Inflammatory Biomarkers in Cardiovascular diseases

By
Ståle Haugset Nymo

for
PhD
Supervisor: Arne Yndestad

Research Institute of Internal Medicine
Oslo University Hospital
University of Oslo

2016
© Ståle Haugset Nymo, 2017

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo


All rights reserved. No part of this publication may be
reproduced or transmitted, in any form or by any means, without permission.

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.
Acknowledgment

The studies this thesis is based on were performed at the Research institute of Internal medicine at Oslo university hospital, Rikshospitalet, Oslo during the years 2010-2015, and I greatly appreciate the outstanding working facilities provided throughout my studies. This has been an excellent place both to work and socialize, and have made my research a lot more fun and interesting.

To me supervisor, Arne Yndestad, I extend my profoundest gratitude. From the very first day I came to the institute, he has always been forthcoming, helpful and available for questions and discussions. While giving me a lot of room to experiment, he has always reined me in when I’ve strayed too far away, and guided my back on track.

I would also extend my deepest gratitude to Pål Aukrust and Lars Gullestad, for having faith in me when I first came to the institute, giving me the responsibility for statistics I might not have been qualified for, and patient while I slowly found my way through the statistical quagmire. Your feedback have been invaluable, and this thesis would have been possible without all help and guidance.

Thor Ueland, thank you for making my time at the office so much more fun, always finding time for discussions, advice, and conversations. Most of my work would not have been possible without your input, and countless hours in the lab.

To all my co-workers and friends at the institute, this would not have been possible, and more importantly, no fun without you!

Last but not least, and I want to thank my wife Kari for always being there, patiently waiting when I’ve had to work long hours, and making my life what it is. And of course I need to thank my daughter Ingrid for smiling most of the times I came home.

Oslo, March 2016

Ståle H. Nymo
Table of Contents

Acknowledgment .................................................................................................................. - 3 -

Table of Contents .................................................................................................................. - 4 -

List of papers ........................................................................................................................ - 7 -

Abbreviations and glossary ................................................................................................... - 8 -

1 Introduction ..................................................................................................................... - 11 -

1.1 Cardiovascular diseases ........................................................................................ - 11 -

1.1.1 Heart failure .................................................................................................. - 11 -

1.1.2 Acute coronary syndrome ............................................................................. - 15 -

1.1.3 Biomarkers in cardiovascular diseases ......................................................... - 17 -

1.1.4 Prognosis assessment in ACS and HF .......................................................... - 19 -

1.2 Inflammation in cardiovascular diseases .............................................................. - 20 -

1.2.1 Initiation of inflammation ............................................................................. - 21 -

1.2.2 Cytokines ...................................................................................................... - 23 -

1.2.3 Neutrophil granulocytes ................................................................................ - 23 -

1.2.4 Resolution of inflammation .......................................................................... - 25 -

1.2.5 Inflammation in chronic heart failure ........................................................... - 26 -

1.2.6 Inflammation in acute coronary syndrome ................................................... - 28 -

1.3 NGAL ................................................................................................................... - 31 -

1.3.1 General properties ......................................................................................... - 31 -

1.3.2 NGAL in disease ........................................................................................... - 31 -

1.3.3 NGAL as a biomarker ................................................................................... - 32 -

2 Aims ............................................................................................................................. - 33 -

3 Material and Methods ..................................................................................................... - 34 -

3.1 Patients .................................................................................................................... - 34 -

3.1.1 The CORONA study ..................................................................................... - 34 -

3.1.2 The PRACSID study .................................................................................... - 34 -
3.2 Blood sampling ................................................................. - 34 -

3.3 ELISA .................................................................................. - 35 -

3.4 Multiplex ............................................................................... - 35 -

3.5 Statistical methods............................................................. - 35 -

4 Results .................................................................................. - 37 -

4.1 Paper I ................................................................................... - 37 -

4.2 Paper II .................................................................................. - 38 -

4.3 Paper III ................................................................................ - 39 -

4.4 Paper IV ................................................................................ - 40 -

5 Discussion .............................................................................. - 41 -

5.1 Analysis of circulating biomarkers in clinical materials ............. - 41 -

5.1.1 Collection of samples ..................................................... - 41 -

5.1.2 ELISA ................................................................................. - 42 -

5.1.3 Multiplex .......................................................................... - 43 -

5.2 Statistical considerations .................................................... - 44 -

5.2.1 The assumptions of the cox model ................................... - 44 -

5.2.2 Missing values ............................................................... - 45 -

5.2.3 A model’s discrimination: Harrell’s C statistics and NRI .......... - 46 -

5.2.4 Validation of prognostic models ....................................... - 47 -

5.3 Inflammation in cardiovascular disease: biomarkers, players, and potential therapeutic targets ............................................. - 48 -

5.3.1 Inflammatory biomarkers in HF ....................................... - 48 -

5.3.2 NGAL; potentials and difficulties ..................................... - 49 -

5.3.3 Neutrophils, an important cell type in CVD? .................... - 50 -

5.3.4 Inflammation in HF: a way ahead? ................................. - 51 -

5.4 Biomarkers, any use beyond prognosis? ............................... - 52 -

5.4.1 Current status ............................................................... - 52 -
5.4.2  Biomarkers as pathophysiological informants .............................................. - 53 -
5.4.3  Biomarkers as source of new hypotheses ..................................................... - 53 -
5.4.4  Should there be a shift of focus in biomarker research? ............................... - 54 -
6    Concluding remarks and future work .............................................................. - 56 -
7    References ........................................................................................................ - 58 -
List of papers

The thesis is based on the following papers, referred to by their roman numerals:

I. The association between neutrophil gelatinase-associated lipocalin and clinical outcome in chronic heart failure: results from CORONA.

II. Serum Neutrophil Gelatinase-Associated Lipocalin (NGAL) is independently associated with mortality in acute coronary syndromes.
Ståle H. Nymo, Marianne Hartford, Thor Ueland, Arne Yndestad, Erik Lorentzen, Katarina Truvé, Thomas Karlsson, Pål Aukrust, Kenneth Caidahl
Submitted manuscript

III. Inflammatory cytokines in chronic heart failure: interleukin-8 is associated with adverse outcome - results from CORONA.
Ståle H. Nymo, Johannes Hulthe, Thor Ueland, John J.V. McMurray, John Wikstrand, Erik T. Askevold, Arne Yndestad, Lars Gullestad, Pål Aukrust
Eur J Heart Fail. 2014; 16:68-75

IV. Limited added value of circulating inflammatory and extracellular matrix biomarkers in multimarker models for predicting clinical outcomes in chronic heart failure.
Submitted manuscript
**Abbreviations and glossary**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>AT2</td>
<td>Angiotensin-2</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>cNRI</td>
<td>Continuous net reclassification improvement</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CRS</td>
<td>Cardio-renal syndrome</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GRACE</td>
<td>Global registry of acute coronary event</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>HFrEF</td>
<td>Heart failure with reduced ejection fraction</td>
</tr>
<tr>
<td>HFpEF</td>
<td>Heart failure with preserved ejection fraction</td>
</tr>
</tbody>
</table>
HR  Hazard ratio
IL  Interleukin
IFN  Interferons
LBBB  Left bundle branch block
LDL  Low density lipoprotein
LPS  Lipopolysaccharide
LV  Left ventricle
MCP  Monocyte chemoattractant protein
mLDL  Modified low density lipoprotein
MMP  Matrix metalloproteinase
MPO  Myeloperoxidase
NET  Neutrophil extracellular trap
NGAL  Neutrophil gelatinase-associated lipocalin
NP  Natriuretic protein
NSTEMI  No-ST-elevation Myocardial infarction
NT-proBNP  N-terminal pro-brain natriuretic peptide
NYHA  New York heart association
PCI  Percutaneous coronary intervention
PDGF  Platelet derived growth factor
PRR  Patter recognizing receptor
RAS  Renin-angiotensin-aldosterone system
ROS  Reactive oxygen species
SLPI  Secretory leukocyte protease inhibitor
SMC  Smooth muscle cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-elevation Myocardial infarction</td>
</tr>
<tr>
<td>sTNF-R</td>
<td>Soluble TNF receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMI</td>
<td>Thrombolysis in myocardial infarction</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UA</td>
<td>Unstable Angina</td>
</tr>
<tr>
<td>ΔC</td>
<td>Change in Harrell’s C statistics</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Cardiovascular disease

Cardiovascular diseases (CVD) are diseases involving the heart, the blood vessels, or both, and are the number one cause of death globally. 17.3 million people died from CVD in 2008, representing 30% of all deaths worldwide, and CVD is projected to remain the leading cause of death in all foreseeable future.\(^3\)\(^,\)\(^4\) While great progress has been made in both diagnostics and treatment of CVD, the increasing number of deaths worldwide underlines the need for further research into both pathophysiological mechanisms and treatment. While many pathological processes are thought to be involved in CVD, several lines of evidence support an important role for inflammation in the development and progression of these diseases. This suggests that inflammation and inflammatory biomarkers could be a source of important prognostic information, as well as potentially representing a therapeutic target. However, due to the complexity of the immune system, and intricacy of the inflammatory response, this potential is yet largely unrealized and further research is needed to entangle the meshwork of consequences of inflammatory activity in CVD.

1.1.1 Heart failure

Definition, epidemiology and etiology

Heart failure (HF) is defined by the European Society of Cardiology as an abnormality of cardiac structure or function leading to failure of the heart to deliver oxygen at a rate commensurate with the requirements of the metabolizing tissues, despite normal filling pressures (or only at the expense of increased filling pressures).\(^5\) It is further defined clinically as a syndrome in which patients have typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles, and displaced apex beat) resulting from an abnormality of cardiac structure or function.\(^5\)

It is estimated that there are 23 million people affected by HF worldwide.\(^6\) Approximately 2% of the adult population in developed countries has HF, with prevalence of up to 10% among patients older than 75 years of age.\(^7\) There also seems to be an increasing prevalence of HF probably due to better treatment of CVD, prolonged life span and improved diagnosis.\(^8\)

There is a significant morbidity and mortality associated with HF. It is not only the most common condition leading to hospital admission for people above the age of 65, the 5 year survival rate is also poor with an estimate of 40-50% mortality.\(^9\),\(^10\) The high morbidity,
multiple hospital admissions and mortality rate lead to HF being a major socioeconomic burden, representing as much as 2% of medical expenditures in the West.\textsuperscript{11, 12}

Functionally, approximately 50\% of patients with HF have a reduced left ventricular ejection fraction (HFrEF), while the remaining half has preserved left ventricular ejection fraction (HFpEF).\textsuperscript{5, 13} While HFrEF mainly encompasses disturbance in systolic left ventricular (LV) function, HFpEF is predominantly a diastolic failure.\textsuperscript{5, 13} In this thesis, our main focus has been patients with HFrEF, and we will mainly discuss features of HF of this etiology. The most common cause of HF in the Western world is ischemic heart disease accounting for about two thirds of the HFrEF cases. Other causes are cardiomyopathy, congenital and valvular heart disease.\textsuperscript{5, 7}

\textit{Pathogenesis of HF}

HF is a progressive disorder initiated by an index event that either damages the heart muscle or disrupts the ability of the myocardium to generate force resulting in a decline in the heart’s pumping capacity (Figure 1).\textsuperscript{14} This index event may be acute such as a myocardial infarction (MI) or acute myocarditis, or it may have a gradual onset as in the case of hemodynamic overload, for example due to hypertension or valvular disease. The decline in pumping capacity leads to activation of mechanisms that compensate for and in many cases restore the myocardial function. These compensatory mechanisms involve increased heart contractility through Frank-Sterling mechanisms, activation of the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS), as well as remodeling of the ventricles (see below). Over time, however,
these compensatory or adaptive mechanisms may turn maladaptive, possibly due to secondary damage to the heart, leading to a transition from asymptomatic to symptomatic and decompensated HF.\textsuperscript{14} In HFrEF, cardiac output decreases, and leads to an increase in angiotensin II (ATII) partly because of reduced renal arterial pressure and increased renal venous pressure leading to renin release. Furthermore, SNS activity is increased due to fall in systemic arterial pressure, and probably through central stimulation of the SNS by a combination of factors including decreasing nitric oxide (NO) and increasing afferent renal sympathetic nerve activity.\textsuperscript{15} In addition there exist positive feedback links between RAAS and SNS, where increasing activity in one system also increases the activity in the other. This is among other factors due to centrally stimulating effects of ATII and SNS on renal blood flow, again increasing RAAS activity. RAAS and SNS both take part in volume retention in HF. Several other factors as well, such as decreasing levels of NO, increased adenosine A1 receptor activation and increased vasopressin-mediated volume control all act synergistically to increase volume.\textsuperscript{15-17} This increased sodium and water retention lead to an expansion of the extracellular fluid that increase preload and afterload of the heart, leading to cardiac dilatation, decrease of function and thereby worsening HF.\textsuperscript{18} This close link between renal and cardiac function in HF have led to the coining of cardio-renal syndrome (CRS), underscoring the importance of dysfunction in one of the organs for the function of the other. In addition to fluid retention through activation of RAAS and SNS as well as different degrees of renal dysfunction, immunologic and inflammatory mechanisms are also suggested to be involved in developing and progression of HF (discussed in chapter 1.2.5).\textsuperscript{19}

**Myocardial remodeling**

An important aspect of the compensatory response to reduced myocardial function is a process commonly referred to as myocardial remodeling.\textsuperscript{20} The remodeling process consists of a set of complex molecular and cellular events that lead to changes in both myocardial structure and function. Neurohormonal activation with increased RAAS and SNS activity is not only a direct compensatory mechanism of reduced cardiac function, but also an initiator and mediator of cardiac remodeling.\textsuperscript{21} Studies suggest that also inflammation may influence this process.\textsuperscript{22} The most prominent feature of myocardial remodeling is increased myocardial mass, primarily due to hypertrophy of individual cardiomyocytes.\textsuperscript{23} On the other hand, cardiomyocyte loss also occurs through necrosis and apoptosis. In addition to increasing its
mass, the ventricles dilate and change to a more spherical shape.\textsuperscript{24} A change in the quantity and quality of the extracellular matrix is another hallmark of remodeling.\textsuperscript{25} This process both contributes to ventricular dilation and also increased collagen deposition and interstitial fibrosis. Finally, myocardial remodeling is characterized by reactivation of a fetal pattern of gene expression.\textsuperscript{26}

The myocardial remodeling is initially thought to be favorable or adaptive and contribute to preservation of the myocardial function. However, continued remodeling leads to progressive impairment of the myocardial structure and become deleterious or maladaptive to the overall function of the heart.\textsuperscript{21} The transition from compensated to decompensated HF is a key event in the pathogenesis of HF, but the exact mechanism as well as the relative importance of the various factors are far from clear, and this process represents an important area of HF research.\textsuperscript{21, 23}

**Diagnosis and management of HF**

Diagnosis of HF is dependent on typical symptoms and signs of HF, as well as objective evidence of reduced systolic or diastolic function and the measurement of biomarkers, in particular the natriuretic peptides. However, due to the non-specific nature of clinical findings, it is not always easy to make an initial diagnosis of HF in early phases of the disease. In addition to chest x-ray, ECG and routine blood work, echocardiogram as well as measurement of brain natriuretic peptide (BNP) or N-terminal (NT)-proBNP are the most useful tests in aiding the diagnosis of the disease.\textsuperscript{5, 27}

The main components of HF treatment have not changed over the last decade and are still based on medical treatment with beta-blockers, angiotensin converting enzyme (ACE) blocker, and diuretics.\textsuperscript{5} For end stage HF, surgical intervention is also warranted with different left ventricular assistance devices, aortic balloon pumps, and finally heart transplantation. Finally, there is increasing evidence for the use of resynchronization therapy in certain forms of HF with left bundle branch block (LBBB), whereby pacemakers are used to optimize the hearts depolarization patterns. All treatment options today except heart transplantation, and to some extent resynchronization therapy, only delays the development of disease, and most patients will suffer from a progressing HF despite optimal medical treatment.\textsuperscript{5}
1.1.2 Acute coronary syndrome

Definition and epidemiology

Acute coronary syndrome (ACS) is caused by coronary artery disease (CAD), and refers to a group of symptoms caused by the sudden full or partial obstruction of one or more coronary arteries. ACS typically comprises one of three conditions, i.e., ST-elevation myocardial infarction (STEMI), no-ST elevation myocardial infarction (NSTEMI) and unstable angina pectoris (UA). The typical symptoms are retrosternal pressure or heaviness (angina) with possible radiation to the left or both arms, neck, or jaw, which may be intermittent (usually lasting several minutes) or persistent. There might also be other symptoms such as diaphoresis, nausea, abdominal pain, dyspnea, and syncope. However atypical presentations are not uncommon. MI from CAD (i.e., STEMI or NSTEMI) is defined as detection of a rise and/or fall of cardiac biomarker values (preferably cardiac troponin) with at least one value above the 99th percentile upper reference limit (URL) and with at least one of the following:

- Symptoms of ischemia.
- New or presumed new significant ST-segment–T wave (ST–T) changes or new LBBB.
- Development of pathological Q waves in the ECG.
- Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.
- Identification of an intracoronary thrombus by angiography or autopsy.

Worldwide, CAD is the single most frequent cause of death, killing more than 7 million people a year. The incidence rate of STEMI has decreased the last decade from about 120/100 000 in 1997, to approximately 80/100 000 in 2005. In the same time span, the incidence rate of NSTEMI increased slightly from 126/100 000 to 132/100 000. Hospital mortality is higher among STEMI patients (7%), than NSTEMI and UA (3-5%). However, at 6 months the mortality for the two groups are similar, at 12% and 13%, and long-term data shows a two-fold higher mortality among NSTEMI and UA patients compared to STEMI patients at 4 years. This however might be due to the difference in profile between the two patient groups as NSTEMI and UA patients are in general older, with more co-morbidities such as diabetes and renal failure.
**Pathophysiology of ACS**

ACS represents a life-threatening manifestation of atherosclerosis. Atherosclerosis is a chronic, multifocal inflammatory, fibroproliferative disease of medium-sized and large arteries mainly driven by lipid accumulation further discussed below.\(^{32}\) In ACS, there is usually not only atherosclerosis, but also an acute worsening of the underlying atherosclerosis caused by thrombosis, vasospasm or both. Thus the symptomatic lesion most often consists of a variable mix of an atherosclerotic plaque as well as a thrombus.\(^{28}\)

Several factors contribute to the development of ACS. Firstly, not all atherosclerotic plaques are equally vulnerable. Certain plaques are more prone to instability and rupture. These plaques often have a thinner fibrous cap and larger lipid core with more inflammatory activity.\(^{32}\) Plaque vulnerability may also depend on wall stress, the size of the plaque as well as the impact of flow on the luminal plaque surface.\(^{28}\) Instead of plaque rupture, there may also be plaque erosion causing a thrombus to form in relation to the surface of the plaque. This may contribute to rapid progression of the plaque and decreased luminal diameter. Autopsy data has demonstrated a central role of thrombosis in ACS.\(^{28,33}\) The thrombus usually develops at the site of the vulnerable plaque when the highly thrombogenic lipid-rich core is exposed by rupture. Thrombosis induced at the site of plaque rupture could increase vessel occlusion dramatically and give a subtotal or complete occlusion of the coronary artery. Spontaneous thrombolysis does however happen, and may explain some of the transient episodes of ACS. There may also be embolization of the thrombus, leading to occlusion of downstream arterioles and capillaries, leading to small areas of infarct as well as release of cardiac markers.\(^{28}\)

**Diagnosis in ACS**

While most patients with ACS present with some sort of chest pain, many patients presenting with this symptom does not have an ACS. ACS is usually characterized by\(^{30}\):

- Prolonged (>20min) angina pain at rest.
- New onset severe angina.
- Recent destabilization of previous angina.
- Post-MI angina.

Prolonged pain is observed in 80% of patients with ACS, while *de novo* or accelerated angina is observed in the remaining 20%. It is not possible to distinguish between STEMI, NSTEMI
and UA based on symptoms alone. The main classification of ACS depends on ECG (presence or not of ST-elevation, or new left bundle branch block in probable STEMI), and the rise or fall in serial measurements of cardiac specific proteins (troponin I or troponin T) with at least one value above the 99th percentile, separating STEMI and NSTEMI from UA.  

### 1.2 Biomarkers in cardiovascular diseases

The idea of using information about a subject to detect subclinical disease states and to predict future health events has great appeal, and the search for such markers in CVD has been blooming for many years. A biomarker may be defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”. Biomarkers are anything that can be measured objectively and consistently and include measurements in a biosample (e.g. blood or urine sample), recording of parameters (e.g. ECG), or data from imaging tests. Biomarkers have several potential functions, which can roughly be divided into indicators of disease trait (e.g. risk factors), disease state (e.g. preclinical or clinical) or disease rate (progression of disease). Some are antecedent, i.e., measured before development of a disease. Such biomarkers can give clues on the risk of developing the disease, work as screening methods recognizing subclinical disease states, be diagnostic and recognize what disease causes the overt symptoms, be used in staging the disease, and finally aid in estimating prognosis of a patient with known disease.

Several factors influence the clinical utility of a given biomarker. First of all, the accuracy of the biomarker is important. One needs to be able to precisely estimate the levels of the biomarker using available methods. Furthermore, the reproducibility of biomarker measurements is important. If the measurement depends heavily on when, by whom, and how it is computed, it will be very difficult to compare levels across time and individuals. Measuring the biomarker must be acceptable for the patient, which partly will depend on the usefulness of the biomarker itself. The acceptance of more invasive sampling techniques would be greater if the biomarker significantly aids treatment. Ideally, the biomarker should be easy to interpret by the clinician, and affect the way the patient is managed. If there is no change in treatment related to biomarker levels, there will be no obvious benefit from measuring the biomarker, only a cost in doing so. The biomarker should also explain a reasonable proportion of outcome in multiple studies independent of already existing
predictors. If other, already implemented markers give the same information, including a new marker do not make much sense as both methods and routines related to the old marker are already established. However, if the cost involved in measuring the new marker is substantially less than the old one, the new biomarker could still be useful.

There have been an abundance of new potential biomarkers in CVD over the last decades. In chronic HF, troponins, galectin-3, pentraxin 3, soluble ST2, as well as soluble TNF receptor (sTNFR) 1 and 2 are some of the biomarkers more widely studied, and often associated with outcome in these patients. However, none of these have yet made it into clinical use, and studies conducted so far show some discrepancies in effect and importance of these variables.

Today, only troponins and the natriuretic proteins have made it into clinical guidelines, and then mostly for diagnostic purposes, not prognostic ones. Furthermore, while natriuretic peptides have been suggested as potential prognostic markers in HF, they do not reflect all underlying pathological processes in the failing heart, and only improve the prognostic power of well-constructed models by a few percent. In an attempt to further improve prognostic models, it has been suggested to implement multi-marker risk models, where a panel of biomarkers reflecting different aspects of the disease process are evaluated together to improve prognostic models. Braunwald suggested selecting biomarkers reflecting seven different aspects of HF pathology; that is myocardial stretch, myocyte injury, matrix remodeling, inflammation, renal dysfunction, neurohumoral activation, and oxidative stress (Figure 2). By including biomarkers covering different areas of dysfunction in HF, the hope is to succeed in improving current prognostic models. Moreover, this approach is appealing from the point of view of pathogenesis, as they could not only improve prognostic abilities of models, but also be able to hint to which processes are driving the development of

Figure 2 Braunwald’s classification of biomarkers in HF. The seven main causes of chronic heart failure pathogenesis.
the disease in a given patient and could help select patients for different treatments. Several biomarkers have been suggested as possible pieces in such a model. For example, galectin-3, a soluble β-galactoside–binding lectin released by activated cardiac macrophages, has been shown to be associated with cardiac fibrosis and remodeling in a rat model of hypertrophic HF. Several authors have suggested it can be used as a fibrosis marker in clinical HF as well. And while NT-proBNP/BNP is thought to reflect myocardial wall stress, troponins have proven a valuable marker of myocyte injury in ACS, and several studies have supported a potential role for troponin in prognostic models of HF as well. However, no panel of biomarkers has been thoroughly validated or reached clinical practice, and their clinical relevance has not yet been tested.

1.2.1 Prognosis assessment in ACS and HF

ACS is an unstable coronary condition prone to complications and recurrences both in short and long term. There is also a wide repertoire of both pharmacological and physical interventions that could help patients, but also have serious side effects. The timing and intensity of interventions should thus depend on the risk of the individual patient. There are several tools to help classify patients according to risk, and based on this assessment select the best treatment options.

Clinical risk assessment, and especially short term risk assessment is important in ACS to select patients for more intensive treatment. In addition to some universal risk markers such as age, diabetes, renal failure, and other co-morbidities, the severity of the initial clinical presentation is important for early prognosis. Factors such as the presence of symptoms at rest, increasing number of episodes preceding the index event, presence of tachycardia, and hypotension are all linked to higher risk, and warrant rapid diagnosis and aggressive management. ECG is also an important source of information, where ST-elevation is the most significant finding which should lead to emergency percutaneous coronary intervention (PCI) or antithrombotic treatment. Other findings, such as ST-depression and abnormal T-waves also signify a higher risk than patients presenting with a normal ECG. In patients with no symptoms at rest after admission, stress ECG may provide further information. Continuous ST-segment monitoring can reveal transient ST-depression in patients with NSTEMI or UA, signifying increased risk. There are also useful biomarkers in the acute setting of ACS, where troponins have gained the most central role over the last decade. Not only does
dynamic change in troponin levels in most cases alone confirm the presence of cardiac
necrosis, but troponin levels are also associated with short term and long term risk of
mortality and recurrent MI.\textsuperscript{31} Also other biomarkers may give additional information to ECG,
clinical presentation and troponins in the diagnosis of ACS. Among others, C-reactive protein
(CRP) and NT-proBNP have been extensively validated.\textsuperscript{56} While CRP seems to give
significant information on short and long term prognosis, NT-proBNP may help separate
cardiac causes from other causes of dyspnea. Also blood glucose and other hematological
parameters such as hemoglobin, platelet count and white blood cell count have been shown to
give additional prognostic information.\textsuperscript{31}

Several risk scores have been developed to facilitate the separation of patients into risk
groups, aiding further follow-up. The two most widely used today are the thrombolysis in
myocardial infarction (TIMI) score and the global registry of acute coronary event (GRACE)
score.\textsuperscript{57} While the TIMI score is easier in use, the GRACE score has shown itself to provide
the most accurate risk stratification. However, as the GRACE score requires a computer to
calculate the final score, it is more complicated to apply in the clinic.\textsuperscript{58}

There are some risk scores available for HF as well, but their predictive power is lower than
those for ACS.\textsuperscript{5, 59} These include the Seattle heart failure score (SHFS), heart failure survival
score (HFSS) and EFFECT model.\textsuperscript{60, 61} Common for these scores are the usage of clinical
data such as age, sex, New-York heart association (NYHA) class and body mass index (BMI)
that gives important prognostic information. Furthermore, echocardiography, chest x-rays, as
well as standard biochemical data on kidney function, blood hemoglobin, white blood cells
are also added to some of the models.\textsuperscript{46, 47, 59, 62, 63} As discussed above, the role of other
biomarkers on risk assessment of HF patients is still only in its infancy, and only the
natriuretic proteins for diagnosing HF is in widespread clinical use.

1.3 Inflammation in cardiovascular diseases

Inflammation is vital for the host to protect against invading pathogens, but also to promote
repair during tissue damage. In response to a pathogen or a sterile injury, a cascade of signals
leads to recruitment of inflammatory cells and activation of the immune system.\textsuperscript{64} The
immune system consists of cellular and humoral components that work in concert in response
to infection or injury in an attempt to maintain homeostasis.\textsuperscript{65} This involves both an ability to
respond effectively against invading pathogens and self-injury, as well as regulatory pathways to keep the inflammatory cascade from spiraling out of control and properly resolve inflammation when the stimulus is removed, and inflammation no longer is needed.65

The immune system is commonly divided into two main components, the innate and the acquired immune system. Although these two systems are highly interconnected, and their separation might not be as clear cut as originally envisaged, this division can still be conceptually useful.66 The innate immune system mainly consists of leukocytes, natural killer cells, complement, and inflammatory mediators such as chemokines, cytokines, and acute phase proteins, which provides immediate host defense based on the recognition of different molecular patterns. The adaptive immune system on the other hand consist of antigen-specific reactions through T and B lymphocytes.66

### 1.3.1 Initiation of inflammation

The innate immune system is usually considered the first responder (Figure 3).66 It responds to general molecular patterns or signals from pathogens as well as tissue damage. These first signals acts trough pattern recognizing receptors such as the toll-like receptor (TLR) family, activating cells such as mast cells, tissue macrophages, neutrophils and others.67 In addition, molecules primarily present in the fluid phase such as the complement system can bind pathogens and activate an immune response.68 An influx of cells of the innate as well as acquired immune system to the site of inflammation is initiated through vasodilation, expression of adhesion molecules by nearby endothelial cells, and chemotactic molecules from endothelial cells as well as tissue residing cells and the activation of fluid phase danger signals.69 Altogether, these pathways lead to upregulation and recruitment of cells and mediators that may be helpful in eradicating the cause of inflammation, and restoring tissue homeostasis. The immune system is not only important in pathogen eradication, but also plays a pivotal role in tissue and wound repair. The inflammatory response to trauma, ischemia-reperfusion injury and chemically induced injury are coined “sterile inflammation” as it is induced in the absence of any microorganisms.64 Cellular damage is sensed by the innate immune system through many of the same pathways as pathogens. Molecules released from dying cells, so called damage associated molecular patterns (DAMPs), are recognized by pattern recognition receptors such as TLRs, Nod-like receptors, RIG-1-like receptors and C-type lectin receptors, as well as circulating molecules such as the complement system.67
This leads to activation of immune cascades recruiting neutrophils and monocytes/macrophages, and production of inflammatory cytokines and chemokines. Activation of the immune system leads to removal of cellular debris, and scar tissue formation. However, the invasion of neutrophils with the release of proteases, growth factors and reactive oxygen species (ROS), also leads to collateral damage with tissue destruction as well as fibroblast proliferation and aberrant collagen accumulation. Hence, the regulation of the response with quick resolution of inflammation when no longer needed is paramount, and unresolved chronic inflammation can have many detrimental effects as exemplified by

**Figure 3 Cardiac injury and sensing damaged tissue.**

The figure shows a coronary artery occlusion (black) that leads to ischemic (US-eng i tekst vs UK-eng i legend) tissue injury (grey zone). From within the ischemic area, cell necrosis, extracellular matrix (ECM) degradation and recruitment of immune cells all lead to the production of specific damage-associated molecular patterns (DAMPs), which are recognized by pattern recognition receptors. This leads to the generation of inflammatory responses to internal injury signals. CpG, CpG dinucleotides; dsRNA, double-stranded RNA; HMGB1, high-mobility group box 1; HSP, heat shock protein; IL, interleukin; IL-1R, IL-1 receptor; NLRP3, NOD-, LRRand pyrin domain-containing 3; P2Y, P2Y purinoceptor; P2X, P2X purinoceptor; RAGE, receptor for advanced glycation end-products; TLR, Toll-like receptor. 2

This leads to activation of immune cascades recruiting neutrophils and monocytes/macrophages, and production of inflammatory cytokines and chemokines.
several inflammatory diseases, such as atherosclerosis, Crohn’s disease, ulcerative colitis, and systemic lupus erythematosus.64

1.3.2 Cytokines
Cytokines are soluble, intercellular signaling molecules produced to a varying extent by virtually all cells. Most are soluble signaling molecules, but some may be membrane-bound.66 They can exert autocrine, paracrine and endocrine functions, producing a wide range of responses such as cell activation, proliferation, differentiation, movement, survival or death. Cytokines are classified in different ways, where the classification into various families based on the structure of their receptors is the most frequently used. According to this classification, the largest family is the hematopoietin receptor family (type I cytokine receptor family) and includes the receptors for most ILs. Other cytokine families include the interferon (IFN), IL-1, TNF, chemokine and transforming growth factor (TGF)-β receptor families.70 Immune cells are major contributors to the cytokine pool, but also other cell types such as endothelial cells and fibroblasts contribute significantly to cytokine production in several conditions. One of the main roles of cytokines is to regulate the activity of immune cells. Hence, some cytokines are coined pro-inflammatory and others anti-inflammatory according to their main effect on immune activity. However, several cytokines may exert both pro- and anti-inflammatory effects, at least partly dependent on co-stimuli and the degree of cellular pre-activation.71 Moreover, the activity and response to cytokines can be modulated by the presence of not only other cytokines, but also of endogenous cytokine modulators such as soluble cytokine receptors and receptor antagonists underscoring the complexity of this cytokine network.70 In addition, cytokines not only exert influence on immune cells, but also play an important role in the interaction between the immune system and other systems, such as the central nervous system.72 Given the importance of neurohormones in HF, the interplay between cytokines and the neuroendocrine system may be of particular relevance to this disease.

1.3.3 Neutrophil granulocytes

Recruitment and phagocytosis
Neutrophil granulocytes play an important role in initiation of an immune response mediated through 1) phagocytosis, 2) release of anti-microbial peptides and proteases, and 3) formation of neutrophil extracellular traps (NETs).73,74 In early stages of infection or tissue damage, a wide range of cytokines are released from resident mast cells and macrophages, as well as
other native tissue cells. Some of these cytokines (e.g., TNF, IL-1β) upregulate adhesion molecules (E-selectin and ICAM-1) on nearby vascular endothelial cells. Circulating neutrophils attach to these adhesion molecules, and diapedeses out of the circulation through spaces between, and sometimes through, the endothelial cells, attracted by powerful chemoattractants such as leukotriene B4, IL-8 and C5a. Outside of the vascular space, they move along concentration gradients of chemokines until they reach the site of inflammation. A major role at the site of inflammation is to phagocytose pathogens. The neutrophils achieve this by engulfing the pathogens in pseudopodia, thus internalizing the pathogens. Once inside the cells, the membrane bound phagosome fuses with lysosomes forming phagolysosomes in which the pathogens are broken down by two main mechanisms. The first depend on creation of toxic oxygen radicals by NADPH oxidase. The other mechanism is independent of oxygen, and dependent on toxic cationic proteins and enzymes such as myeloperoxidase (MPO) and lysozyme. The ingestion and killing of pathogens are greatly amplified if the particle is opsonized with complement or specific antibodies, binding to specific receptors on the neutrophil, enhancing its uptake and priming the cell for efficient removal and digestion.

**Other functions**

Neutrophils do not only phagocytose pathogens at the site of inflammation. They play several other decisive roles, helping to resolve inflammation, but at the cost of self-damage. While some neutrophils find and digest pathogens at arrival on site, others release their granulas into their surroundings. These serve many different purposes. The first granulas to be released are the peroxidase-negative granulas containing among other proteins several matrix metalloproteinases (MMPs; MMP-8, -9 and -25), as well as neutrophil gelatinase-associated lipocalin (NGAL), LL-37, lactoferrin and many others. Neutrophils proceed to release their α-granulas containing four α-defensins and MPO. The combination of lactoferrin, an iron binding protein, and NGAL, a siderophore binding protein, help starve invading bacteria of needed iron. The MMPs liquefies the extracellular matrix, making it easier for immune cells to invade the area, and might hamper with the bacteria’s ability to escape the site of infection. Finally MPO released from the primary granules converts the relatively innocuous H₂O₂ into more powerful antiseptics, thereby potentiating the toxic soup.

Recently, another role of the neutrophils has been suggested. Instead of undergoing apoptosis, neutrophils have the ability to instead undergo “NETosis”, in which it releases its nuclear
DNA as an extracellular trap (NETs). These NETs may have a multitude of function, of which one seems to be the ability to trap pathogens in tissue or capillaries, preventing them from disseminating to other areas of the body. In addition, the NETs also contain some of the cytotoxic enzymes found in neutrophil granulas, which might help in quickly killing attached pathogens.

1.3.4 Resolution of inflammation

The problem with the immune system is not how often it is activated, but rather how often it fails to subdue. While the immune system is activated as a response to inflammatory stimuli, the resolution of inflammation is to a large extent dependent on signals from the immune system itself as well as removal of the original cause of inflammation. One important factor in resolving inflammation, are the inflammatory cells themselves. Neutrophils attracted to a site of inflammation will at some point undergo apoptosis. Apoptotic cells are mainly ingested by macrophages, in a process termed efferocytosis, and this triggers these cells to release anti-inflammatory cytokines such as TGF-β and IL-10. The phagocytosis of apoptotic cells is further enhanced by glucocorticoids, and thus enhances the release of anti-inflammatory cytokines from macrophages. Factors prolonging the life of neutrophils, or inhibiting the phagocytosis of the apoptotic cells, can thus prolong the inflammatory process. Many soluble anti-inflammatory molecules have also been discovered in the last few decades. Some are byproducts of the inflammatory process itself and thus constitute a negative feedback system (e.g. oxygenated or nitrogenated lipids), while others are released by cells of the immune systems. For example, secretory leukocyte protease inhibitor (SLPI) is secreted by macrophages late in the response to an inflammatory stimulus. SLPI suppresses the ability of neutrophils to be activated by TNF, and indirectly inhibits the release of IL-8 from epithelial cells. While nucleosomes main role seem to be in the initiation of inflammation, they may also play an essential part in resolving the acute inflammatory response. Not only can neutrophils produce pro-resolving lipid mediators (e.g., lipoxin A4, resolvin E1 and protectin D1) inhibiting further neutrophil recruitment, they also function as cytokine scavenging cells, removing several important cytokines present, further decreasing the inflammatory activation signals. Moreover, neutrophils may release cytokine antagonists, e.g., IL-1 receptor antagonist and also express cytokine decoy receptors, e.g., IL-1RII. The resolution of inflammation is an incredibly complicated process where we are only starting to understand some of the pathways involved. Its importance is however evident, as illustrated below.
1.3.5 Inflammation in chronic heart failure

Levine et al. reported more than 20 years ago that patients with HF had increased levels of the inflammatory cytokine TNF. Since then a wide range of papers have demonstrated the immune activation in this patient group, shown increased levels of several cytokines and other inflammatory markers both systemically and locally in the heart, and suggested its involvement in initiation and progression of HF. Several animal models have also shown the potential adverse effects of increased inflammatory activity, and how this could contribute to HF. However, others studies have also shown the potential harmful effect of inhibiting the immune system by certain pathways, demonstrating the difficulty in untangling the complicated inflammatory meshwork.

Causes of immune activation in HF

While studies suggest there is a persistent immune activation in HF patients, the causes of this activation in developing HF are still not entirely understood. There are several theories on how this persistent activation comes about. Several have suggested that increased endotoxin levels circulating in blood could be the cause. Volume overload followed by mesenteric venous congestion leads to edema in the bowel wall. Gram negative bacteria could then translocate from the intestinal space to the circulation with release of endotoxins. Lipopolysaccharide (LPS) is a strong activator of the immune system, and leads to a robust increase in the production of TNF and many other cytokines. This theory is supported by studies showing increased endotoxin levels in patients with peripheral edema, and that these levels decrease with diuretic treatment. Systemic levels of endotoxin was also found to be reduced after resolution of acute decompensated episodes.

Intravascular congestion could itself also lead to cytokine production. Endothelium stimulated by stretch produced endothelin-1 and TNF within hours of exposure, and studies suggest that markers of inflammation such as cyclooxygenase-2 and inducible nitric oxide synthase expression are elevated in venous endothelial cells harvested from patients with clinical signs of congestion during decompensation of chronic HF, while production decreased to normal levels after resolution. Hence, endothelial stretch itself during acute phases may be an independent source of inflammatory cytokines.

Increased activation of the neurohormonal system may also contribute to immune activation. In HF there is increased activation of the RAAS as well as the SNS. Studies in mice as well as humans suggest that ATII, a central effector in RAAS, increases production of
inflammatory cytokines in cardiomyocytes, and AT2 receptor blocking reduces circulating levels of TNF and IL-6.\textsuperscript{108-110} Also chronic SNS activation increases the expression of cytokines in myocardial cells as well as cardiac blood vessels, and beta-adrenergic receptor blockade reduces TNF and IL-6 expression in the myocardium, suggesting that this treatment might also have an anti-inflammatory component.\textsuperscript{111}

Finally, the heart itself may constitute an important source of cytokines in HF (Figure 3).\textsuperscript{112} Oxidative stress, DAMPs from necrotic and apoptotic cells, as well as oxidized LDL may work as immune activating ligands, and increase expression of several inflammatory cytokines in cardiomyocytes as well as other resident cells in the heart.\textsuperscript{113-115} Pattern recognition receptors (PRRs) such as TLRs are probable candidates of initiating cytokine production and activating the immune system in the heart.\textsuperscript{67, 116, 117} TLRs are activated by several ligands, both proteins, RNA and DNA of different types. While their main function seems to be the recognition of exogenous ligands such as LPS from gram negative bacteria, they have also been shown to recognize endogenous molecules such as heat shock molecules, ROS, and self DNA and RNA.\textsuperscript{67, 116, 117}

The immune cascade in the heart could potentially be activated from a combination of local stimuli from necrotic cells, ROS and other endogenous ligands in the heart, as well as exogenous ligands from the gut. These lead to activation of leukocytes, but potentially also heart specific cells such as cardiomyocytes and fibroblasts which respond to these ligands by cytokine production.\textsuperscript{118, 119}

\textbf{Consequences of immune activation in HF}

The inflammatory status of chronic HF patients has potentially several detrimental effects and influence heart function substantially. Inflammatory cytokines may directly affect cardiac contractility.\textsuperscript{90} TNF, IL-6 and IL-2 can reduce contractility in a dose dependent manner, through inhibiting Ca\textsuperscript{2+} release from the sarcomeres, and thus limiting Ca\textsuperscript{2+} concentration in systole. Furthermore, they also produce an indirect decrease in contractility through NO-dependent attenuation in myofibrillar sensitivity to Ca\textsuperscript{2+}.\textsuperscript{90}

Inflammation plays a role in the response to tissue injury as discussed above, but may also play an important role in other aspects of HF development. Both in models of volume overload in rats, as well as pressure overload in mice, inflammation plays an important role in the functional changes of the heart that follows. TNF knockout mice showed improved
cardiac function, and less fibrosis, hypertrophy, MMP-9 activity and inflammatory response compared to their wild type counterparts, suggesting a potential involvement of inflammation in the induced pathology.\textsuperscript{120} Equally, volume overload in rats leads to activation of MMPs and degradation of extracellular matrix (ECM), preceded by inflammatory activity.\textsuperscript{121} Importantly, TNF inhibition as well as removal of mast cells in rat models of volume overload also lead to attenuation of adverse eccentric LV remodeling and slow down the progression of HF suggesting an inflammatory component in developing of HF in volume overload as well.\textsuperscript{120, 121}

While treatment directed against neurohormonal activation and renal function are among the fundamental principles of HF therapy today, attempts at therapeutically influencing inflammation has so far been unsuccessful.\textsuperscript{26, 122} Several reasons for this failure are possible. Firstly, HF and inflammation could only be correlated with no causal link between them. In this case targeting the inflammatory process would do little to ameliorate heart function. Secondly, several competing, and potentially redundant, processes could be involved in the development of HF, then targeting only one of these would not be helpful. Finally, the immune system is an inherently complicated system with a meshwork of interconnected pathways and signaling systems. So far attempts at immune modulation have been directed at limiting overall inflammation by tumor necrosis factor (TNF) or interleukin (IL)-1 inhibition, and immune modulating therapies such as intravenous immunoglobulins (IVIG) or methotrexate treatment. In these approaches, the potential benefit of anti-inflammatory therapy could have been limited by unintended side effects hampering the homeostatic role of the immune system in HF.\textsuperscript{26} These reasons are of course not mutually exclusive, and a combination of them could be the cause of the lack of effect of anti-inflammatory treatment in HF so far.

1.3.6 Inflammation in acute coronary syndrome

The immune system plays important roles in ACS as well. Firstly, research over the last two decades has shown us the importance of the immune system in the development of atherosclerosis, a central component of ACS. Secondly, in the case of cardiac necrosis, the immune system is the main player in removing dead tissue, and stimulating the scar formation necessary to keep the organ functionally intact.
**Atherosclerosis**

Atherosclerosis is a chronic inflammatory condition in the vessel wall, involving lipids, thrombosis, fibrosis, and immune cells.\(^{123}\) The vessel wall is not a static environment, but rather serves a very important function in inhibiting coagulation and cellular adhesion, and regulating blood flow. In addition, the vessel wall plays an important role in local injury where it produces cytokines and upregulates adhesion molecules leading to the infiltration of leukocytes.\(^{124}\) In atherosclerosis the normal function of the vessel wall is interrupted, and the wall itself plays an important role in the formation of the atherosclerotic plaque.\(^{124}\)

**Fatty streaks and plaque progression**

While plaque formation is a complex interplay of many different mechanisms over many years, it can broadly be divided into three main processes: formation of the fatty streak, plaque progression and plaque disruption.\(^{123}\) Fatty streaks appear as yellow discolorations in the artery walls that do not protrude into the lumen.\(^{123}\) An important factor in forming the fatty streaks is dysfunction of the endothelial lining of the vessel. Disrupting the normal homeostasis of the endothelium leads to an activated state with increased permeability, release of cytokines, decreased antithrombotic function and upregulation of adhesion molecules.\(^{123, 125}\) The decreased barrier function of the endothelium leads to the entry of LDL particles into the subendothelial space.\(^{126}\) Here, the particles bind to proteoglycans, and may be modified by reactive oxidants both from the endothelium itself, but also from infiltrating macrophages, as well as glycosylated in states of increased blood glucose such as diabetes.\(^{123}\) Modified LDL (mLDL) stimulates the endothelium to produce more cytokines, promoting further recruitment of leukocytes, in particular monocytes.\(^{127}\) Monocytes entering the early plaque differentiate into phagocytosing macrophages, and imbibe LDL from the subendothelial space by phagocytosis mediated by scavenging receptors that show high affinity for mLDL.\(^{127, 128}\) This process may initially be beneficial by removing the pro-inflammatory mLDL, however the imbalance between low efflux and high influx of macrophages leads to a buildup of cells in the plaque, forming of foam cells (fat-laden macrophages), and increased apoptosis and necrosis of these foam cells as the plaque develops.\(^{124}\)

As the plaque progresses, smooth muscle cells (SMCs) infiltrates the intima, where they proliferate and start to form ECM.\(^{123}\) While the mentioned factors lead to plaque growth, resident cells also produce factors leading to the breakdown of the ECM, and inhibition of
SMCs. Notably foam cells stimulated by cytokines from T-lymphocytes produce MMPs, that actively disrupts the ECM, and other cytokines from the T-lymphocytes inhibit ECM production by SMC.\textsuperscript{123, 129} Thus the thickness of the fibrous cap protecting the core of foam cells, lipids and cellular debris from the circulation depends on the balance between ECM synthesis and degradation, again determined by the balance of signal molecules produced by the resident cells.

**Plaque rupture**

Plaques can continue to develop over decades. Death of foam cells and SMC will however lead to a slow buildup of cellular debris, and lipids to the plaque core. As the growing plaque protrudes more into the lumen of the vessel, there is increased wall stress especially in the borders between normal and dysfunctional endothelium, called the “shoulder” of the plaque.\textsuperscript{130} In addition, there is evidence for an increased concentration of T-lymphocytes as well as foam cells in these areas potentially leading to increased production of MMPs and disruption of the ECM\textsuperscript{123}. This may lead to increased vulnerability of the plaque in these areas, and potentially a plaque rupture where the circulating blood gets in contact with the plaque core activating coagulation cascades and clot formation. The partial or complete occlusion leading to ACS mainly comes from these events.

**Inflammation in cardiac ischemia**

When an atherosclerotic plaque ruptures, blood flow can be altered in downstream areas, leading to ischemia, and if perfusion is not reestablished, infarction. This leads to sterile inflammation, with influx of neutrophils and macrophages (See section 1.3.1). Once at the site of injury, neutrophils and macrophages have multiple roles both in sustaining and resolving inflammation (see section 1.3.3 and 1.3.4). Traditionally, monocyte/macrophages present in the heart during sterile inflammation have been assumed to stem from circulating monocytes recruited to the tissue in the acute setting or in the non-acute setting as tissue residing macrophages. However, recent studies have shown that residing macrophages actually consist of at least three different sub-populations described by combination of level of expression of MHC II receptors, as well as presence or absents of chemokine receptor 2 (CCR2).\textsuperscript{2} Most CCR2\textsuperscript{−} cells, seem not to stem from circulation monocytes, but rather derived from embryonic progenitors, and renew \textit{in situ}. Furthermore, the function of different sub-populations are not entirely the same, and while recruited monocytes/macrophages to a larger extent produce inflammatory cytokines, residing, embryologically derived macrophages seem
to be more involved in the resolution of inflammation and initiation of scar formation. When macrophages, and in particular cardiac residing macrophages, ingest apoptotic cells, this induces the production of anti-inflammatory and pro-fibrotic cytokines, initiating the transition from acute inflammation to fibrosis.\textsuperscript{131} In addition, regulatory T-cells probably play an important role in this transition, and residing fibroblasts and cardiomyocytes could play a part as well.\textsuperscript{2} When the acute inflammation is resolved, residing fibroblasts start the formation of scar tissue by deposition of extracellular matrix proteins, and in this way preserving the structural integrity of the tissue.\textsuperscript{131}

\subsection*{1.4 NGAL}

\subsubsection*{1.4.1 General properties}
NGAL is a 25kDa molecule of the lipocalin family, first isolated from neutrophils.\textsuperscript{132} NGAL is produced by a wide range of different tissues, most notably epithelia in different organs as well as macrophages and fibroblasts.\textsuperscript{133} Systemic levels of NGAL are greatly increased in several conditions, most notably in cardiac disease, inflammatory diseases and particularly in kidney injury.\textsuperscript{134-137} The marked and rapid increase in acute kidney injury has made NGAL a promising biomarker in this condition.\textsuperscript{136, 138} Others have however, shown that NGAL has potential as biomarker in other diseases as well, both with and without kidney components, such as HF, some forms of cancer, and chronic obstructive pulmonary disease.\textsuperscript{139-142}

\subsubsection*{1.4.2 NGAL in disease}
Several potential roles of NGAL have been suggested, most connected to its ability to bind bacterial siderophores and thus influence of the iron homeostasis.\textsuperscript{137, 143-145} A study by Flo \textit{et. al.} showed that NGAL is important in limiting bacterial infection in mice due to its iron binding properties.\textsuperscript{137} NGAL may also influence apoptosis rate in cells, and can affect necrosis, but if this is due to its iron-binding capacity or other mechanisms is not clear.\textsuperscript{145, 146}

NGAL can bind MMP-9 inhibit the inactivation of MMP-9 and thus increase its activity.\textsuperscript{147} This has been suggested as a potential role for NGAL in CVD, in particular in atherosclerotic plaques where NGAL is upregulated and associated with increased plaque vulnerability.\textsuperscript{147, 148} There is also an increased NGAL/MMP-9 complex concentration in vulnerable plaques co-localized with macrophages, and there is an elevated level of these complexes in plaques with intra-plaque hemorrhage or thrombus.\textsuperscript{147, 149}
NGAL is produced by many tissues and found both in urine and circulation in healthy individuals as well as in disease. Yet the sources of NGAL may differ depending on condition. NGAL is freely filtrated by the kidneys, but is to a large extent reabsorbed in the proximal tubuli. However, with kidney injury, NGAL is produced by epithelial cells in the distal tubuli and directly secreted in the urine. Thus, urinary NGAL may mainly stem from local production, and to a much lesser extent to be filtrated NGAL from other sources. Circulating NGAL on the other hand is produced by other organs in response to kidney injury or other conditions. Thus while both circulating and urinary NGAL increases in many conditions, they may not always reflect the same pool, and their significance may vary in different conditions.

1.4.3 NGAL as a biomarker

As mentioned, NGAL has been thoroughly studied as a potential biomarker in acute kidney injury (AKI) over the last decade, showing promising results. In particular, urinary NGAL shows a robust increase in levels as quickly as two hours after AKI, and thus might help in early diagnosing this condition. Previously, creatinine has been the main biomarker of AKI, but several days can pass from the initial insult to the increase in circulating creatinine. NGAL could therefore help in substantially decreasing time from injury to treatment. However the picture is not yet clear, and further research is needed before adoption of NGAL in the clinic is warranted. In other conditions, NGAL is much less studied, and the results more diverse. Some studies show a potential role as a biomarker in acute and chronic HF, ACS, CKD, sepsis, chronic obstructive pulmonary disease as well as a wide range of other conditions. However in these fields, there are a lot more discrepancies in the findings and NGAL seems to be a weaker marker with less clinical application than in AKI. One of the main problems with NGAL as a biomarker is its induction in a wide range of normal conditions, such as most inflammatory states. Its specificity for a given disease is thus limited, especially in the elderly who often have several comorbidities.
2 Aims

Our overall hypothesis is that the immune system and inflammation are involved in the pathogenesis of CVD. Taking a prime interest in the innate immune system, and in particular neutrophils, our main aim was to shed light on the potential role of inflammatory biomarkers as biomarkers in this condition.

In two large patient cohorts, we aimed to see if inflammatory cytokines in general and NGAL in particular, were associated with outcome in two important and connected groups of diseases; chronic HF and ACS. In this way we wished to both investigate the potential role as a biomarker for these proteins, as well as further elucidating the differentiated involvement of the immune system in both conditions.

Our specific aims were to:

1. Investigate if NGAL was a suitable biomarker for mortality and morbidity in HF using material and data from the CORONA study.
2. Investigate if NGAL was a potential biomarker of mortality in ACS using material and data from the PRACSID study.
3. Investigate if other prototypical cytokines, namely IL-8, TNF, sTNF-RI and II, and MCP-1 were associated with morbidity and mortality in HF, and could be potential biomarkers in this disease using data from the CORONA study.
4. Investigate if a panel of biomarkers increased prognostic abilities of established prognostic models in HF patients from the CORONA population.
3 Material and Methods

3.1 Patients

3.1.1 The CORONA study
The design and principal findings of the CORONA study have been described previously by Kjekshus et al. and is also described more in detail in the method section of paper I and paper III. Briefly, elderly patients (>60 years of age) with chronic HF of ischemic cause, NYHA class II–IV disease and left ventricular ejection fraction (EF) <40% (35% for NYHA II) were eligible as long as the investigator considered that they did not need treatment with a cholesterol-lowering drug. In our studies, we included approximately 1400 patients from a sub-study of the original 5011 patients included in the CORONA study. The trial was approved by the ethics committees at each of the participating hospitals, and patients provided written informed consent.

3.1.2 The PRACSIS study
The design of the PRACSIS study is described in detail elsewhere, and further details can also be found in the method section of paper II. Patients with ACS, admitted to the coronary care unit of the Sahlgrenska University Hospital, Gothenburg, Sweden from September 1995 to February 2000 were eligible for participation. Patients who consented to blood sampling were included consecutively. The primary outcome measure of the study was all-cause mortality from the time of inclusion in the study to September 15, 2001. The study protocol was approved by the regional ethics committee before the initiation of the study. Informed consent was obtained from all participating patients.

3.2 Blood sampling
Blood samples from the CORONA and PRACSIS studies were drawn from peripheral venous blood into pyrogen-free blood collection tubes without any additives and allowed to clot before centrifugation. Serum samples were stored at -70°C and thawed less than three times before measuring the biomarkers.
3.3 ELISA

To measure NGAL, we used an enzyme-linked immunosorbent assay (ELISA). Due to its ease of use, and high sensitivity and specificity, ELISA has been extensively used to measure concentration of circulating biomarkers. The standard ELISA makes use of a double antibody sandwich principle. In step one, a microtiter plate is coated with a primary antibody specific for the molecule at interest. After saturating the unspecific binding capacity of the plate with a non-specific protein, in our case bovine serum albumin, the plasma or serum was added to sample wells, at the same time as a solution with a known concentration of the molecule at interest was added in separate wells. At this step the molecule at interest will bind the primary antibody. For NGAL, serum samples diluted 1:200 in PBS with 1% albumin were used at this step. The wells were then washed to rinse of superfluous samples, and a second antibody specific for the molecule at interest was added. This binds the molecule at a different epitope (or several different epitopes). The secondary antibody was biotinylated, and an enzyme conjugated to streptavidin binds it, which after addition of a substrate solution catalyzed a change of color proportional to the concentration of the molecule at interest. After stopping the enzyme reaction with acid, the color intensity were read by a spectrophotometer, and compared with the intensity of color of the standard solutions analyzed at the same time. Concentration was then calculated from color intensity and dilution factor.

3.4 Multiplex

In paper II, the cytokines were measured using cytokine panel 1 on Evolution multiplex technology from Randox Laboratories (Northern Ireland). The methods are very similar to ELISA, but instead of only measuring one substrate, several antibodies to a range of molecules are attached to a biochip, allowing measurements of several molecules in parallel from the same sample. For all the cytokines, the detection limits are given in the method section of paper II, and at these levels, the intra- and interassay coefficient of variance were less than 20%.

3.5 Statistical methods

In our studies, all our main results come from standard application of the cox proportional hazard ratio model, or cox model for short. The cox model is a semi-parametric model where
time is modeled non-parametrically, while all independent variables are modeled parametrically. The model enables the investigation of the association between a variable and an outcome in a population with censoring. To test the assumptions of the cox model, we used log-log plots, a formalized test using Schoenfeld residuals, as well as cubic spline plots. In addition we used DF-beta plots to check if any outliers had a disproportionate influence on our results. To assess the clinical relevance of our findings, we mainly used two additional methods; the change in Harrell’s C-statistics (ΔC) as well as continuous net reclassification improvement (cNRI). In addition, in paper IV, we included the change in Gönen and Heller’s K statistics (ΔK). To calculate prognostic scores based on estimated models, we multiplied each variable in the model with the natural log of its HR for each patient. For missing values in paper IV, multiple imputation was used to generate 20 new datasets, and chained regression analysis was used to fill in missing values. All models were estimated on the collection of this data using Rubin’s combination rule for combining results into one estimate. All statistics in paper I, III and IV were done using Stata version 11 to 14 (StataCorp LP, College Station, TX, USA), while analyses for paper II were done using SAS version 9.2 (SAS Institute, Cary, NC, USA).
4 Results

4.1 Paper I

*The association between neutrophil gelatinase-associated lipocalin and clinical outcome in chronic heart failure: results from CORONA*\(^{158}\)

In paper I we explored the prognostic value of NGAL in chronic HF of ischemic etiology using data from 1415 patients in the CORONA study.

Our main findings, using NGAL as a continuous, log-transformed variable were:

- NGAL added significant information when adjusting for clinical variables, but was no longer significant when further adjusting for Apolipoprotein (Apo) A-1, eGFR, CRP and NT-proBNP.
- Belonging to the highest NGAL tertile was associated with more frequent hospitalization, even after adjusting for clinical variables, GFR and ApoA-1, but not after adjusting for CRP and NT-proBNP.
- There was no interaction between rosvastatin treatment and NGAL.

**Conclusion:** NGAL added no significant information to NT-proBNP and GFR in a multivariate model for primary and secondary end-points
4.2 Paper II

*Seraum Neutrophil Gelatinase-Associated Lipocalin (NGAL) is independently associated with mortality in acute coronary syndromes*

In paper II we tested the hypothesis that circulating NGAL levels could add prognostic short- and long-term information among patients with a range of ACS using data from 1122 patients with ACS from the PRACSIS study. We also investigated whether any gene polymorphism was associated with NGAL levels.

Our main findings were, all models adjusted for GRACE score, EF, CRP and BNP, and comparing NGAL levels in top quartile with quartile 1 through 3 if not otherwise specified:

- NGAL was associated with adjusted hazard ratios of 1.41 (95% confidence interval (CI): 1.05-1.89; p=0.02) for mortality and 1.45 (95% CI: 1.20-1.81; p=0.006) for the combined endpoint cardiovascular (CV) death, MI or HF during long-term follow-up (median 91 months).
- NGAL was also significantly associated with short-term (3 months) and late long-term (median 129 months) mortality, with adjusted hazard ratios of 2.76 (CI: 1.35-5.65; p=0.005) and 1.51 (CI: 1.18-1.94; p<0.001).
- Relative change from baseline to 3 months, but not to 25 months, was predictive of long-term mortality (before 2011), and patients with relative change in the top quartile (more than 22.6% increase) had a hazard ratio of 1.60, CI: 1.06-2.40; p=0.02.
- Combining NGAL with GRACE score in a combined score improved the AUC of the model significantly from 0.681 (0.653,0.709) to 0.713 (0.682,0.743), p=0.0002 for comparison with GRACE alone.
- Change in NGAL levels from baseline to three months was associated with a genomic region on chromosome 2, and the most significantly associated single nucleotide polymorphism was located within an intron for MYO7B, a gene mainly expressed in the proximal tubule of the kidney.

**Conclusion:** NGAL is associated with both short and long-term prognosis in patients with ACS independently of GRACE score, BNP, CRP and EF. NGAL could therefore improve the selection of patients needing closer follow-up after admission to hospital for ACS.
4.3 Paper III

Inflammatory cytokines in chronic heart failure: interleukin-8 is associated with adverse outcome - results from CORONA163

In paper III we investigated the ability of prototypical inflammatory cytokines to predict clinical outcomes in a large population of patients with chronic systolic HF from the CORONA study.

Our main findings in this paper were:

- TNFα, sTNFR 1, sTNFR 2 and IL-8, but not MCP-1, were independent predictors of all endpoints except the coronary endpoint in multivariable models including conventional clinical variables.
- After further adjustment for eGFR, ApoB/ApoA-1 ratio, NT-proBNP and CRP, only IL-8 remained a significant predictor of all endpoints (except the coronary endpoint), while sTNFR 1 remained independently associated with CV mortality.
- Adding IL-8 to the full model led to a significant improvement in net reclassification for all-cause mortality and CV-hospitalization, but only a borderline significant improvement for the primary endpoint, CV mortality and the composite endpoint hospitalization for worsening HF or CV mortality.

Conclusion: IL-8 was associated with the primary endpoint, all-cause and CV mortality, as well as CV-hospitalization when adjusting for a range of clinical parameters, hsCRP and NT-proBNP. Our findings further support the involvement of inflammation and immune activation in the progression of HF.
4.4 Paper IV

*Limited added value of circulating inflammatory and extracellular matrix biomarkers in multimarker models for predicting clinical outcomes in chronic heart failure*

In paper IV we investigated the ability of a panel of biomarkers to predict clinical outcomes in a large population of patients with chronic systolic HF from the CORONA study.

Our main findings in this paper were:

- Adding a panel of biomarkers to the CORONA risk model previously published does not add significant information to the risk model’s prognostic abilities.
- While a panel of biomarkers does improve prognostic abilities of Seattle heart failure model scores in the CORONA population, most of the added predictability can be gained by including NT-proBNP in the model.
- Three months statin treatment did not lead to significant changes in inflammatory biomarker levels compared with placebo. There might however be increased survival with statin treatment of patients having lower levels of biomarkers.

**Conclusion:** Several authors have suggested that a multimarker approach may be the way ahead to improve prognostic models in chronic HF. We were however not able to construct such a panel from a broad range of inflammatory biomarkers in elderly patients with systolic HF of ischemic etiology. Nor do our findings support a general anti-inflammatory effect of statins. However, as suggested by previous studies, there seems to be some improvement of survival with statin treatment in patients with lower biomarker scores.
5 Discussion

5.1 Analysis of circulating biomarkers in clinical materials
Numerous factors can influence the results of serum or plasma analyses, and several pitfalls need to be avoided both in the collection and analysis of samples.

5.1.1 Collection of samples
Firstly, contaminated collection tubes may activate blood cells and heavily influence cytokine concentration. Hence, usage of pyrogen-free collection tubes is essential for reliable results and was used for all blood samples in the two studies. Secondly, for many inflammatory parameters the type of anticoagulant and the choice of plasma or serum have a potential huge impact on final data. Heparin and citrate have been shown to influence levels of some circulating cytokines such as IL-6 and TNF, while EDTA seem to be the most reliant anticoagulant for cytokine measurements. Furthermore, many cytokines and inflammatory biomarkers are released from circulating immune cells as well as platelets when activated. There is also a certain binding of inflammatory molecules to some of the proteins involved in the coagulation cascade, and these molecules could be sequestered if blood is allowed to coagulate as when preparing serum. For example, while highly correlated, NGAL levels from serum and plasma cannot be directly compared, necessitating specification from what sample type a study is conducted. Thirdly, storage of blood may influence concentration of several biomarkers. Particularly in larger biomarker studies, samples are often stored for several years before analysis, potentially influencing findings significantly. NGAL has been found to be relatively stable throughout several freeze-thaw cycles as well as during storage at -70°C, however some studies have shown a potential influence of freeze-thaw cycles on cytokine concentration. Since several of the samples were stored for more than two to three years, and thawed up to three times, we cannot exclude some influence of the storage conditions on biomarker concentration. While storage times were unequal for samples due to different inclusion times, all samples were stored under equal conditions and thawed an equal number of times, limiting the potential bias. In our study, the cytokines in paper III were measured on samples not previously thawed, thus limiting this potentially influencing factor. Finally, other factors such as time of day, fasting state, physical activity, stress and recent weight loss or gain have been shown to influence several cytokines such as TNF, IL-6 and IFN-γ. Blood samples for the CORONA study were not fasting, or at any particular time of the day. This is a potential source of error in our studies. However, there is no particular
reason to suspect that this would introduce any systematic bias. This could however attenuate the association between the biomarkers and outcome, and thus a more strict protocol could have given other results.

5.1.2 ELISA

ELISA is a popular method for biomarker measurement. However some important caveats must be noted. Firstly, while ELISA is considered to have good sensitivity and specificity in general, the individual kit properties depend upon which antibodies it uses, and their properties. For the measurement of NGAL in paper I, we used a non-commercial ELISA kit, developed by Trude H. Flo and previously described elsewhere. The primary antibody was a rabbit affinity-purified anti-NGAL antibody developed by Trude H. Flo. The secondary antibody was a commercially available biotinylated mouse monoclonal antibody (no. HYB211-05; Antibody Shop, Gentofte, Denmark). The detection limit of the ELISA was estimated to 0.06 ng/mL, and intra- and interassay coefficient of variability was <6%. For paper II, a commercial available ELISA kit from R&D Systems (Minneapolis, MN) was available. As the usage of commercial available systems makes it easier for others to attempt to reproduce the data, we opted to change to this kit. The detection limit of this kit was 0.04 ng/mL, and intra- and interassay coefficient of variability was <8%. However, while intra- and interassay properties were good for both ELISA kits, the exact levels of NGAL must be considered with care. Different kits for NGAL measurement are not standardized, so the exact levels may vary between kits. One reason for difference in NGAL measurements across different kits is the specificity of the antibodies. Often the molecules at interest, such as NGAL, do not only exist in one configuration in the circulation. NGAL exist in several different configurations, among others as a monomer, a homodimer, as well as in complex with MMP-9 and potentially with bacterial siderophores. In addition, antibodies of one particular kit only bind one epitope of the molecule of interest. Which epitopes of a molecule is available for binding may differ according to presence of other binding molecules such as inhibitors, also influencing the measured quantity of the molecule of interest. However, in this type of study, the main interest will be the relative level of NGAL from patient to patient, and the exact concentration is of less importance as long as all samples are measured using the same kit. It is not however possible to be sure that the measured quantities do reflect total NGAL levels in a patient, but could only reflect the pool of NGAL in certain configurations or complexes. Thus one can argue that concluding on the abilities of NGAL as such as biomarker is somewhat erroneous, and should be limited to NGAL measured by a certain kit.
Secondly, with relatively large studies like CORONA and PRACSIS, it is often practically difficult to do measurements of a biomarker on all samples the same day. While the use of a standard curve of known concentration should limit measurement differences from day to day, the number of steps, and complexity of the ELISA methods makes it difficult to exclude the potential for systematical higher or lower levels from one day to another. To alleviate this problem, we have attempted doing the measurements in as few days as possible, and have also included control samples on every plate which makes it possible to adjust plate measurement levels in case of significant difference between different days. Thirdly, ELISA has a relatively small analytic range compared with multiplex technology, and biomarkers with huge variation, such as often is the case with cytokines and other inflammatory parameters, it can be difficult to find a dilution where all samples fit within the same standard curve. This was not a problem for NGAL with less than 20 fold change from lowest to highest value, but for some of the other biomarkers such as sTNF-R2 with more than a 100-fold difference from bottom value to highest value, this could influence the final results.

5.1.3 Multiplex

Compared to ELISA, Multiplex methodology has several advantages, and a few difficulties. Firstly, the dynamic range of multiplex kits is usually much greater than ELISA, allowing the difference in concentration of one biomarker from sample to sample to be much larger. This is especially important for cytokines where there is often a long upper tail of the distribution, with some samples having thousand to several thousand fold higher levels than others.

However, the kit applied in paper III did suffer from relatively low sensitivity for some of the biomarkers such as TNF, resulting in a lot of the samples having non-detectable levels. Several newer kits have higher sensitivity, detecting levels ten folds lower than the kit used in our study and could potentially have given different results for paper III. In addition, while the dynamic range of multiplex is greater than for ELISA, there is less room to optimize conditions as samples for all biomarkers in the multiplex assay need to be treated equally.

Using ELISA, incubation time and temperature, as well as addition of calf serum, albumin or other reagents to dilution mediums can be optimized for each biomarker, potentially increasing sensitivity. For example, while IL-1β often is below detection limit in current multiplex kits, as was the case for the multiplex used for paper III, it can often be measured by high sensitive ELISA kits. Secondly, since several biomarkers are measured in parallel in the same sample, smaller quantities of sample are needed for analysis, and a large number of biomarkers can be measured in much shorter time than with ELISA. However, the ease at
which measurements of several biomarkers can be made makes the importance of proper statistical handling of the resulting data of uttermost importance.

5.2 Statistical considerations

5.2.1 The assumptions of the cox model

For the use of the cox model to be appropriate, some assumptions need to be met by the population studied, and the variables investigated. Firstly, the cox model requires non-informative censoring. This means that the reason why people drop out of the study must not be associated with the outcome investigated. Secondly, the model requires the risk associated with independent variables to be time independent. There should be a uniform association over time between the independent variable and risk, which does not depend on which time point you are at. Thirdly, it assumes the risk of all variables to be linear and additive.

While non-biased censoring is a very important assumption, there are few tests that can assess to what extent this is the case. However, the study design for both populations used in our studies seems to limit this problem. There were very few dropouts early in both studies, and the PRACSIS study also used register data to gather information on endpoint status of individuals which assured less censoring on these endpoints, and limits the potential for bias considerably.164 Furthermore, while the CORONA study does randomize patients to receive rosvastatin, the dosage used is widely used in other patient groups and is considered to give very few, serious side effects, and should therefore lead to few dropouts from medication use.162, 177

In our studies, we mainly used a formal test utilizing the Schoenfeld’s residuals, as well as log-log plots, to test the proportional hazards. This was deemed sufficiently satisfied for all variables investigate.

Finally, linearity is often a problem with biomarkers. Due to their often skewed distribution, and not necessarily linear risk association, we did several tests to assure that the variables were transformed appropriately when needed. However, there is a risk of over-fitting variables to the population, thus necessitating some care while transforming the data. We have chosen two main approaches for transforming the variables. Firstly, as the distributions were skewed for all the biomarkers investigated, we did log-transformation to make them more normalized, and thus limiting the problem with outliers significantly influencing the analysis. This was also checked using DF-beta plots to assure that no one observation
significantly changed the associations estimated. When there was evidence of a non-linear association with the endpoints, we categorized the data. This approach has some disadvantages by loss of potential information. In addition, it can be significantly affected by the cutoff values chosen. The latter problem was amended by using the 33% or 66%, or alternatively 25%, 50% and 75% percentiles as predefined cut-offs. We intentionally chose not to try to find optimal cutoffs for the population considered, as this could over-estimate the association severely. While not finding the optimal cutoff, as well as losing some information when categorizing continuous data may lead to potentially lower prognostic value, it does increase the robustness of the findings.

From the cox model, you obtain hazard ratios (HRs) associated with the variable at interest. Hazard ratio, similarly to the odds ratio obtained in logistic regressions, describes the change in risk associated with a one unit change in the variable. For interpretation of HRs, the unit chosen for the variable is therefore very important. To make HRs more comparable across different biomarkers, we have chosen to standardize the variance of all non-categorical variables of interest where appropriate, so that their standard deviation is one.

5.2.2 Missing values
Missing values could be a problem when analyzing biomarkers in clinical material. First of all, the normal approach to missing values is to exclude these patients from analysis. This naturally leads to a decrease in power of the statistical model, especially when many patients lack data on one or several variables. Furthermore, there is always a risk of the data missing not being randomly distributed; that is data on certain variables for certain patients are missing for reasons associated with factors relevant to the purpose of the model. In this case, excluding patients with missing values could bias the results of the study. For paper I-III, there were relatively few missing values, and the risk of bias, and the loss of power by excluding patients with missing values were therefore considered to be relatively low. However, for paper IV, where several biomarkers where included, some of them missing in a significant proportion of the patients, the loss of power as well as the risk of bias had to be taken into consideration. To avoid these pitfalls, we used multiple imputations. This method gives unbiased estimation of both coefficients and variance of coefficients without loss of power, as long as the pattern of missing data is not associated with non-observed, relevant variables. In our studies, missing values where mainly missing because of lack of samples, or procedural errors while measuring biomarkers, and could therefore be assumed to be missing.
at random. In hindsight, one could argue that paper I-III as well could have benefited from similar approach; this was however not considered at the time of writing.

5.2.3 A model’s discrimination: Harrell's C statistics and NRI

The statistical significant association with chosen endpoints is only the first step in identifying a potential useful new biomarker. While a statistically significant association suggests that there could be an association between the variable and the outcome, it does not say whether this association is clinically significant or not, and it says little about the discriminatory properties of the variables between events and no-events. Today there is no agreed upon measure to estimate the clinical significance of a new marker. Several statistical methods have been developed to this end, but none of them are perfect.

Harrell’s C-statistics is a measurement of concordance between model and outcome, and is similar to the area under the curve statistics from case-control studies. It is a number between 0-1, where 0.5 means no addition to the model whatsoever, and the closer to 1, the better the model. It is calculated by comparing all suitable pairs of individuals in the study population, that is, all pairs where not both were censored. Then the proportion of pairs where the individual with the highest estimated risk reaches an endpoint first is estimated. To compare two different models, the difference in Harrell’s C, or for nested models, change in Harrell’s C (ΔC) is then used. While widely used, the ΔC has several limitations. Firstly, it greatly depends on the already existing model. The higher C-statistic of the original model, the more difficult it is for any new parameter to improve it to any degree. Secondly, very small ΔC may be statistically significant, but their clinical relevance is not certain. Thirdly, biomarkers that could be clinically significant can lead to not significant ΔC. Fourthly, while it compares all suitable pairs, it does not take into account the degree of difference between the pairs; thus a difference of 0.1% estimated risk is accorded the same importance as a difference of 50%. If a large group of patients have similar estimated risk, and reaches the endpoint at similar time points, the exact order of which the patients reach the endpoint influences the final C statistics substantially.

Another measure argued to be closer to the clinical reality is net reclassification improvement (NRI). Originally, it was developed for estimating relevance of models where there already existed clinically used risk thresholds. It measures the proportion of patients who are reclassified to a better risk group when the new biomarker is added to the model. NRI thus tests a clinically meaningful reclassification of patients to a better risk group. However, for
conditions where no previous risk categories exist, NRI is very sensitive to the selection of risk cutoffs, and several authors have therefore suggested a different approach in these cases. The most widely used today is a continuous NRI (cNRI) where instead of looking at only patients reclassified to a new, pre-specified risk group, one looks at the direction of change in risk, and compare it with the outcome. Thus we measure what proportion of patients with higher estimated risk has an event, as well as how many with lower estimated risk have no events. This continuous measure does not then depend on already existing risk groups. There is however certain limitations with this method. As it is relatively new, there is no consensus on what NRI should be considered a clinical meaningful NRI. Furthermore, the way it is currently implemented, any change in risk score is measured, meaning that a change in 0.1% count as much as a 5% change. While a 0.1% change would have no clinical relevance, a 5% change would probably have. cNRI thus therefore suffers from some of the same problems as ΔC. However, while none of the measures are perfectly suited to judge clinical relevance of a new biomarker, they both add to the information provided by the cox model, and thus may help in selecting suitable candidates that warrant further investigation.

5.2.4 Validation of prognostic models

The approach mostly widely used for testing association of new, potential biomarkers with outcome, is including these biomarkers in a model, adjusting for known prognostic factors. This way, the statistical significance of the included biomarker, as well as improvement of discrimination and calibration of the new model in comparison with a model only including known prognostic factors, can be estimated. This was the approach we used in paper I to III. However, while methodologically simple, this method leads to overly optimistic estimates since the estimations are fitted and evaluated in the same population. Results therefore need to be very carefully interpreted. As a method of hypothesis generation, this method could work reasonably well, however for clinical models intended for clinical use, a more rigorous approach is needed, and validation of models is necessary. While the gold standard of testing new prognostic models is external validation, a first and useful step is internally validating the model. Several approaches have been suggested, including different methods of subdividing the population into a training set and validation set, as well as using methods based on jackknife or bootstrapping data. To retain maximum power, for example a jackknife approach, where a prognostic score is generated based on a model estimated for all other patients, is possible. However, we chose to randomly divide the CORONA...
population into a training set and validation set in our study in paper IV, with some loss of power. Since building a prognostic model was part of our aim, this approach avoided a bias towards more optimistic results from over-fitting when the same population is used for variable selection and model evaluation. However, due to the loss of power, one could argue that a jackknife approach would have been as appropriate, if not more so, for Model 2 in paper IV, where variables were not selected based on the CORONA population. While this could have led to some of the non-significant results becoming statistically significant, the clinical relevance of the change in discrimination would still in our opinion be very limited.

It is also important to mention that while internal validation of models are of some use, external validation is the only way of separating useful from not useful models, and often there is a substantial decrease in discrimination and calibration of models when applied to a different population.187, 189

5.3 Inflammation in cardiovascular disease: biomarkers, players, and potential therapeutic targets.

5.3.1 Inflammatory biomarkers in HF
Since the discoveries of Levine et al, an abundance of cytokines have been investigated and found to be upregulated in HF.89, 190-192 This is some of the strongest evidence for the low-grade, persistent inflammation assumed to be occurring in these patients. In our study we have evaluated some of these as biomarkers. Previously, especially TNF and its receptors have been studied extensively, but there are some discrepancies between these earlier studies and our findings. While earlier, TNF, and TNF receptors were found to be associated with outcome, this was not the case in our study.192-194 This could be due to the difference in the two populations studied, or the variables adjusted for in the model. Also, TNF circulates at low levels requiring methods with high sensitivity for reliable detection. TNF was not detectable in a large portion of the samples, and TNF measurement could therefore have benefited from a more sensitive, customized assay. TNF receptors were significantly associated with outcome in our study before we adjusted for NT-proBNP levels which many of the previous studies did not adjust for. Our patient group was also a selected group where all patients had systolic HF of ischemic etiology, and the findings in other populations could differ. Nevertheless, HF of ischemic origin is the most prevalent type today, and the inability
to reproduce earlier findings suggests that TNF and its receptors are not likely candidates as biomarkers for chronic HF in general. Equally, no other cytokine or inflammatory marker has managed the transition from early findings of upregulation in HF, to become a biomarker for this condition. Of the several we have studied, only IL-8 proved statistically significant in the model, and its clinical relevance remains doubtful. Studies of more specialized cytokines, with the aim to find specific biomarkers unique for HF, have had no significant success either, and we are not much closer to finding a good inflammatory biomarker of HF today than we were a decade ago. While several authors have suggested a multimarker approach to aid in prognostication of HF patients, we were not able to build a multimarker model from biomarkers available in the CORONA population that to any clinical significant degree added to the discriminatory power of a prognostic model built on clinically available data.\textsuperscript{40, 48} While some recently suggested biomarkers such as GDF-15 was not available in our data, this still suggests that a multimarker approach might not necessarily be an easy solution, and if such an approach is applied, the biomarkers included need to be meticulously selected, and models carefully validated. Of course, this does not mean that no such panel of biomarkers can be constructed, but suggest that better, more specific markers are needed if this approach is to succeed.

5.3.2 NGAL; potentials and difficulties.

The growing interest in NGAL over the last decade primarily as biomarker, but also a mediator, may be partly ascribed to its well documented association with kidney function. Several studies have been published on its prognostic abilities in both chronic and acute HF.\textsuperscript{134, 157, 158, 195-197} While some previous reports have suggested there may be an association between NGAL levels and outcome in both conditions, we were not able to reproduce these findings in our study of chronic HF. However, there are a number of important factors separating these studies, such as whether NGAL is measured in the circulation or in urine, as well as the number of patients included and how many variables are adjusted for in the analysis. As an example, a similar study to ours on NGAL in chronic HF concluded that NGAL was significantly and independently associated with outcome.\textsuperscript{198} They measured NGAL in urine, and did not control for NT-proBNP, possibly explaining our different findings. As discussed in section 1.4, the source is likely different for urinary and circulating NGAL, and while they often are closely correlated, they could be differentially regulated in several conditions.\textsuperscript{150} This is important to bear in mind for other studies as well, and could explain some of the discrepancies on NGALs prognostic abilities found in the literature.
Furthermore, whether or not NGAL in chronic HF is a marker for a systemic inflammation as such, or rather a more specific marker of kidney function is not yet certain. In our study in chronic HF, NGAL was closely associated with kidney function, and adjusting the prognostic models for eGFR removed any prognostic power of NGAL. This is further supported by another small study suggesting the kidney function is the most important reason for NGAL increase in patients with systolic HF.\textsuperscript{156} Importantly, we only studied patients with systolic HF secondary to ischemic heart disease – the role of NGAL in HF of other etiologies is still unclear. Interestingly, in the ACS population NGAL was still strongly associated with eGFR, but including eGFR in the models did not remove NGAL’s prognostic power suggesting other causes of increased NGAL levels may be relevant. For ACS in general, the findings on NGAL are a bit more consistent, and suggest an association between NGAL levels and outcome.\textsuperscript{160, 199-201} Nevertheless, further studies are needed to support our findings, and risk models need to be externally validated. While there are plausible reasons why NGAL could be linked to outcome in this patient group as discussed in section 1.4, many of the difficulties with NGAL as a biomarker is equally valid for this group as for HF patients. Its increased circulating level in a range of different diseases, suggest that there would be a lot of “noise” when using NGAL as a biomarker for a specific condition.\textsuperscript{161} NGAL’s better prognostic abilities in the ACS population could be due to these patients in general being younger, and having fewer comorbidities, but it cannot be excluded that it also reflects a closer link between NGAL levels and the development of the disease.

5.3.3 Neutrophils, an important cell type in CVD?

In our studies, we have among other evaluated IL-8 and NGAL, two proteins linked with neutrophils. One of the main functions of IL-8 is as a chemoattractant for neutrophils, and NGAL is actively secreted by neutrophils partly in complexes with MMP-9. Similarly, other studies have found upregulation of MPO, another neutrophil-secreted enzyme, in the circulation of acute and chronic HF patients as well as patients with ACS, and that the concentration reflects the severity of the disease.\textsuperscript{202-205} Others have found worse prognosis for patients with higher levels of circulating neutrophils, or higher neutrophil to lymphocyte ratio in patients with ACS as well as acute and chronic HF further.\textsuperscript{206} Together these findings suggest that the neutrophils and neutrophil activity is associated with, and could be involved in the development of CVD. However, this is of course no evidence for neutrophil involvement and furthermore, even if assuming they are involved in the pathogenesis, there is no way from these results to conclude if their influence is positive or negative. Some recent
findings do however suggest that neutrophils do more than simply correlate with disease in these patients. As mentioned previously, NGAL and MMP9 are correlated with increased vulnerability of atherosclerotic plaques (See section 1.4.2). Recent findings have also demonstrated the presence of pro-thrombotic NETs in culprit lesions in STEMI, suggesting that neutrophils may not only play a role in the destabilization of plaques, but also in forming the thrombus when a plaque ruptures. Furthermore, a recent study has also shown that the quantity of NETs in culprit lesions in STEMI correlates negatively with ST resolution and positively with size of infarction further supporting this notion. Our findings together with others do suggest that neutrophils may be one cell group that warrants a closer examination in relation to CVD.

5.3.4 Inflammation in HF: a way ahead?

Inflammation is characterized by the complexity of the system, multiple redundancies, and molecules serving several different roles according to situation and other stimuli present. We find that some inflammatory markers such as IL-8 are more closely linked to outcome than others. While our findings do not support a role as biomarker for any of the molecules studied, they do suggest that a more nuanced view on inflammation in HF is needed. The discrepancies of findings between our studies and other studies also suggest that HF populations are not equal, and cytokine levels do not necessarily have the same importance in all of them. There could be subgroups where higher levels of some cytokines are more linked with outcome than others. The activation of the immune system in chronic HF is well established. However, what part of, and when this activation is advantageous or counterproductive are only partly understood. It is even likely that some parts of the system may do both, according to the degree of activation, and balance between inflammatory and anti-inflammatory signals. It is interesting to note that different cytokines seems to be differential regulated, and while some like IL-6, TNF and CRP are closely correlated (data not published), others like IL-8 and CRP are not, suggesting that there are differences in immune regulation and activity among patients. One could speculate that mapping several cytokines, and looking at the cytokine profile of patients would permit further classification of patients according to inflammatory profile. In addition, further understanding of the role of inflammation in HF could help towards finding more useful inflammatory biomarkers, or subpopulations where already suggested biomarkers are more powerful, and could aid in clinical decision-making.
So far, only anti-inflammatory treatments have been attempted in HF patients. Resolving inflammation is, however, an active process and could be another potential target for therapy. While many of the inflammatory cytokines are closely investigated, and their potential role in HF mapped to some extent, this is not equally the case for molecules involved in resolving inflammation. The persistent inflammation seen in HF might be due to the lack of removal of an inflammatory stimulus, but could just as well be due to failure of mechanisms in resolution of inflammation itself. This is another path that warrants exploration, and where findings could both be inspired by, and help further research into inflammatory biomarkers.

5.4 Biomarkers, any use beyond prognosis?

5.4.1 Current status
As mentioned above, a good biomarker should be possible to measure accurately and repeatedly, preferably to a reasonable cost and turnaround time. Furthermore, it should provide additional information to already existing models, and help medical decision making. None of the biomarkers investigated in our studies comes close to fulfilling all these requirements. Indeed, few if any of the suggested biomarkers for HF in the literature do. The best candidates so far have been the natriuretic peptides (NPs), notably NT-proBNP and BNP. These have consistently been significantly and independently associated with outcomes in many studies of chronic HF, and there are some promising studies on NP-guided therapy, suggesting that these may fulfill the above mentioned criteria, at least in subgroups of HF patients hospitalized for HF related reasons. While there have been plenty of suggested inflammatory biomarkers in HF, few of these have been verified in large enough studies, or against already existing models. In particular, few have been verified to give clinically significant data in addition to the NPs. As discussed in paper III, relatively well-studied inflammatory biomarkers such as TNF, and sTNFR-1 and -2, while associated with outcomes independent of regular clinical markers, loses this association when adjusting for NT-proBNP. In this respect, the results of the extensive research on inflammatory biomarkers in HF are disappointing.

In particular when investigating prototypical inflammatory cytokines, as we do in paper III, their relevance as potential prognostic biomarkers may be further diminished as these are
cytokines upregulated in most diseases with immune activation. While we do show that one of these, IL-8, demonstrate some potential, it is an important caveat that the typical population investigated in these studies are from clinical trials, often only including patients without any other serious comorbidities. However, as HF patients often are elderly, in an unselected group of patients, there are good reasons to suspect the presence of other conditions that influence immune activation as well. A reduction of the prognostic abilities of such prototypical inflammatory markers should thus be expected in unselected populations, at least for cause specific CV outcomes.

5.4.2 Biomarkers as pathophysiological informants

Part of what makes predicting a likely progression to HF so difficult, is that so many different processes can lead to its emergence. Several have suggested that, while inflammatory as well as other biomarkers may not turn out to be strong biomarkers in HF as such, they may provide significant information on the pathophysiology of the condition. Braunwald proposed six broad categories, later extended to seven, for the classification of researched biomarkers into classes of different pathological systems. These categories – inflammation, neurohormonal activation, oxidative stress, matrix remodeling, myocyte injury, myocardial stress and later added renal dysfunction – illustrate the possible usefulness of biomarkers to give information on some of the potential pathological causes of HF. While earlier approaches to the classification of HF has focused on the pathological cause (i.e. coronary heart disease, cardiomyopathy etc.), and the pathophysiological characteristic (i.e. preservation or reduction in EF), biomarkers may add a third dimension to this characterization scheme, reflecting different pathways involved in progression of the condition. The potential usefulness is not only limited to the experimental setting, but also underlines the fact that HF patients are not identical, and different underlying etiologies may profit from tailored treatment and prognostication.

5.4.3 Biomarkers as source of new hypotheses

Findings from biomarker studies may not only suggest pathways involved in the disease, but also be an inspiration to further research into these pathways. An example may be the realization that TNF was increased in HF in the early 90s, leading to a wide range of studies on the influence of TNF on the cardiac system as such. While later trials on anti-TNF therapy may have failed, illustrating the complexity of immune involvement in progression of HF, this research has given important insights. Furthermore, the failure of TNF therapy to
significantly improve HF prognosis, does not prove that this approach to finding new potential targets for HF therapy could not work in the future. \textsuperscript{103} Biomarker studies could therefore be regarded as potentially important sources of hypotheses. It is also important to notice that in our study, TNF, as well as the TNF receptors where not independently associated with most of the outcomes. If only biomarkers fulfilling the characteristic above where of scientific interest, the last decades of TNF and HF research would potentially not have been initiated.

\textbf{5.4.4 Should there be a shift of focus in biomarker research?}

The seven categories mentioned above also illustrate another important aspect. While many of the parameters included in prognostic models of HF today increases power of the models, many of them reflect the symptoms and results of HF deterioration, rather than the causes. This is the case of among others EF, NYHA-class, as well as partly for NT-proBNP and troponins. While independence of these variables is important when considering the potential usefulness of a new clinical prognostic biomarker, this is not as evident when using biomarker studies as an approach to further understand the development of the disease. A multi-marker approach to study HF patients could be useful even if it did not improve on already existing prognostication models if it leads to new ways to categorize HF patient, and potentially aid in therapy selection. After all, choice of therapy is not only a question of how likely a patient is to die, but also how that patient is likely to respond to the treatment. This does not necessarily follow from the prognosticated risk. Therefore, while investigating whether or not a new biomarker adds clinical significant value to existing prognostic models is important, limiting biomarker research to this aspect may preclude us from important discoveries. One could for example also consider therapy response and development of disease according to baseline levels of markers. This could be an argument for putting more focus on this potential aspect of biomarkers in further studies, and put less weight on the pure prognostic aspect.

While investigating the seven proposed categories of biomarker could be useful, assuming all inflammatory markers in the inflammation category give the same type of information is somewhat premature. Inflammation is an incredibly complex process, and the outcome is often decided by the balance of different pathways. Looking at only one inflammatory biomarker may not be enough, and several biomarkers may be needed to entangle the effect. It is not necessary that the same level of an inflammatory cytokine in one patient will have
the same effect as an equal level in another patient, depending on levels of other cytokines and activity of other systems. This interconnectedness makes the search for inflammatory biomarkers inherently difficult, and the use of panels of cytokines, or more complicated systems approaches may be needed.88, 190
6 Concluding remarks and future work

The results presented in this thesis have further extended our knowledge on the role of some important inflammatory markers in heart disease. We have assessed the potential as biomarkers of NGAL in both ACS and HF, as well as several cytokines such as TNF and related soluble receptors, and IL-8 in HF. Our main conclusions may be summarized as follows:

- Circulating NGAL is not independently associated with mortality and morbidity in elderly patients with systolic HF of ischemic etiology. While there is a significant association between NGAL and outcomes when adjusting only for clinical parameters, including eGFR, CRP and NT-proBNP in the model reveal that NGAL does not seem to be a good prognostic biomarker for this condition.

- However, circulating NGAL at the time of hospitalization for ACS is independently associated with both short and long term mortality. This is true even when adjusting for GRACE score as well as other relevant cardiac and inflammatory markers of disease (EF, CRP and BNP). NGAL leads to a small, non-significant increase in C-statistics, but a significant increase in NRI. Its potential role as a prognostic biomarker thus seems to be somewhat limited. However, our findings support further research into NGAL’s potential role in development and progression of ACS.

- When looking at IL-8, MCP-1, TNF and sTNF-R1 and -2, only IL-8 was independently associated with mortality and morbidity in elderly patients with systolic HF of ischemic etiology. All variables were however associated with several of the outcomes when only adjusting for clinical variables. While IL-8 was independently associated with all outcomes, only a minor non-significant increase in NRI and C-statistics suggest that IL-8 may not be a very good prognostic biomarker for this condition. However, our findings support the involvement of inflammation in chronic HF, and suggest that inflammatory pathways may be differentially regulated. While others have suggested that TNF and its receptors may be potentially prognostic biomarkers in chronic HF, our findings do not support this.

- While there was some improvement in discriminatory power of the models when including a panel of multiple biomarkers in addition to previous prognostic models in HF, the gains were modest and the clinical relevance doubtful. Our findings do not support the notion that adding biomarkers representing different aspects of HF...
pathology improves the prognostic abilities of existing risk scores. We cannot, however, exclude that other panels of biomarkers or similar panels of biomarkers in other more heterogeneous HF population would result in different results. We also found no correlation between changes in inflammatory and ECM-related biomarkers and treatment with rosuvastatin, suggesting that statin treatment in this population has limited anti-inflammatory effects. There was, however, a tendency for patients with lower biomarkers scores at baseline to have beneficial effects of rosuvastatin treatment.

CVD is the leading cause of death in the world today, and there has been some progress in our understanding of these diseases over the last few decades. In this thesis we have argued that inflammation, and in particular the innate immune system spearheaded by neutrophils may be involved in development and progression of both HF and ACS. In particular, neutrophils could play an important role in plaque vulnerability, and their potential role in destabilizing atherosclerotic plaques needs further research. While the search for new, prognostic biomarkers in HF and ACS may not have been very fruitful so far, these studies may still play a role in mapping systems and molecules potentially involved in the pathogenesis of the diseases. Further exploitation of this potential could increase the utility of these studies and help bring research on CVD forward.
7 References


96. Torre-Amione G. Immune activation in chronic heart failure. *Am J Cardiol*. 2005;95:3C-8C; discussion 38C-40C.


