with the following traits:\textsuperscript{14}

- HF symptoms; typically shortness of breath and/or fatigue
- Signs of fluid retention such as pulmonary congestion or ankle swelling
- Objective evidence of an abnormality of the structure or function of the heart at rest

HF is a progressive disorder characterized by the dysregulation of several physiological systems. The first step is usually damage to the myocardium with ensuing remodeling, pump dysfunction, neurohormonal activation and salt-water retention. Both neurohormonal activation and hydrosaline retention are compensatory mechanisms that initially increase stroke volume and peripheral resistance, i.e. have inotropic effects to help maintain the mean arterial perfusion pressure. Later in the process, however, these mechanisms prove deleterious. The heart is subjected to structural changes: the loss of microfilaments, apoptosis and the disorganization of the cytoskeleton, disturbances in calcium hemostasis, alterations in receptor density, signal transduction and collagen synthesis and the degradation of the matrix by matrix metalloproteinases (MMPs).

**Recurrent MI**

In the UK-Belgian cohort of the GRACE, about 18.2\% of the patients with an index diagnosis of ACS experienced one or more MIs during 5 years of follow-up.\textsuperscript{7} On average, each patient was rehospitalized 1.6 times for suspected ACS, less than 10\% of those hospitalizations, however, resulted in a new diagnosis of MI.

**Stroke**

According to the definition used by the World Health Organisation (WHO), a stroke is “a neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours”. There are two main categories of stroke: ischemic and hemorrhagic, with ischemic being the most common. Ischemic strokes result from the interruption of blood supply by a clot, either formed in the arteries; locally (thrombotic) or elsewhere in the body
(embolic); or in the veins. Hemorrhagic stroke is caused by the rupture of a blood vessel. Stroke is, after ischemic heart disease and cancer, the third leading cause of death in Sweden. In the GRACE publication mentioned above, about 12.9% of ACS patients sustained one or more strokes during 5-year follow-up.7

Clinical risk stratification

A large number of models for risk stratification in patients with an ACS have been proposed; they are composed of clinical variables, laboratory markers or both. Many of the risk scores have been derived from large clinical trials, such as Thrombolysis In Myocardial Infarction (TIMI), perhaps the one most widely used, even though the GRACE score has been shown to be superior at least in NSTEMI and UAP.15, 16 Moreover, clinical trials tend to have strict criteria for inclusion and exclusion, making the results less generalizable than those from a registry such as GRACE (for further information see below).

Clinical risk factors

The Framingham heart study17 first introduced the term “risk factor” in modern medical literature. The CV risk increases with age, a family history of CV disease and male gender, ie factors that are not possible to modify. There are, however, risk factors that can also be controlled and risk factors we still have some hope of being able to treat.

Smoking is known not only to accelerate atherosclerosis but also to increase the risk of an acute event and smokers are more likely to present with STEMI than non-smokers.18 In areas where bans on smoking have been evaluated, rates of MI have decreased,19-21 even in areas were MI rates were increasing at the time.22 Smokers actually have a better prognosis when having an MI, but this is explained by the fact that they are more likely to have an MI at a younger age.23, 24 In several large, observational studies, smoking cessation in patients with known coronary artery disease (CAD) is associated with a substantial reduction in mortality compared with those that continue to smoke. In one study, a 36% reduction in the crude relative risk (RR) of mortality for patients with CHD who quit compared with those who continued smoking (RR, 0.64; 95% CI, 0.58-0.71) was observed.25 Diabetes and its precursor, reduced glucose tolerance, are strong risk factors, but they are possible to affect or even prevent by regular exercise and maintaining a normal weight. Mortality is high in diabetics after a first MI,26 and women younger than 65 years with diabetes have a poorer outcome than
men after MI. Obesity in itself, particularly abdominal obesity, increases the risk of CAD. Hypertension, especially high systolic pressure, is a risk factor for CV events and antihypertensive drug treatment reduces the risk. Dyslipidemia, one of the dominant risk factors for atherothrombosis, can be a result of both hereditary factors and lifestyle. Dyslipidemia, although often referred to as “hypercholesterolemia”, refers not only to high circulating levels of LDL, the “bad cholesterol” and lipoprotein a, but also to decreased levels of HDL and to increased levels of triglycerides. The lowering of cholesterol with statins improves survival and reduces CV events in patients with CAD. Statin treatment also reduces mortality after MI. Even mildly impaired renal function is associated with an increased risk of MI and mortality in the general population. The Cockcroft-Gault method for estimating creatinine clearance is better than the Modification of Diet in Renal Disease (MDRD) method at predicting outcome after MI, but cystatin C could be a better measure than creatinine. Obesity as measured by waist-to-hip ratio is also a major risk factor.

Dietary factors play a role; the dietary approaches to stop hypertension (DASH) diet reduces CV risk compared to both a diet rich in fruit and vegetables and a control diet while a diet rich in fruits and vegetables reduces CV risk compared to controls. What food is rinsed down with matters, too; drinking too little is related to increased coronary risk, while high alcohol consumption (> 14 drinks a week if you are a man and > 9 drinks if you are a woman) is associated with increased mortality and a risk of alcohol-related health problems. Hyperhomocysteinemia, which may be caused by hereditary factors, renal impairment and lack of vitamins B12, B6 and folic acid, is associated with an increased risk of CAD. Since there is a mechanistic explanation in that homocysteine appears both to damage the endothelium, thereby facilitating plaque formation, and to be pro-thrombotic, it was believed that homocysteine might be a treatable risk factor, by supplementing the above-mentioned vitamins. According to three recently published randomized trials, however, folate and B12 supplementation are probably neither friend nor foe. Lack of physical activity is a major risk factor and aerobic training like walking seems to be protective. Depression is associated with a poorer prognosis in ACS. Personality is also of importance. Those people who have a propensity towards psychological stress run a higher risk, and so do those displaying cynical hostility. The effects seen in the hostile, however, appear to be mediated by behavioral risk factors: they are not nice to themselves either.
**Scoring systems**

A number of scoring systems have been developed for risk stratification in the general population (Framingham) and in coronary disease patients (TIMI, GRACE). For ACS patients, GRACE is perhaps the most frequently applied scoring system and it appears to have some advantages.\textsuperscript{15, 16} The Global Registry of Acute Coronary Events (GRACE) is a large multinational registry of ACS patients\textsuperscript{49} from which a risk prediction model has been developed and further elaborated on. The original GRACE score was created as a prediction tool for in-hospital mortality in ACS\textsuperscript{50} and it is sometimes referred to as the Granger score. The model consists of 8 parameters: age, Killip class, systolic blood pressure, ST-segment deviation, cardiac arrest during presentation, serum creatinine level, positive initial cardiac enzyme findings and heart rate and it has a very good discriminatory ability. The C-statistics were 0.83 and 0.84 in the derived and confirmation GRACE data set respectively and 0.79 in the GUSTO-IIb database that was used for external validation. Another version of the model, tailored to predict 6-month mortality across the spectrum of ACS, used 9 variables: age, history of MI, history of HF, increased pulse rate at presentation, lower systolic blood pressure at presentation, elevated initial serum creatinine level, elevated initial serum cardiac biomarker levels, ST-segment depression on the presenting electrocardiogram and not having a percutaneous coronary intervention performed in hospital. The C-statistics were 0.81 for the development cohort and 0.75 for the validation cohort.\textsuperscript{51} Another version of the score uses 9 parameters to predict death and the composite variable of death and MI over the 6 months after presentation with an ACS:\textsuperscript{52} age, development or history of HF, peripheral vascular disease, systolic blood pressure, Killip class, initial serum creatinine concentration, elevated initial cardiac markers, cardiac arrest on admission and ST segment deviation. The discharge version of the score has been shown to predict mortality over up to four years of follow-up\textsuperscript{53} and, in a recently published article, up to six years.\textsuperscript{54} The Granger score predicted HF as well as mortality in a recent publication.\textsuperscript{55} The C-statistic was GRACE 0.663 (95\% CI 0.554-0.773), p=0.010, as compared to that of BNP: 0.689 (95\% CI 0.565-0.812), p=0.003. However, in that respect, the thrombolysis in MI risk index (TRI) did even better: TRI 0.714 (95\% CI 0.609-0.819), p= 0.001.

**Risk markers**

Risk determinants are risk factors (the players) and markers (the innocent bystanders).
Introduction

Since the number of research articles on new CV markers is growing rapidly, Morrow and de Lemos postulated three criteria in Circulation in 2007\textsuperscript{56} for the appraisal of their clinical value:

(1) Can the clinician measure the biomarker?
(2) Does the biomarker add new information?
(3) Will the biomarker help the clinician to manage patients?

With the publication of INTERHEART, a large case-control study that concluded that more than 90\% of CV disease can be attributed to 9 conventional risk factors,\textsuperscript{57} those opposed to further research on risk determinants appeared to be right – could laboratory markers really add anything of real value to that? It appears, however, that they actually can. C-statistics nearing 1 are not the only measures of a good risk marker in CVD/CHD.\textsuperscript{58} Also, INTERHEART did not specifically study ACS patients. Khan et al. actually showed that NT-proBNP is better than the TIMI risk score at predicting death after MI.\textsuperscript{59}

i. Markers of myocyte necrosis

In 1954, aspartate aminotransferase (AST), a non-specific marker of cytoplasmatic constituents, was introduced as the first biomarker of myocardial injury. Then came measurements of enzyme activity, followed by markers more specific to the heart, such as CK-MB measured by immunoassay. Only 20 years ago, these markers of myocyte necrosis were the only risk markers known and used in ACS patients. Now, the most commonly used markers are the troponins – in the heart, three types exist: TnT, TnI and TnC, but only I and T are heart specific and commercially available as markers. With troponins, the opportunity not only to diagnose myocardial injury but also to identify patients running a high risk of myocardial injury made its appearance. Since several clinical conditions apart from MI cause an increase in circulating troponin levels, the diagnosis of MI now requires troponin elevation within an adequate clinical context. Both TnT\textsuperscript{60-62} and TnI\textsuperscript{63, 64} have been shown to predict mortality in ACS.

ii. Markers of cardiac function

B-type natriuretic peptide (BNP) is commonly used as a blood test for cardiac function and neurohormonal activation. It is a natriuretic hormone that is produced by myocytes in the ventricle wall of the heart in response to LV stress. ProBNP, consisting of 108 amino acids, is its precursor. It is cleaved into BNP, a 32 amino acid polypeptide, 3.5 kDa in size, and NT-
proBNP, which is 76 amino acids long and 8.5 kDa in size, biologically inactive and has a longer half-life than BNP, approximately 120 minutes and approximately 20 minutes respectively. Since BNP levels are significantly higher in patients with HF than in healthy subjects, or subjects with a cause for dyspnea other than HF, it is frequently used as a diagnostic test for ruling HF in or out. BNP is an indicator of LV ejection fraction (EF) and predicts mortality after MI. It is also predictive of mortality in other ACS patients. Serum levels of BNP and proBNP are lower in obese subjects with HF. Reduced levels are seen in treatment with angiotensin converting enzyme inhibitors (ACE-I), angiotensin receptor blockers (ARB), aldosterone antagonists, and diuretics may reduce BNP beyond hemodynamic effects. Levels may be elevated in conditions other than HF, such as LV hypertrophy, myocardial ischemia, tachycardia, right ventricular overload, cirrhosis, low glomerular filtration rate (GFR), diabetes and severe infection, and the levels are generally higher in women than in men and also increase with age. BNP is eliminated from plasma by natriuretic peptide receptors and degraded by neutral endopeptidases, but also through glomerular filtration, the only route of elimination of importance for NT-proBNP. It appears that BNP has greater variability, both within hour and within week, than NT-proBNP, at least in stable HF patients. So which marker should be used? So far, no difference of any real importance relating to prognosis potential has been demonstrated.

**Echocardiographic measurement of LVEF** is not only a diagnostic tool in HF, it is also a prognostic marker in ACS patients and provides additional prognostic information to the TIMI risk score. In PRACSIS, we have demonstrated an association between LVEF and mortality (dichotomized, LVEF <40% and ≥40%) that was significant after adjustment for clinical information.

**Pathophysiology and search for new risk markers**

Atherosclerosis is the underlying process in MI, angina, stroke and ischemic heart HF. The pathophysiology of atherosclerosis has attracted enormous interest over time and it has become evident that inflammation is a key factor. Lately, therefore, efforts to detect not only vulnerable plaque but also the vulnerable patient have increasingly involved inflammatory and other biomarkers.

The word atherosclerosis is Greek and consists of the word *ather* which actually means “porridge” but is used to describe the focal accumulation of lipids, and *sclerosis*, meaning “hardening” that refers to the thickening of the arterial intima.
Rudolf Virchow was perhaps the first to consider inflammatory changes in the artery to be of primary importance in atherosclerosis. This belief got him into a controversy with pathologist Carl von Rokitansky who thought the inflammatory changes were secondary. Recent research\(^8\) and modern investigations of Rokitansky's specimens\(^8\) however, appear to prove Virchow right. Atherosclerosis is now regarded as an inflammatory disease triggered by the accumulation of LDL in the arterial wall.

The atherosclerotic lesion

The normal artery wall is composed of three layers; the intima, the innermost layer which includes the endothelium and is separated from the other two by the internal elastic lamina, the media is the middle layer, consisting of smooth muscle cells (SMCs), and the adventitia is the outermost, connective tissue layer. Large arteries are supported by small vessels, the vasa vasorum, in the adventitia, which is where the nerves enabling vasomotor activity (dilation and constriction) are also located.

A diffuse intimal thickening (DIT) seems to be a prerequisite for atherosclerosis.\(^8\) The first morphological change seen in early atherosclerosis is the fatty streak, an extracellular deposition of lipids in the outermost layer of DIT. LDL particles are retained in the extracellular matrix, where they are then oxidized, in response to elevated plasma levels of LDL.\(^8\) LDL then releases phospholipids that activate the endothelial cells (ECs) into expressing leukocyte adhesion molecules such as VCAM-1.\(^8\) Leukocytes adhere to the endothelium and migrate into the intima in response to cytokines such as CX3CL1 (fractalkine), CCL5 (RANTES) and CCL2 (MCP-1). Macrophage-colony stimulating factor (M-CSF) induces the differentiation of monocytes into macrophages, while scavenger receptors (SRs), such as CXCL16 and Toll-like receptors (TLRs) are upregulated on the cell surface. SRs mediate the uptake of oxidized LDL (oxLDL) particles by macrophages, turning them into foam cells. The TLRs initiate the activation of inflammation, which may result in the release of several vasoactive molecules, such as leukotrienes and nitric oxide (NO). SMCs are recruited from the media to the cap surrounding the lipid core, which, apart from SMCs, also contains a collagen-rich matrix. As the person ages and the disease becomes more advanced, fibrotic and calcific layers add to the plaque. Inside the plaque, the immune cells that are seen are predominantly macrophages and T-cells.\(^8\) Macrophages and vascular cells in a forming plaque produce CCL5 (RANTES), CXCL10 and CXCL11, all of which attract T-cells. They also express CXCL16, and CCL11 - a mast cell attractant. Activated T cells differentiate into Th1 effector cells that produce interferon-\(\gamma\) (IFN-\(\gamma\)). IFN-\(\gamma\) in turn activates
macrophages and vascular cells, resulting in vascular inflammation that propagates atherosclerosis.\textsuperscript{80, 89, 90} The effect of plaque growth on the lumen diameter tends to be counteracted by the enlargement of the artery, usually referred to as Glagov’s phenomenon.\textsuperscript{91}

\textbf{Figure 1.} Evolution of atherosclerosis. a. EC dysfunction and activation under pro-inflammatory conditions of hyperlipidemia leads to early platelet aggregation and leukocyte adhesion and increased permeability of endothelium. b. Monocytes accumulate lipids and transform into macrophages or foam cells, which results in fatty streaks. c. Apoptosis of macrophages and other plaque cells creates a necrotic core, and a fibrous cap that consists of matrix and an SMC layer forms. Neovascularization can occur within the plaque and from the adventitia, and leakage of fragile vessels can lead to plaque hemorrhage. d. Thinning and erosion of the fibrous cap in unstable plaques, for example, owing to matrix degradation by proteases, ultimately results in plaque rupture, with release of debris, activation of the coagulation system and plaque thrombosis of the artery. This leads to arterial occlusion and MI or stroke. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol 2008;8:802-15, copyright 2008.
When a plaque ruptures or is eroded, platelets and the coagulation cascade are activated by substances inside the plaque and a thrombus is subsequently formed. As the thrombus grows or embolizes into narrower parts of the artery, the arterial occlusion causes ischemia, followed by the necrosis of dependent tissue. Since a large proportion of MIs are caused by plaque rupture,\(^92\) which in its turn is more affected by the vulnerability of the plaque than the size of the plaque or its effect on the lumen diameter, it is not easy to predict which plaques are potentially dangerous, at least not from imaging techniques alone. In sudden coronary deaths, approximately two-thirds of coronary thrombi are organizing, particularly in young individuals and in women.\(^93\)

**Inflammation in cardiovascular disease**

Since the hypothesis that inflammation contributes to CV disease was first formulated, numerous publications have confirmed the relationship.\(^79, 80, 94\) There is also an association between CV disease and other inflammatory diseases; for example, the incidence of MI is increased in subjects with SLE,\(^95\) RA,\(^96\) gout\(^97\) and psoriasis.\(^98\) Heart rate variability, an independent predictor of mortality in ACS,\(^99, 100\) is inversely related with inflammatory markers in subjects with CV disease as well as in healthy subjects.\(^101\)

**i. Immune system in atherosclerosis**

Inside the plaque, macrophages are the dominant cells, followed by T-cells. Neutrophils are few, suggesting chronic, not acute, inflammation. B-cells are also few in number, suggesting that humoral immunity is not important. CD4\(^+\) T-cells (CD4\(^+\) being a cell-surface glycoprotein that acts as a co-receptor for the T-cell receptor) help determine the extent – and nature – of the immune response, mainly through cytokine secretion. CD4\(^+\) T-cells coordinate and take part in the defense against external pathogens. Initially naive, the CD4\(^+\) T-cells differentiate into effector T-helper cells upon stimulation by antigen presenting cells (APCs). Cytokines determine if the T-helper cell will become type I (immunity against intracellular pathogens by secretion of interleukin-2, (IL-2), IL-12 and IFN-\(\gamma\)) or type 2 (clearance of extracellular pathogens and secretion of IL-4, IL-10 and IL-13). The cellular immune response to modified LDL antigen plays a crucial role in atherosclerosis. IL-10 and TGF-\(\beta\) exert anti-atherogenic effects. T-cells promote atherogenesis. In a plaque, about 40% of cells express macrophage markers, 10% are CD3+ T-cells, while the rest mostly appear to be SMCs, even if small numbers of B-cells, DCs and mast cells have also been demonstrated.\(^80, 81, 102, 103\)
The inverse correlation between HDL levels and the risk of CHD is well documented. The anti-atherogenic effect of HDL can be attributed to its ability to remove cholesterol by cholesterol efflux from foam cells, as well as SMCs and ECs inside the arterial wall. Infiltration of the atherosclerotic plaque by macrophages and activated T-cells lead to matrix degradation, apoptosis of cells in the arterial wall and to increased production of pro-inflammatory cytokines, all increasing the risk of plaque rupture.\textsuperscript{85, 104}

Cytokines are small protein molecules that are secreted by a variety of cells in response to stimuli such as apoptotic cells, bacterial endotoxins and other cytokines. They usually act over short distances in a paracrine, juxtacrine or autocrine way. When expressed strongly in abundance, however, cytokines may spill over into the circulation and exert endocrine effects. Chemokines are chemotactic cytokines, a large protein family whose common denominator is their influence on leukocyte chemotaxis. They are small proteins, typically 8-14 kDa, that act via chemokine receptors. Quite often, the same chemokine has been “discovered” by several research groups and ended up with several names. To bring order to this chaos, a consensus agreement was reached, dividing chemokines into four subfamilies. C, CC, CXC and CX3C (based on the identification of a conserved cystine residue pattern).

\textbf{ii. Circulating inflammatory markers}

A number of inflammation-related substances are present in the circulating blood and can be analyzed by immunometric techniques. Some, particularly C-reactive protein (CRP), have been studied in detail. The circulating levels of proinflammatory cytokines are increased in patients with ACS and in HF, where circulating levels correlate with functional class.\textsuperscript{105-108} This thesis evaluates the prognostic power of one representative of the tumor necrosis factor (TNF)-superfamily (osteoprotegerin, OPG), another from the CXC cytokine subfamily (CXCL16), as well as a granin (chromogranin A, CgA) with metabolites active in communication between the neuroendocrine and immune systems.\textsuperscript{109}

\textbf{C-reactive protein (CRP)} was the first acute-phase protein to be described. It was found in sera from patients acutely ill with lobar pneumonia and was named for its ability to precipitate C-polysaccharide of Streptococcus pneumoniae.\textsuperscript{110} It is synthesized in hepatocytes\textsuperscript{111} in response to inflammatory cytokines, particularly IL-6, with or without the support of IL-1.\textsuperscript{112, 113} CRP belongs to the pentraxin protein family, which is usually divided into two subfamilies based on size: the short pentraxins to which CRP belongs are 25 kDA while the long
pentraxins typically are 40-50 kDa. In atherosclerosis, IL-6 and IL-1, the cytokines that stimulate CRP production, are produced by macrophages in transition to foam cells. Originally rather crude, tailored as they were to being used in infections, the assays for CRP have been fine tuned into the highly sensitive (hs-)CRP, available today. Hs-CRP detects even mild inflammation, from values nearing zero up to 10 mg/L. Since CRP was shown to predict CV risk in patients with manifest atherosclerosis,\textsuperscript{114-116} as well as in healthy subjects,\textsuperscript{117} several studies have attempted to figure out whether CRP in itself could be pro-atherogenic\textsuperscript{118,119} or is merely an innocent bystander in vascular disease. The data suggest that CRP may have effects on plaque stability, endothelial function, coagulation, fibrinolysis and LDL oxidation.

The fact that several Mendelian randomization studies have failed to demonstrate concordance between CRP genotypes, CPR concentrations and CV risk has been used as an argument against causality and a recently published paper by Elliot et al.\textsuperscript{120} contradicts a causative role.

In 1997, Ridker with coworkers showed that prophylactic treatment with salicylic acid, an inhibitor of both platelet aggregation and inflammation, is more effective in subjects with higher CRP levels\textsuperscript{117} and they went on to demonstrate that rosvustatin lowered CRP levels – and the risk of MI by 54% – in subjects with normal lipids but elevated CRP. The latter finding was deemed interesting enough to make no 2 on TIME Magazine’s “Top 10 medical breakthroughs” list for 2008.

**Osteoprotegerin (OPG),** also known as osteoclastogenesis inhibitory factor (OCIF), is a basic secretory glycoprotein that consists of 401 amino acid residues with 7 distinct structural domains. It exists in two forms: the monomeric one has a molecular weight of 60 kDa, while a homodimer linked by a disulfide bond also exists\textsuperscript{121} and is biologically more active. Amino terminal domains 1-4 are cysteine rich and have osteoclastic inhibitory properties, while, at the carboxy-terminal end, domains 5-6 have apoptosis-mediating death domain-homologous regions and domain 7 has a free cysteine residue (enabling disulfide bond and hence dimerization) and a heparin-binding region.\textsuperscript{122}
Figure 2. The RANKL-OPG-RANK axis is illustrated schematically within the vessel wall. 

A. Regulation of osteoclastogenesis: Soluble and membrane bound RANKL on osteoblasts stimulate: 1. proliferation into preosteoclasts 2. differentiation into prefusion osteoclasts 3. fusion into multinucleated osteoclasts that resorb bone, and 4. prevent apoptosis of mature osteoclasts. B. OPG blocks the effects of RANKL by preventing binding to its receptor RANK. Proinflammatory cytokines upregulate RANKL on 1. ECs 2. activated T cells 3. vascular (V-)SMCs undergoing osteogenic differentiation and 4. ECs in contact with CD-44-expressing VSMCs. C. Function of RANKL in atherosclerotic vascular calcification. RANKL may play a role in the atherosclerotic process through: 1. triggering EC survival and proliferation 2. stimulating monocyte and lymphocyte transmigration and activation of T cells 3. maturation and activation of dendritic cells (DCs) 4. increased matrix MMP activity from monocytes and VSMCs that may promote plaque rupture and thrombus formation, and 5. promotion of osteogenesis, leading to synthesis of bone proteins and matrix calcification.


OPG is a soluble decoy receptor for two members of the TNF receptor superfamily, RANKL (receptor activator of nuclear factor-kappaB ligand) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). By binding RANKL it can inhibit the interaction between RANKL and RANK. It also has more classical cytokine effects. ECs, vascular SMCs and osteoblasts express OPG, which is colocalized with von Willebrand factor within secretory granules called Weibel-Palade bodies inside ECs. RANK is expressed on the surface of osteoclasts, monocytes and DCs, while RANKL is expressed on osteoblasts, T-cells and stromal cells.
OPG inhibits vascular calcification in mice and, like RANKL, it is an important regulating molecule in bone turnover; it inhibits osteoclastogenesis by binding RANKL. Paradoxically, in clinical studies, it appears that serum OPG levels increase with the progression of CAD\textsuperscript{125} and are related to coronary artery calcium and aortic plaque,\textsuperscript{126} the number of plaques,\textsuperscript{127} as well as being increased in STEMI\textsuperscript{128} and UAP.\textsuperscript{129} OPG is also detected in human atherosclerotic lesions.\textsuperscript{130} In the general population, OPG levels are associated with CV risk factors and atherosclerotic plaque burden, and predict plaque growth in women but not in men.\textsuperscript{131} Elevated circulating levels are associated with the increased prevalence and severity of coronary artery disease, CV disease and peripheral vascular disease. In a nested case-control study of 254 patients with ischemic stroke and 254 controls within a prospective cohort of 57053 Danish men and women who had OPG drawn at baseline, no association was found between OPG plasma concentrations and the risk of ischemic stroke.\textsuperscript{132} OPG is elevated in type II diabetics with microvascular complications. OPG perhaps regulates RANKL-mediated extracellular matrix remodeling (e.g. in myocardium in HF development and in atherosclerotic plaques).

In a study of post-MI HF patients, elevated OPG concentrations were associated with an increased risk of death, even after correction for BNP. Further, the prognostic value of OPG in relation to both all-cause mortality and CV events was actually stronger at one month and one year after inclusion than that of the baseline value.\textsuperscript{133} In patients with severe aortic stenosis, with or without HF, Helske et al. demonstrated increased OPG levels in HF due to LV pressure overload that decreased after successful valve replacement.\textsuperscript{134} In stable HF patients, OPG levels are associated with mortality, independently of conventional CV risk factors.\textsuperscript{135}

Most animal studies support a protective role for OPG in the vasculature. OPG-deficient mice develop vascular calcifications.\textsuperscript{136} Bone loss in oophorectomized mice was prevented by OPG.\textsuperscript{137} Transgenic mice that overexpress OPG develop osteopetrosis.

Osteoporosis appears to be related to atherosclerosis, especially vascular calcification, in elderly people, postmenopausal women and persons with autoimmune disease. It has been suggested that OPG could be the link between bone metabolism and vascular calcification that explains this relationship, but data exist that argue against this.\textsuperscript{138,139}
CXCL16 is a cytokine that combines chemokine functions with those of an SR and an adhesion molecule. Also known as SR-PSOX (SR for phosphatidylinerine and oxidized LDL/lipoprotein), it is a chemokine involved in inflammation, lipid metabolism and matrix degradation that is expressed by T-cells, macrophages, DCs, ECs and cytokine-stimulated SMCs. CXCL16 is different in that it has a mucin-like stalk, as well as cytoplasmic and transmembrane domains that are not present in other CXC chemokines. It is cleaved off from the cell membrane by ADAM10, a disintegrin and metalloproteinase, and the chemokine domain of the released protein functions as a chemoattractant for cells bearing its specific receptor, CXCR6 or Bonzo, ie T, NK, NKT, B and DCs. CXCL16 is upregulated in macrophages in response to atherogenic lipids and, by acting as an SR for oXLDL, it aids the transition from macrophages into foam cells. It works as a chemoattractant for SMCs and also stimulates their proliferation and transition into foam cells and has pro-inflammatory effects. CXCL16 is present in both human and murine atherosclerotic plaques and is upregulated by IFN-γ. Sheikine et al. found significantly lower levels of CXCL16 in 40 patients with stable angina than in a healthy control group and saw a (non-significant) tendency towards lower levels in 17 UAP/NSTEMI patients and 387 survivors of a first MI before the age of 60 compared with healthy subjects. Mitsuoka et al. found that CXCL16 was lower in ACS than in stable angina patients undergoing coronary angiography, but the study had few (17) ACS patients and non-ACS (89) patients. These studies had fewer subjects and follow-up was shorter than in our study, and some published data contradict their findings. In a nested case-control study of acute and chronic CAD, circulating levels were associated with chronic CAD and ACS after adjustment for established risk factors including CRP. In another study, even though no significant differences were

### Table 1. The OPG/RANK/RANKL/TRAIL axis

<table>
<thead>
<tr>
<th>Produced by</th>
<th>OPG</th>
<th>RANK</th>
<th>RANKL</th>
<th>TRAIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECs, VSMCs, osteoblasts</td>
<td>ECs, VSMCs, osteoclasts, monocytes, DCs</td>
<td>T-cells, stromal cells, osteoblasts</td>
<td>Normal organs and tissues, cancer cells</td>
<td></td>
</tr>
<tr>
<td>Actions</td>
<td>Decoy receptor for RANKL and TRAIL</td>
<td>Receptor for RANKL</td>
<td>Activates osteoclasts</td>
<td>Membrane bound form cleaved off by MMPs</td>
</tr>
<tr>
<td></td>
<td>Inhibits RANK-RANKL interaction</td>
<td></td>
<td></td>
<td>Induces apoptosis in cancer cells by binding death receptors DR4 and DR5</td>
</tr>
</tbody>
</table>
seen between acute and stable CAD, CXCL16 levels were significantly higher in CAD patients.\textsuperscript{150}

**Figure 3.** CXCL16 in the vessel wall. Membrane bound CXCL16 1. is upregulated on macrophages in response to atherogenic lipids 2. acts as an SR for oxLDL, aiding the transition of macrophages and SMCs into foam cells 3. attracts cells bearing its specific receptor, CXCR6, eg. SMCs and monocytes 4. is shedded, in its soluble form, by ADAM10. *Original illustration by Thor Ueland.*

Increased serum levels of CXCL16 have been demonstrated in patients with cardiomyopathy-induced HF in whom its expression is also upregulated in the cardiomyocytes\textsuperscript{151}, suggesting a possible role in HF development. Vascular SMCs that are stimulated with CXCL16 show enhanced MMP expression, suggesting that CXCL16 could have matrix-degrading properties.\textsuperscript{150}

**Chromogranin A** is a 49 kDa acidic polypeptide and a member of the granin family, eight proteins, CgA-C, SgIII-VI and VGF, all of which are found as major components of the soluble core of dense-core secretory granules in neuroendocrine cells throughout the body. The granins range from 27 to 100 kDa in size and function as pro-hormones, giving rise to diverse biologically active peptide fragments with an array of effects on the vascular system and the heart, for example. From CgA, catestatin, parastatin, pancreastatin, vasostatin and
chromatin – to mention a few – can be cleaved off and have antibacterial and antifungal effects, activate the adhesion of fibroblasts, the relaxation of constricted vessels and the reduction of intracellular calcium. Catestatin has antimalarial as well as antimicrobial effects and induces chemotaxis. It appears that CgA – or rather a cleaved-off peptide from CgA – promotes the generation of secretory vesicles and possibly controls sorting\textsuperscript{152}. CgA is co-secreted with peptide hormones and amines such as noradrenaline. In healthy subjects, the serum levels of CgA are, however, not related to circulating levels of norepinephrine and epinephrine\textsuperscript{153}. CgA levels are markedly elevated in neuroendocrine tumours (NETs), such as pheochromocytoma\textsuperscript{154}, carcinoid\textsuperscript{155} and neuroblastoma\textsuperscript{156}. It is therefore a frequently used marker of activity in different kinds of NETs and is also considered to be the best available biomarker for the diagnosis of NETs. As opposed to the catecholamines, with a half-life in the circulation of just a few minutes, CgA is fairly stable. In an animal model of secondary hypertension, CgA levels were elevated\textsuperscript{157}, a finding that is corroborated by observations in humans.\textsuperscript{157} CgA levels are also more likely to be elevated in inflammatory diseases such as rheumatoid arthritis,\textsuperscript{158} Crohn’s disease and ulcerative colitis.\textsuperscript{159}

The circulating levels of CgA are strongly influenced by renal function – patients with renal failure have been shown to have levels up to 22 times those of healthy subjects and patients with liver cirrhosis also have increased levels\textsuperscript{160}. Proton pump inhibitors increase the levels of chromogranin A substantially\textsuperscript{161}. Levels also increase with age. Chromogranin A is produced by human endocardium in patients with cardiomyopathy but not in controls and exerts negative inotropic effects on mammalian heart\textsuperscript{162} and predicts mortality post-MI\textsuperscript{163,164} but not in stable HF patients after adjustment for clinical risk markers\textsuperscript{165}. Levels are increased in HF and also correlate with the severity of HF with levels in NYHA class IV patients as high as those seen in pheochromocytoma and other neuroendocrine tumors.\textsuperscript{166} CgA is believed to reflect neurohormonal activation and to provide a link between sympathetic tonus and inflammation.
Rationale

We hypothesized that OPG, CXCL16 and CgA could add valuable information on the prognosis in ACS concerning mortality and morbidity due to

- their presence in failing myocardium
- the elevated circulating levels seen in patients with HF
- the possible role of OPG and CXCL16 in atherosclerosis and plaque destabilization
- the presence of CgA in neurohormonal activation

They are all easy to quantify in a clinical context, which makes them suitable biomarker candidates.
Aims

The overall aim of this thesis was to study the feasibility of using some different biochemical markers, all supposedly involved in inflammation, for prognostication in ACS. We sought to evaluate the prognostic merit of OPG, CXCL16, a combination of OPG and CXCL16 and CgA in patients hospitalized due to an acute MI or UAP.

- To assess OPG, CXCL16 and a combination of the two as markers of mortality in ACS patients
- To evaluate OPG, CXCL16 and a combination of the two for predicting rehospitalizations due to HF, MI and stroke in ACS patients
- To examine whether baseline values of CXCL16 and OPG are equally predictive of mortality and morbidity as levels obtained in direct relationship to an ACS
- To describe the pattern of serum concentrations of OPG and CXCL16 after an ACS
- To examine the association between CgA and mortality and rehospitalizations in ACS
Methods

Study population and data collection

Patients admitted to the coronary care unit (CCU) at Sahlgrenska University Hospital, Gothenburg, Sweden from September 1995 to March 2001 with an ACS, i.e. a diagnosis of UAP, NSTEMI or STEMI were eligible for prospective inclusion in PRACSIS, Prognosis and risk in acute coronary syndrome in Sweden, Figure 4. The main exclusion criteria were age < 18 or ≥ 80 years, non-coronary artery disease associated with an expected life expectancy of < 1 year, residence outside the hospital’s catchment area, unwillingness to participate and prior admission resulting in inclusion in the study. During the inclusion period (5.5 years), 2335 patients were included in the PRACSIS program. Until November 1995, only clinical information was recorded and no consecutive blood sampling was performed. From then on, blood was drawn on the first morning in the CCU in patients who had, by then, received a diagnosis of MI or UAP. Serum was then frozen at -70° for later analysis. Of the patients included in PRACSIS, 612 were transferred to the CCU from either an internal medicine ward (usually after having been admitted there due to uncertainty about the ACS diagnosis) or from the intensive care unit (where they had been admitted due to a need for mechanical ventilation). For logistical reasons, the majority of these 612 patients did not have blood sampled in the morning. During some weekends, no blood collection was possible even though patients were included and, finally, some patients

Figure 4. Proportions of index diagnoses in PRACSIS.
were undergoing angiography on, or died prior to, the first morning. These patients were enrolled in the PRACSIS program but were not included in the biomarker substudy.

A diagnosis of ACS had to be supported by ECG changes (defined below) on admission, cardiac biomarkers increased above the upper reference levels (CK-MB above 5 µg/L or troponin T above 0.04 µg/L) or previously recognized coronary artery disease such as MI, prior coronary artery bypass grafting (CABG) or prior percutaneous coronary intervention (PCI), prior angina pectoris with significant changes on coronary angiography or a stress test suggestive of ischemia.

ECG changes were defined as

* ST elevation ≥ 0.1 mV (0.2 mV in V1-V4) or
* ST depression ≥ 0.1 mV or
* T-wave inversion in at least two adjacent leads
* Q-wave ≥ 0.03 sec and ≥ 25% of the amplitude of the following R-wave
* LBBB

The inclusion criteria as stated on the inclusion form were:
1) Patients ≤ 79 years receiving thrombolysis or PCI due to a certain or uncertain diagnosis of MI; included on arrival
2) Patients ≤ 79 years with a suspected MI who were not being treated with thrombolysis or PCI and patients ≤ 79 years with suspected UAP. Included when the criteria below are met.

Symptoms
a) Accelerating angina upon exertion during the last four weeks
b) Angina at rest during the last four weeks but not the last 48 hours
c) Angina at rest during the last 48 hours

Objective findings
a) Elevation of CK-MB or troponin after admission
b) New ST depression or T-wave inversion at admission or during hospitalization
c) ST depression on exercise ECG before hospital discharge
d) Previous objective evidence of ischemic heart disease such as previous MI, pathological exercise ECG, pathological coronary angiography
Patients below the age of 75 years received an invitation to an outpatient follow-up visit 3 months after discharge. Those who accepted had blood drawn on this visit as well as completing the Cardiac Health Profile, a disease-specific quality of life questionnaire.

**Echocardiography**
An echocardiographic investigation was performed by an experienced investigator within 5 days of hospital admission. The biplane LVEF was calculated by the disc sum method and tracings were checked in the motion mode for accuracy.

**Blood sampling procedures**
Peripheral venous blood was obtained within 24 hours of admission and on an outpatient visit approximately 90 days after the index admission by direct venipuncture of an antecubital vein after the patients had been resting for at least 30 minutes. Blood samples for OPG, CgA and CXCL16 determination (serum) were drawn into serum tubes and centrifuged at room temperature within 1 hour. Blood samples for the determination of CRP and BNP/proBNP (plasma) were drawn into pyrogen-free tubes with EDTA as the anticoagulant, immediately immersed in ice water and centrifuged at -4° within 1 hour. All plasma and serum samples were stored at -70°C and thawed < 3 times prior to analysis.

**Biochemical analysis**
Both serum OPG and plasma CXCL16 were quantified by an enzyme immunoassay using commercially available matched antibodies (R&D Systems, Minneapolis, MN). The intra- and interassay coefficient of variation was 3.6% and 10.6% for OPG and the intraobserver coefficient of variation for CXCL16 was 3.3 ± 2.2% (mean±S.D.). The sensitivity for OPG, defined as ±3 SD of the 0 standard, was determined as 15 pg/mL and the detection limit for CXCL16 was calculated as 11 pg/mL. CgA was measured by a commercially available ELISA assay (DakoCytomation, Glostrup, Denmark). The detection limit of the assay was 7.0 U/L and the intra- and interassay coefficients of variation were < 5% and 10% respectively. According to the manufacturer, the upper limit is 18 U/L. Troponin T (TnT) and creatinine kinase MB (CK-MB) fractions in serum were measured on a modular platform (Roche Diagnostics, Mannheim, Germany). CRP, TnI, BNP and proBNP were measured using immunofluorescent assays calibrated with spiked plasma (Biosite Inc, San Diego, CA). CRP for paper III was quantified in Oslo, Norway by an enzyme immunoassay using commercially available matched antibodies (R&D Systems, Minneapolis, MN). Samples for CRP analyses were diluted (factor 1600) in order to bring the concentration into the measurable range. The
minimal detectable concentration – upper range was 400-30,000 pg/mL for proBNP, and 0.3-100 mg/L for CRP. All samples were run in duplicate in a blinded fashion. Creatinine and total cholesterol concentrations in serum were determined by routine laboratory methods.

### Table 2. Coefficient of variation for the studied biomarkers

<table>
<thead>
<tr>
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<th>OPG</th>
<th>CXCL16</th>
<th>CgA</th>
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<tbody>
<tr>
<td>Detection limit</td>
<td>15 pg/mL</td>
<td>11 pg/mL</td>
<td>7.0 U/L</td>
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<tr>
<td>Intraassay CoV</td>
<td>3.6%</td>
<td>&lt;5%</td>
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<tr>
<td>Interassay CoV</td>
<td>10.6%</td>
<td>&lt;10%</td>
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<tr>
<td>Intraobserver CoV</td>
<td>3.3±2.2%</td>
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<td>CoV freeze-thaw x 3</td>
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<tr>
<td>CoV circadian variation</td>
<td>8.8±3.0%</td>
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<tr>
<td>CoV food intake</td>
<td>9.7±3.9%</td>
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</table>

### Assessment of endpoints

The primary outcome measure was all-cause mortality. Survival confirmation and date of death were obtained from the Swedish National Population Registry. Patients who emigrated from Sweden and were lost to follow-up were censored alive on the day of emigration. For the papers on CXCL, CgA and on the OPG and CXCL combination, a total of 11 patients were lost to follow-up due to emigration. In the smaller OPG study, 5 of the emigrated patients were included.

Secondary outcome measures were the incidence of acute MI (International Statistical Classification of Disease, Ninth Revision (ICD-9) code 410 or ICD-10 code I21 or I22), HF (ICD-9 code 428 or ICD-10 code I50) and stroke (ICD-9 codes 431, 432, 433, or 436 or ICD-10 codes I61, I62, I63, or I64), as obtained from the Swedish Hospital Discharge Registry, and CV mortality (ICD-9 codes 390-459 or ICD-10 codes I00-I99), as obtained from the Swedish National Cause of Death Register. In study I, II and IV, we used the endpoints all-cause mortality (long-term) and rehospitalization due to HF, MI or stroke. In study III, we studied all-cause and CV mortality and rehospitalization due to HF or MI, since we had already concluded that neither OPG nor CXCL16 was predictive of stroke rehospitalization.
Potential confounders

Patients were prospectively classified according to maximum Killip class on admission and during the index hospitalization. Electrocardiographic findings on admission were classified according to the presence or absence of ST-segment elevation and ST-segment depression. Presenting signs and symptoms, biochemical variables, medical treatment and procedures and in-hospital complications were recorded. Based on hospital records and personal interviews, patients were classified as having or not having a history of MI, angina pectoris, HF, diabetes mellitus, hypercholesterolemia or arterial hypertension. The GFR in ml/min was estimated using the Cockcroft-Gault formula \([(140\text{-age}) \times \text{weight (kg)/serum creatinine (umol/L)}]\) multiplied by a constant of 1.23 in men and 1.04 in women.

Table 3. Variables adjusted for in the studies

<table>
<thead>
<tr>
<th>Variables adjusted for</th>
<th>Paper I OPG</th>
<th>Paper II CXCL16</th>
<th>Paper III CXCL16 OPG</th>
<th>Paper IV CgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Gender</td>
<td>x</td>
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<td>Index diagnosis</td>
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<tr>
<td>Prior hypertension</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Prior CHF</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Prior diabetes</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Prior angina</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior MI</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Smoking status</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST-depression</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Heart rate</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Systolic BP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Killip class (&gt;1)</td>
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<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>No in-hospital PCI</td>
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<td></td>
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<tr>
<td>Est. GFR</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline creatinine</td>
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<td></td>
<td></td>
<td>x</td>
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<tr>
<td>Elevated cardiac markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak CK-MB</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>TnI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TnT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Pro-BNP</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>BNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEF</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Ethics

Informed consent was obtained from all individuals. The study protocol was approved by the Regional Ethics Committee at the Sahlgrenska Academy, Gothenburg University.

Statistical analysis

We used 95% confidence intervals to indicate the precision of estimated HRs.  
**The Mann-Whitney U test** was used to test the associations between the different markers and the dichotomous baseline demographic variables and the CV risk factors.  
**Spearman’s rank correlation** statistics were used to determine the association between the markers and continuous variables.  
**Fisher’s exact test** was used in Paper III to test any difference between the four different combinations of OPG and CXCL16 quartiles and the dichotomous baseline demographic variables and the CV risk factors.  
**The Kruskal-Wallis test** was used in Paper III to test any difference between the four different combinations of OPG and CXCL16 quartiles and continuous variables.  
**Wilcoxon’s signed rank test** was used in Paper III for paired testing, i.e. the difference in marker levels between time points.  
**Pearson’s correlation** was used to examine the extent to which different variables were related.  
**Cox proportional hazards regression analysis** was used to calculate crude and adjusted risk estimates associated with a 1-SD increase in logarithmically transformed CXCL16 levels for the different endpoints. In the multivariate analyses, we adjusted for the potential confounders, using the same set of variables previously described in Papers I, II and IV and the GRACE score variables in Paper III.  
**Kaplan-Meier curves** were generated to visualize the relationship between the markers in quartiles (in Paper III, we used the four quartile combinations OPG q4/CXCL16 q4, OPG q4/CXCL16 q1-3, OPG q1-3/CXCL16 q4, OPG q1-3/CXCL16 q1-3) and mortality.  
**The log-rank test** was used for comparisons of the resulting curves.  
**C-statistics** were used for exploring the sensitivity and specificity of the different markers in predicting mortality. C-statistics equal the “area under curve”, AUC, in receiver operating characteristic (ROC) curves, i.e. they are a measure of model discrimination for binary outcomes.
Results

Study I

In the first paper, we examined circulating OPG concentrations in a population of 897 patients for whom we also had data on CRP, BNP and TnI in addition to clinical risk predictors. The median age was 66 years and 29% of the patients were female. During a follow-up period of 89 months, 261 patients (29%) died. Patients with higher OPG levels were more likely to be older and to have a lower body mass index. OPG levels were associated with a previous history of HF but not with prior MI. Higher OPG levels were associated with a number of indices of acute thrombotic occlusion, myocardial damage and LV dysfunction, such as ST-segment elevation, treatment with thrombolysis/primary PCI, CK-MB and TnI, acute signs of HF and LVEF. Serum levels of OPG were strongly associated with long-term all-cause mortality and the development of HF, irrespective of the index diagnosis. The hazard ratio (HR) per 1 SD pg/mL increase in the natural logarithm of OPG were significant for mortality and HF, and remained so after adjustment for clinical variables, BNP, CRP and TnI (Table 4). These associations remained even including LVEF (hazard ratio for mortality 1.4; 95 % CI 1.2-1.7; p < 0.0001 and for HF 1.6; 95% CI 1.2-2.1; p < 0.0001). According to univariate analysis, the baseline OPG concentration was associated with the recurrence of MI but not after adjustment for confounders.

In a cohort of 724 of the patients, echocardiographic determination of LVEF had also been performed and after further adjustment for LVEF, OPG was still predictive of mortality and HF development. C-statistics showed that OPG provided significantly better prognostic information: AUC 0.68 (95% CI 0.64-0.73) than TnI: AUC 0.55 (95% CI 0.50-0.60), p < 0.001 and CRP: AUC 0.59 (95% CI 0.54-0.63), p = 0.002) and was of similar value to BNP: AUC 0.70 (95% CI 0.66-0.74), p = 0.28) and LVEF: AUC 0.71 (95% CI 0.66-0.76), p = 0.09.

Table 4. Associations between OPG concentrations and events during follow-up

<table>
<thead>
<tr>
<th>OPG</th>
<th>Unadjusted</th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Mortality</td>
<td>1.7 (1.5-1.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HF</td>
<td>2.0 (1.6-2.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Recurrent MI</td>
<td>1.3 (1.0-1.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>Stroke</td>
<td>1.2 (0.9-1.6)</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Figure 5. OPG and long-term mortality

Figure 6. C-statistics for OPG and other markers, Paper I.
Study II

CXCL16 levels were assessed in 1351 patients, 377 of whom died during a median follow-up of 81 months. Patients with higher levels of CXCL16 were more likely to be older, to be female, to have a history of hypertension and to be current smokers. There was no significant association between CXCL16 and prior history of MI or HF. Higher CXCL16 levels were associated with STEMI and variables related to acute myocardial injury, such as CK-MB, TnT, thrombolysis, Killip class and LVEF. CXCL16 predicted long-term mortality and the relationship remained after adjustment for clinical and laboratory risk markers CRP, TnT and proBNP as well as index diagnosis and LVEF. Those with 4th quartile levels were 3 times as likely as those with 1st quartile levels to be hospitalized due to HF and twice as likely to be rehospitalized due to MI. After adjustment for clinical risk factors, the associations were still significant. However, after further adjustments were made for LVEF, CRP, proBNP and TnT, in a subgroup (n= 714) where these data were available, there was no remaining association between CXCL16 levels and readmission for MI or HF. When analyzing data for subgroups, we found a significant association between CXCL16 levels at inclusion and prognosis in patients with STEMI undergoing thrombolysis and primary PCI, as well as in non-ST-elevation ACS not undergoing revascularization. This association remained significant after adjustment for clinical variables and, in the non-ST elevation ACS group not undergoing revascularization, also after adjustment for LVEF, TnT, pro-BNP and CRP. In STEMI, as well as in non-ST-elevation-ACS, a univariate association was seen between baseline CXCL16 levels and prognosis, but it was attenuated after further adjustments.

We found a significant interaction between CXCL16 and CRP even though CXCL16 predicted mortality after adjustment for CRP. Having 4th quartile levels of CXCL16 but not of CRP gave an HR of 1.4 (95% CI 1.0-1.9), p = 0.06, which was of the same order as the HR of having 4th quartile levels of CRP but not of CXCL16, 1.6 (95% CI 1.1-2.2), p = 0.005, when compared with patients with neither CXCL16 nor CRP in the 4th quartile. A considerably higher HR was noted for patients with both markers in the 4th quartile, 3.3 (95% CI 2.3-4.6), p < 0.0001.

We observed a high stability of CXCL16 with little variation after three cycles of freezing and thawing, minor circadian variation and little effect of food intake on serum levels. We also compared the mean and median CXCL16 levels from the first half of the study inclusion period with the second half, with neither showing a difference over time.
Table 5. Associations between CXCL16 concentrations and events during follow-up.

<table>
<thead>
<tr>
<th>CXCL16</th>
<th>Unadjusted HR (95% CI)</th>
<th>P value</th>
<th>Adjusted HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>1.33 (1.21-1.46)</td>
<td>&lt;0.0001</td>
<td>1.21 (1.09-1.36)</td>
<td>0.0006</td>
</tr>
<tr>
<td>HF</td>
<td>1.31 (1.12-1.52)</td>
<td>0.0005</td>
<td>1.25 (1.05-1.48)</td>
<td>0.01</td>
</tr>
<tr>
<td>Recurrent MI</td>
<td>1.25 (1.08-1.44)</td>
<td>0.002</td>
<td>1.18 (1.01-1.38)</td>
<td>0.04</td>
</tr>
<tr>
<td>Stroke</td>
<td>1.09 (0.86-1.39)</td>
<td>0.48</td>
<td>0.98 (0.77-1.25)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Figure 7. CXCL16 and long-term survival, Paper II.
Study III

A combination of OPG and CXCL16 for risk prediction was assessed in 1322 patients, 30 % of whom were female and the median age was 67 years. During the first 3 months of follow-up, 110 patients, i.e. 8.3% died, 34 (2.6%) during the index hospitalization. All the deaths were from CV causes. During the same period 223 patients developed HF, 198 of them during index hospitalization, and 75 patients had new MIs, 18 during index hospitalization. Using patients with both OPG and CXCL16 in quartiles 1-3 as a reference, having 4th quartile levels of both markers was associated with an almost fivefold increase in total mortality and, after adjustment for the GRACE score, a threefold mortality increase remained and was significant. Developing HF was approximately four times as likely in those with the highest OPG and CXCL16 levels and, after adjustment for the GRACE score, almost three times as likely as in those with the lower quartiles of both. There was, however, no association between high levels and new hospitalizations due to MI.

![Figure 8. 3-month morbidity and mortality, Paper III](image)

The median, (25th, 75th percentile) total follow-up period was 91 months (70, 110 months). During that period, there were 411 deaths (31% of patients), of which 287 were from CV causes, while 329 patients were hospitalized with HF and 235 with acute MI. 635 patients also had OPG and CXCL16 sampled at 3 months after the index admission.
Study IV

The CgA concentration was available in 1268 patients. 389 patients (31%) died during follow-up (median 92 months). Patients with higher CgA levels were more likely to be older, to have a lower body mass index and to be on treatment with diuretics, angiotensin-converting enzyme blockers, angiotensin receptor blockers, statins or salicylic acid. CgA was also associated with a history of MI and HF as well as acute signs of HF and a low LVEF, but not with any variables related to the acute infarct process. Patients with higher CgA levels were also more likely to have a low GFR and a significant correlation between GFR and CgA was seen (R_s = -0.43, p < 0.001). No gender-related differences were seen. Since no significant interaction was found between index diagnosis and CgA in terms of outcome, we did not analyze the data for subgroups. CgA predicted mortality and hospitalizations for HF after adjustments for conventional risk factors, including TnT, with HR 1.27 (1.13-1.42), p < 0.001, for mortality and HR 1.23 (1.01-1.49), p = 0.04, for HF hospitalizations. After further adjustment for LVEF and proBNP, CgA was still predictive of mortality, 1.18 (95% CI 1.01-1.37), p < 0.04. CgA predicted recurrent MI in univariate analysis but the association was attenuated and no longer significant after adjustment for clinical risk factors. However, an independent association between CgA and recurrent MI was found in the subgroup where TnT was available. We also assessed the relationship between CgA and stroke but found no significant associations even in univariate analysis.
Table 6. Associations between CgA concentrations and events during follow-up

<table>
<thead>
<tr>
<th>CgA</th>
<th>Unadjusted HR (95% CI)</th>
<th>P value</th>
<th>Adjusted HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>1.57 (1.44-1.70)</td>
<td>&lt;0.001</td>
<td>1.28 (1.15-1.42)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HF</td>
<td>1.54 (1.35-1.76)</td>
<td>&lt;0.001</td>
<td>1.24 (1.04-1.47)</td>
<td>0.02</td>
</tr>
<tr>
<td>Recurrent MI</td>
<td>1.27 (1.10-1.47)</td>
<td>&lt;0.001</td>
<td>1.15 (0.96-1.36)</td>
<td>0.12</td>
</tr>
<tr>
<td>Stroke</td>
<td>1.16 (0.93-1.46)</td>
<td>0.19</td>
<td>0.96 (0.73-1.26)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Figure 10. CgA and long-term survival.
General discussion

The prognosis for people developing ACS varies a great deal; some patients will not leave the hospital alive, others die within a few months and some can look forward to decades of life in good health. PRACSIS was conceived to study predictors of prognosis in a consecutive series of ACS patients. This thesis focused on the study of three biochemical markers and their relationship with prognosis relating to both mortality and CV morbidity.

Methodological considerations

PRACSIS comprises a prospective, single-center cohort of ACS patients. A prospective design usually entails several advantages compared with a retrospective design; sources of bias and confounding are generally fewer. In Sweden, all citizens have a personal identification number, the national registration number, based on the year, month and date of birth combined with four digits at the end that make the number unique to the individual. All deaths are reported, using this number as a unique identifier, to the Swedish National Population Registry. In our study, only 11 patients were lost to follow-up, all of them due to emigration. All hospitalizations are recorded with diagnoses in the Swedish Hospital Discharge Registry, again using the national registration number. This is a strength of all clinical research in Sweden, as it enables an almost complete follow-up at least of mortality (with the exception of those who emigrate during follow-up) and probably does fairly well concerning hospitalizations in Sweden even though Swedish people travel quite a lot – in 2009, 6.8 million trips abroad were made by the 9.3 million population of Sweden.168

Did we use the optimal time point for blood sampling of the markers in ACS? When it comes to OPG, there are data that suggest that OPG is elevated as early as 1 hour into an ACS.128 Since many patients present more than 1 hour after symptom onset and more time passes before blood can be sampled, obtaining OPG levels before a possible increase caused by an ACS is not feasible. An alternative approach in the study of risk prediction is population studies as exemplified by the Framingham cohort, where OPG in the general population predicted future events.169 In this case, several years can elapse between blood sampling and event, and we do not know the OPG level in direct connection with the event. At any rate, as we have demonstrated in Paper III, there is little change from day 1 to day 4, suggesting there may be a window of opportunity for sampling OPG at least during the first four days.
Regarding CXCL16, the variation is smaller, but there was a significant increase between day 1 and day 4. In terms of BNP, there is now evidence to suggest that repeating the measurement weeks to months after the ACS could enhance risk stratification beyond the information provided by the baseline BNP.\textsuperscript{170,171} When adjusting for CRP it could be argued that using values obtained at 3 months after the event would have been better, as there are data suggesting that CRP levels in the acute phase after an MI are not predictive of mortality, while chronic levels are.\textsuperscript{116} The most likely explanation would be that the acute-phase response to myocardial necrosis masks the baseline CRP level which better corresponds to risk.

It may be argued that the cycles of thawing and refreezing could have affected the levels of what was being analyzed; OPG, CgA, CXCL16, CRP and BNP. One study of OPG found significant differences between morning and early afternoon and after five freeze-and-thaw cycles compared with before.\textsuperscript{172} It seems that the available commercial ELISAs for OPG have marked variations in calculated serum concentrations.\textsuperscript{173} CgA is stable \textit{in vitro} at room temperature. Plasma levels are not affected by repeated thawing-refreezing cycles but are elevated in liver and renal failure as well as in several neuroendocrine tumors.\textsuperscript{174} In Paper II, the stability of CXCL16 in serum and the small variation observed in relation to food intake and time of day would make CXCL16 easy to use clinically if other studies were to confirm our results relating to its association with prognosis.

In Paper III, we made adjustments for the risk factors that make up the GRACE score instead of staying with the conventional risk factors that we used in Papers I, II and IV. Since we actually corrected for more variables than the GRACE score, it would seem that we attenuated the relationships more in Papers I, II and IV than in paper III. Since the GRACE score is used around the world and is well documented,\textsuperscript{175} we felt this made our findings easier to judge from a contemporary clinical perspective.

The way we have defined new HF is not the same in all four papers. In Papers I, II and IV, HF was defined as rehospitalization with a primary ICD-9 code 428 or ICD-10 code I50, but, in Paper III, we also included patients who developed HF symptoms, Killip class > 1, during the index hospitalization.

The definition of MI used in the four papers is the one that was in use during the inclusion period. The new definition of MI, where a lower cut-off for infarction markers has been established, has naturally increased the number of people receiving a diagnosis of MI each year, while also reducing the diagnosis-related mortality. For our studies, this means that some patients who were diagnosed with UAP in relation to inclusion and data collection in
the study would today receive a diagnosis of NSTEMI instead and some patients who did not fulfil the inclusion criteria for UAP would do so now, with the more sensitive troponin assays. This has to be borne in mind when relating the data to contemporary data and patients. Also, treatment has changed in patients with MI, with more patients receiving revascularization today which explains part of the decrease in mortality since the inclusion period of PRACIS. With the continuing improvement of treatment, we will (thankfully) never be able to base our risk estimates for the patient having an ACS today, on patients treated exactly them. Over time, the “golden standard” of statistical measures used in articles on prognostic markers has changed. From mere demonstrations of a difference in circulating concentration between those suffering a certain event and those who do not (using all study subjects or only case control), to a comparison of how much a marker contributes, in addition to clinical variables in C-statistics with complicated goodness of fit testing for the model, as is possible today. It would naturally be interesting to be able to analyze our data further.

Findings

Prediction of HF

In Paper I, OPG was related to HF rehospitalizations, even after adjustment for conventional risk factors, LVEF, TnI, CRP and BNP. This suggests that its association with HF goes beyond only predisposing factors such as hypertension and diabetes, a large MI with corresponding high maximum levels of markers of myocyte necrosis and pre-existing or developing myocardial dysfunction (as assessed by LVEF and BNP). OPG has been shown to reflect the activity of the OPG/RANK/RANKL axis, which is involved in matrix degradation and remodelling. Serum levels of OPG are increased in patients with LV pressure overload due to aortic stenosis and decreased after valve replacement. Both serum levels and myocardial expression are increased in HF. In Paper II, CXCL16 was associated with HF rehospitalizations after adjustment for conventional risk factors. We found an association between CXCL16 and disease severity evaluated by ECG abnormalities (ST elevation and Q-wave on admission), maximum levels of markers of myocardial injury (TnT and CK-MB) and indices of systolic dysfunction (LVEF and proBNP). Like OPG, it is expressed in the myocardium of HF patients and levels are increased in HF patients where they correspond to disease severity. CXCL16 is implied in matrix degradation as it increases MMP.
expression.\textsuperscript{150} Not surprisingly, given the results in Papers I and II, the combination of CXCL16 and OPG predicted HF both during the index hospitalization and during long-term follow-up, also after adjustment for the GRACE score. It has been suggested that OPG and CXCL could be mediators, and not just markers, in the development of HF. Our data lend further support to these hypotheses. In paper IV, CgA also predicted HF rehospitalizations. Like OPG and CXCL16, CgA is produced in the myocardium of HF patients who also have higher circulating levels than controls.\textsuperscript{162} For CgA, part of the explanation of the relationship could be the increase in sympathetic tone seen after MI.\textsuperscript{180}

**Prediction of recurrent MI**

Generally, MI appears to be more difficult to predict than HF, perhaps since the development of HF is usually a slower and steadier process than the plaque rupture that is so often the pivotal point in the transition from stable atherosclerosis to an acute event. Of the biochemical markers studied in this thesis, only CXCL16 predicted recurrent MI after adjustment for clinical risk factors. OPG and CgA were associated with the recurrence of MI in univariate analysis but not after adjustment. There was no relationship with a history of MI for OPG or CXCL16, while there was for CgA. Both the OPG/RANK/RANKL axis as represented by OPG, and CXCL16 play possible roles in the transition from a stable to a vulnerable plaque, through their matrix-degrading effects as well as via pro-inflammatory effects.\textsuperscript{150, 179} There may also be an effect by the RANK/RANKL/OPG axis in plaque calcification\textsuperscript{126, 181, 182} and CXCL16 is involved in SMC migration and lipid metabolism.\textsuperscript{146, 183}

**Prediction of mortality**

OPG, CXCL16 and CgA levels all predicted mortality in univariate analysis and, as shown by multiple regression analyses, they added independent prognostic information to clinical risk factors and, in the case of an OPG/CXCL16 combination, to the GRACE score. In our patients, CV mortality was, as expected, the leading cause of death. The association with HF development and the subsequent risk of malignant arrhythmia, and the risk of sudden death due to ischemia, perhaps following plaque rupture, help explain those deaths. For the non-CV mortality, several associations of these biomarkers with diseases associated with a shortened life expectancy have been observed. Higher levels of CgA have been demonstrated in several conditions with higher mortality, such as breast, prostate,\textsuperscript{184} lung, uterus, pancreas, GI and
Discussion

head and neck cancers, hematological malignancies, neuroendocrine tumors, renal failure and liver cirrhosis, as well as Parkinson’s disease and rheumatoid arthritis. OPG can inhibit the apoptosis-inducing activity of TRAIL and thereby possibly aid the survival of cancer cells expressing OPG. Elevated OPG levels are increased, and associated with a poorer prognosis, in several cancer forms such as bladder carcinoma, gastric carcinoma and prostate cancer. Serum CXCL16 levels are elevated in systemic sclerosis, multiple sclerosis (MS) and systemic lupus erythmatous (SLE). We did not, however, investigate the specific cause of death in patients who died due to non-CV causes. This may be of interest for future research, as IL-18 was recently found to be unexpectedly associated with non-CV mortality in the PRACSIS study.

Eggers and coworkers demonstrated a C-statistic for the GRACE score of 0.78 for 6-year mortality in chest pain patients. However, to the best of our knowledge, Study III is the first published article to evaluate the GRACE score in a long-term population of ACS patients.

The finding that OPG + CXCL16 concentrations obtained in the acute phase and 3 months later are similarly predictive of mortality and HF development is interesting. It indicates that high circulating levels, whether caused by an acute inflammatory state or chronic inflammation in atherosclerosis, are indicative of prognosis.

Prediction of stroke

Carotid atherosclerotic plaque as detected by ultrasound is a risk factor for ischemic stroke. OPG is present in, and predicts progression of, carotid atherosclerotic plaques and is also positively correlated with plaque echolucency, which is believed to mark the rupture-prone plaque and correlates with risk factors including inflammation and endothelial dysfunction. CXCL16 expression is increased in carotid atherosclerotic plaque compared to normal vessel wall and an association between CXCL16 levels and ischemic stroke was recently demonstrated. CgA is increased in hypertension, an important risk factor for stroke. However, none of the biomarkers we studied was predictive of stroke in our patients.
Concluding remarks

Could these three markers that we studied be of value for the clinician in risk assessment of the patient with ACS?

When using the three criteria postulated by Morrow and de Lemos in Circulation in 2007 to appraise OPG, CXCL16, a combination of the two and CgA, we can say “yes” to the first two.

1. Can the clinician measure the biomarkers? Yes, as they are quantified by an ELISA using commercially available antibodies (OPG, CXCL16) and by a commercially available ELISA assay (CgA).

2. Does the biomarker add new information? Yes, even after adjustment for conventional risk markers, serum levels of OPG, CXCL16 and CgA provide information on both long-term mortality and rehospitalizations due to HF in patients with ACS.

3. Will the biomarker help the clinician to manage patients? More data on risk prediction in other ACS patient populations are needed before this question can be answered. Further studies should include reclassification rates and evaluation of the frequency of false-positive and false-negative errors.

Will these markers, or combinations of them, then change the way we tailor treatment for ACS patients? At the moment this is not likely, since there are available tools for risk prediction that are at least as effective that are still not being used in everyday clinical work. Moreover, it can be argued that the cost of providing additional risk information with markers for individual patients is too high and the potential benefit too small – which will naturally be true unless the risk model is allowed to influence treatment, which will require further studies. Even if new biomarkers have improved risk prediction in ACS, it is important to underscore that, so far, none of them has been proven to alter the outcome of interventions. In previous studies that have looked at adding different markers to clinical prediction models, the improvement of risk stratification was most obvious among the subjects initially classified as being at intermediate risk. Several studies evaluating multimarker strategies for risk prediction had been published when the Atherosclerosis Risk in Communities Study (ARIC) was published and intensified the discussion of the value of markers. It assessed 19 novel risk markers, including CRP, in addition to conventional risk factors, in the prediction of HD.

CRP did not add significantly to the C-statistic and neither did most of the other markers that were evaluated. It may be argued that there is no need for more laboratory markers for risk
Discussion

stratification since INTERHEART showed that more than nine in every ten MIs were associated with nine easily measurable clinical risk factors. Perhaps, in order for patients to receive the additional benefits of more individualized treatment that improved risk stratification could make possible, we need a simpler schedule for the clinician to use. This is where I believe there is a place for a multimarker strategy, containing both clinical data and a proven set of valuable biomarkers. In prognostication, as in life, it is probably best to rely on more than just one source of information.
Conclusions

- OPG, CXCL16 and CgA independently predict mortality in ACS patients.
- OPG, CXCL16 and CgA independently predict rehospitalizations due to HF, but not due to stroke. All three predict hospitalizations due to MI in univariate analysis, but only CXCL16 does so after adjustment for clinical risk factors.
- Baseline values of CXCL16 and OPG, as obtained in a stable phase 3 months after the acute event, are equally predictive of mortality and morbidity as levels obtained in direct relationship to an ACS.
- Levels of OPG and CXCL16 are increased in relation to an ACS. While CXCL16 levels increased between day 1 and day 4, there was a concurrent non-significant decrease in OPG.
- CXCL16 is a stable marker, only slightly affected by circadian and postprandial variation, as well as by the freezing and thawing of serum.
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