Low-Density Lipoprotein Oxidation and Renal Dysfunction

New Markers of Poor Prognosis in Patients with Unstable Coronary Artery Disease

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Abstract

In patients with unstable coronary artery disease (CAD) biochemical markers are emerging as useful tools in clinical management. In this thesis we studied the use of markers of low-density lipoprotein (LDL) oxidation and renal function.

Our study populations consisted of unstable CAD patients included in the Fast Revascularisation during Instability in Coronary artery disease (FRISC)-II trial and healthy controls. Patients were followed for 2 years regarding death and myocardial infarction (MI).

Using receiver operating characteristic curve analysis, we found that oxidized low-density lipoprotein (OxLDL), especially when combined with high-density lipoprotein, compared to traditionally measured lipids/lipoproteins, and a new lipoprotein marker, lipoprotein associated-phospholipase A2, was better at discriminating between healthy controls and CAD patients. In patients, OxLDL was found to be an independent prognostic marker associated with an increased risk of MI, of particular use in patients with no evidence of myocardial necrosis.

In our study on the effects of an early invasive treatment strategy in unstable CAD patients with mild to moderate renal dysfunction (i.e. creatinine clearance <90mL/min) we found that in patients randomized to invasive treatment, the rates of death/MI and MI alone were significantly lower than in patients randomized to non-invasive treatment. In patients treated invasively, no detrimental effects were seen on renal function at follow-up at 6 months.

In healthy controls, we investigated new markers of renal (cystatin C) and cardio-renal function (N-terminal probrain natriuretic peptide, [NT-proBNP]) regarding reference levels and physiological determinants. We found that cystatin C is influenced by age whereas NT-proBNP is influenced by age and gender.

Our studies suggest that OxLDL and renal dysfunction are associated with a poor prognosis in unstable CAD patients and that these markers demonstrate potential for clinical use. In the search for new markers related to renal function we have contributed with reference levels of cystatin C and NT-proBNP.

Keywords: myocardial infarction, unstable angina, oxidized low-density lipoprotein, renal function, prognosis

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To my family, friends and colleagues

“Individual accomplishments reflect successful teamwork”
List of papers

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


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Abbreviations

ACS   acute coronary syndrome  
AUC   area under curve  
BNP   brain natriuretic peptide  
BMI   body mass index  
CABG  coronary-artery bypass surgery  
CAD   coronary artery disease  
CKD   chronic kidney disease  
CI    confidence interval  
CrCl  creatinine clearance  
CRP   C-reactive protein  
CV    coefficient of variation  
ECG   electrocardiogram  
ELISA enzyme-linked immunosorbent assay  
FRISC-II  Fast Revascularisation during Instability in Coronary artery disease II trial  
GFR   glomerular filtration rate  
HDL   high-density lipoprotein  
LDL   low-density lipoprotein  
Lp-PLA2 lipoprotein associated phospholipase A2  
MI    myocardial infarction  
NKF   National Kidney Foundation  
NSTEMI non-ST elevation myocardial infarction  
NT-proBNP N-terminal pro-brain natriuretic peptide  
OxLDL oxidized low-density lipoprotein  
PCI   percutaneous coronary intervention  
ROC   receiver operating characteristic curve  
STEMI ST-elevation myocardial infarction  
SWISCH Sweden, Women and men and ISChemic Heart disease study  
TC    total cholesterol  
TG    triglycerides  
TnT   troponin T  
URL   upper reference limit
Introduction

“With respect to the treatment of this complaint, I have little or nothing to advance: Nor indeed is it to be expected we should have made much progress in the cure of a disease, which has hitherto hardly had a place or a name in medical books. Quiet and warmth, and spirituous liquors, help restore patients who are nearly exhausted, and to dispel the effects of a fit when it does not soon go off. Opium taken at bed-time will prevent the attacks at night. I knew one who set himself a task of sawing wood for half an hour every day, and was nearly cured. In one also the disorder ceased of itself. Bleeding, vomiting, and purging, appear to me to be improper”.

Excerpt from William Heberden’s description of angina pectoris in the History and Cure of Diseases 1772.

In 1768 when William Heberden first presented his description of angina pectoris to the Royal College of Physicians, prescribing treatment for this condition was simple and straightforward: alcohol, opium and physical work. The treatment focused on limiting symptoms, as the cause of the disease remained obscure. In the two centuries since, light has been shed on the pathophysiological mechanisms contributing to coronary artery disease (CAD) and present treatment paradigms target both the cause and symptoms, and moreover, aim at preventing future cardiac events and mortality.[1]

Patients afflicted with CAD, however, constitute a heterogeneous population with regard to background risk factors, underlying pathophysiology, response to treatment and prognosis. Consequently, risk assessment has come to play a central role in clinical decision making in the management of these patients. Fundamental components of assessment include clinical history, physical examination, electrocardiography and increasingly, the measurement of biochemical markers that reflect an array of the different pathophysiological mechanisms involved in the disease process.[2] In this thesis, in patients with unstable CAD, we have investigated the clinical use of markers reflecting low-density lipoprotein (LDL) oxidation and renal function, both physiological processes associated with accelerated atherosclerosis.
Background

Definition of unstable coronary artery disease

Unstable CAD is a major global public health problem. Despite significant improvements in management over the past decades, it remains one of the leading causes of mortality and morbidity in Western countries.[3] Unstable CAD encompasses the clinical conditions known as unstable angina and nonST-elevation myocardial infarction (NSTEMI). Unstable angina describes a syndrome that is intermediate between stable angina and myocardial infarction (MI) characterized by a "crescendo" pattern of chest pain that lasts longer than stable angina, or occurs with less exertion or at rest, or is less responsive to medication.[4] NSTEMI refers to MI without findings on the electrocardiogram of ST-segment elevation. The distinction from ST-elevation myocardial infarction (STEMI) is based on differences in underlying pathophysiology and treatment strategies. Unstable angina and NSTEMI are considered acute coronary syndromes (ACS) and are only differentiated from each other by the detection of biochemical markers indicating myocardial infarction.[1]

Pathophysiology of unstable coronary artery disease

Atherosclerosis is the most common cause of CAD. The term “atherosclerosis” comes from the Greek words athero (meaning “gruel” or “paste”) and sclerosis (meaning “hardness”). The name describes the findings of deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances in the inner lining (intima) of an artery. Atherosclerosis usually affects medium and large size arteries. It is a progressive, complex disease, often associated with the aging process, and is characterized by alternate phases of clinical stability and instability.

Although the exact mechanisms of atherogenesis remain uncertain, it is believed that early lesion development begins with endothelial injury that may be induced by all the major risk factors for CAD such as age, diabetes, smoking, hypertension and hypercholesterolemia.[5, 6] Endothelial function is critical to maintaining blood flow and vascular integrity. A healthy endothelium tends to favor vasodilation, antithrombosis, fibrinolysis and monocyte disadhesion. Vascular injury alters this balance and may lead to an in-
creased adhesiveness of the endothelium for monocytes and platelets. The recruited monocytes and platelets release a number of cytokines, vasoactive agents and growth factors that promote a local inflammatory reaction. Within the arterial vessel wall, low-density lipoprotein (LDL) binds to matrix proteoglycans and may be oxidatively modified. The monocytes recruited to the arterial wall take up oxidized LDL (OxLDL) to form lipid-laden macrophages also known as “foam cells”, the hallmark of atherosclerotic lesions (Figure 1).[7] After its uptake by scavenger receptors, OxLDL is processed by the macrophages and fragments derived from LDL are presented on its surface activating an immune response through the stimulation of T-cells.[8] This in turn may perpetuate the inflammatory reaction. Smooth muscle cells proliferate and migrate towards the endothelial surface and form a fibrous cap in attempt to seal off and stabilize the growing plaque. The plaque remains stable as long as the endothelial surface and fibrous cap remain intact. When denudation of the endothelium or rupture of the cap occurs, the plaque core contents are exposed resulting in thrombus formation with partial or complete occlusion of the blood vessel. The ensuing reduction in myocardial oxygen supply may then manifest as an acute coronary event.

Oxidized low-density lipoprotein

Historical perspectives on cholesterol research

As early as the 19th century, a German pathologist by the name of Virchow described findings at autopsy of lipid accumulation in human atherosclerotic lesions in coronary arteries.[9] In the century that followed Anitschkow, a Russian pathologist, elegantly demonstrated that hypercholesterolemia was a cause of experimental atherosclerosis in rabbits.[10] However, although it was becoming recognized that cholesterol accumulated in atherosclerotic lesions scepticism prevailed among clinicians as to the usefulness of measuring blood cholesterol. The major argument against this was that serum cholesterol must be unimportant since most MIs occurred in patients with well below the “normal” levels of cholesterol in the population. In the 1950’s results from the Framingham Heart Study provided unarguable evidence that individuals with higher blood cholesterol levels were more likely to experience an MI.[11] At about the same time Gofman and his co-workers, described the full spectrum of lipoproteins and presented studies showing a strong correlation between the different lipoprotein classes and CAD risk. [12] In the 1970’s, through their discovery of the LDL receptor and furthermore the scavenger receptor on macrophages, Brown and Goldstein, solved the long standing question of how LDL entered cells and contributed to foam cell formation. [13, 14] Hence, they provided a long awaited fundamental mechanistic link between hypercholesterolemia and cardiovascular disease.

In the 1980’s the results from the Coronary Primary Prevention Trial were published demonstrating that medical intervention was important in cholesterol reduction. This study showed that treatment with a bile acid binding resin reduced major coronary events in hypercholesterolemic men.[15] This laid the groundwork for the first guidelines to be published concerning cholesterol management in the prevention of CAD.[16] With the later development of more efficient lipid-lowering agents, i.e. statins, several landmark clinical trials in the 1990’s demonstrated that decreasing plasma LDL levels was a potent way to reduce the risk of cardiovascular events. [17, 18] Yet, it was also made clear that among individuals with apparently similar LDL levels that the expression of disease and clinical events varies widely. This has led to the search for lipid/lipoprotein markers that better add to CAD risk discrimination. In recent years, OxLDL has become the focus of attention as
experimental evidence has implicated the oxidative modification of LDL as a pivotal event in atherosclerosis.

What is oxidized low-density lipoprotein?

LDL is a large spherical particle, with a molecular weight of about $3 \times 10^6$ Da, a diameter of 22-28 nm, and density between 1.019-1.063 g/ml (Figure 2). The hydrophobic core is composed mainly of cholesterol esters (CE) and a small amount of triglycerides (TG). Surrounding the core is a hydrophilic monolayer of phospholipids (PL) together with free cholesterol (FC) and a single molecule of apolipoprotein B-100 (Apo B-100). Embedded in the surface are the lipophilic antioxidants vitamin E and β-carotene.

OxLDL is a mixture of heterogeneous LDL-particles modified with various oxidation products ranging from small conformational changes in surface lipids to the breakdown of the peptide chain.[19] The assay used in our study utilizes the monoclonal antibody mAb-4E6, which is directed at a conformational epitope in the Apo B-100 moiety. In other studies on OxLDL different antibodies have been used (FOH1a/DLH3 or EO6) which are both directed at epitopes in oxidized phospholipids. The available assays as such measure different variants of so-called minimally oxidized LDL.[20]
Biological properties

All of the oxidative modifications to the “native” LDL particle also lead to an alteration of its biological properties (Figure 3). At present, numerous different proatherogenic effects have been ascribed to OxLDL.[21] These include pro-inflammatory, pro-thrombotic and immunogenic properties. Currently it is not clear which of the measured OxLDL epitopes are most relevant to the different stages of atherosclerosis.
Origin and clearance

It is generally believed that the oxidation process occurs in the arterial wall rather than the circulating blood as LDL in plasma is well protected from oxidation due to the robust antioxidant defense systems associated with several plasma proteins as well as HDL and LDL-bound antioxidants.[22] In the intimal space of the arterial wall, a number of possible pathways and enzyme systems from resident cells such as macrophages, endothelial cells and smooth muscle cells exist which may contribute to the oxidative modification of LDL. It is then assumed that OxLDL is released into the bloodstream from the atherosclerotic plaque.

Although the clearance mechanisms for OxLDL are not well defined, it appears that OxLDL is predominantly cleared by way of a scavenger receptor located on the cell membrane of macrophages, endothelial cells, smooth muscle cells, platelets, and adipocytes. In addition, OxLDL may also be cleared by way of immunological mechanisms through the formation of antigen-antibody complexes.[23]

Oxidized low-density lipoprotein and coronary artery disease

Since the development of immunoassay procedures for the measurement of plasma levels of OxLDL, several studies have shown that levels of circulating OxLDL are often elevated in patients with CAD compared to healthy subjects.[24-26] The significance of OxLDL as a diagnostic marker for CAD compared to traditional markers of dyslipidemia has however, not yet been determined. It has also been suggested that elevated levels of OxLDL are associated with a higher risk of subsequent cardiac events in a general population as well as in patients with stable CAD.[27-30] In patients with unstable CAD only one study has been reported which found baseline levels of OxLDL not to be predictive of outcome at short-term follow-up.[31] However, no studies have investigated the prognostic value of circulating OxLDL levels at long-term follow-up in patients with unstable CAD. Furthermore, little is known about the kinetics of OxLDL during and after an acute coronary event.

Closely associated with LDL is phospholipase A2 (Lp-PLA2). Lp-PLA2 is a macrophage derived enzyme that circulates in the blood bound to LDL. The key role of Lp-PLA2 in atherogenesis is its hydrolysis of OxLDL to produce the proinflammatory by-products, lysophosphatidylcholine (Lyso PC) and oxidized free fatty acids (Figure 3). Initial studies have suggested that Lp-PLA2 may be a risk marker for CAD as well as a prognostic marker for future cardiovascular events.[32-36] No studies have so far examined the utility of Lp-PLA2 as a diagnostic marker of CAD.
Renal function and coronary artery disease

Magnitude of the problem

Chronic kidney disease (CKD) has reached epidemic proportions in many parts of the world driven by a rise in the occurrence of obesity and diabetes mellitus. A definition of CKD and staging according to the National Kidney Foundation (NKF) is provided in Table 1.[37] Patients with CKD have a high prevalence of CAD, estimated at 40% in dialysis patients.[38] The prognosis after a MI in these patients is poor compared to those without kidney dysfunction.[39] In fact, patients with end-stage renal disease have the highest mortality after an acute MI of any chronic disease population.[40]

Table 1. Definition and stages of chronic kidney disease* according to the National Kidney Foundation

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (mL/min/1.73 m²)</th>
<th>Cystatin C† (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal GFR</td>
<td>≥ 90</td>
<td>&lt; 0.89</td>
</tr>
<tr>
<td>2</td>
<td>Mild decreased GFR</td>
<td>60-89</td>
<td>1.22-0.90</td>
</tr>
<tr>
<td>3</td>
<td>Moderate decreased GFR</td>
<td>30-59</td>
<td>2.11-1.23</td>
</tr>
<tr>
<td>4</td>
<td>Severe decreased GFR</td>
<td>15-29</td>
<td>3.70-2.12</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>&lt; 15 (or dialysis)</td>
<td>&gt; 3.70</td>
</tr>
</tbody>
</table>

* Chronic kidney disease is defined as either kidney damage or GFR <60mL/min/1.73 m² for ≥ 3 months. † Level of cystatin C which approximates GFR level calculated from GFR=77.24cystatin C⁻¹.²⁶³, according to reference 56.

The management of CAD in patients with kidney dysfunction is complicated by substantial co-morbidities and unique metabolic and physiological disturbances.[41] Patients with renal dysfunction have often been excluded from randomized clinical trials for safety reasons. The evidence to base the selection of the most appropriate treatment in this patient group is therefore mostly retrospective and experimental in nature. The available studies cite excessive bleeding, heightened risk of contrast-induced nephropathy in conjunction with angiography and acute renal failure after surgical revascularization as well as higher risk of restenosis following PCI.[42-45]. This has led clinicians to hesitate in applying current practice guidelines in this patient population when suffering from an acute coronary event.
Recently data has been published from several clinical trials on patients with unstable CAD citing an increased risk of mortality associated with even mild to moderate renal dysfunction. [46-48] This has stimulated renewed interest in the assessment of renal function and a demand for studies contributing recommendations for clinical management of these patients. One such study by Januzzi et al. found an early invasive management in patients with unstable CAD and mildly to moderately impaired renal function to be superior to a more conservative approach in the reduction of future cardiovascular events at 6 months follow-up.[48] No studies have however, evaluated the effect of an early invasive vs. conservative treatment strategy on long-term outcome in patients with unstable CAD and mild to moderate renal dysfunction.

Biochemical markers of renal function

Creatinine
In the past 40 years, the most commonly used marker of overall renal function in clinical practice has been plasma creatinine concentration. Creatinine is a product of muscle tissue metabolism, derived from creatine and phosphocreatine. It is freely filtered by the glomerulus. Although it is not reabsorbed by the proximal tubules, some tubular secretion of creatinine into the blood does occur. In addition, the creatinine concentration is affected by many factors other than the level of kidney function and varies markedly with age, gender, muscle mass and diet.

Cystatin C
The limitations of creatinine have led to the search for other endogenous substances for the estimation of renal function, one of which is cystatin C. Cystatin C is a 122-amino acid protein that is a member of the cysteine proteinase inhibitors.[49] It is produced by all nucleated cells at a constant rate and freely filtered by the glomerulus. Although not secreted, it is reabsorbed by the tubular epithelial cells and subsequently catabolized so that, unlike creatinine, it does not return to the blood flow. Although several studies have measured cystatin C in healthy individuals, most of them have been limited by small sample sizes or samples consisting of healthy blood donors or mainly younger individuals.[50-54] As such, studies examining cystatin C in healthy elderly individuals, including factors that may influence levels, are lacking.
Estimates of the glomerular filtration rate

A more precise estimation of renal function can be obtained using estimates of the glomerular filtration rate (GFR). GFR is defined as the volume of plasma cleared by the kidneys in a unit of time. The “gold standard” for determining GFR is to measure the clearance of exogenous substances such as inulin, iohexol, Cr-EDTA among others. These techniques are however time-consuming and costly. Therefore a variety of methods have been used to estimate GFR.

The Cockcroft–Gault formula, (140-age) × (weight in kilograms ÷ serum creatinine in milligrams per deciliter) × 72 multiplied by 0.85 for females, is the best validated and therefore at present one of the most widely used in clinical practice.[55] This equation comprises several anthropometric variables to compensate for the inadequacies of creatinine as a marker of GFR.

Recently cystatin C based formulas for the determination of GFR have been introduced (i.e. \( GFR = 77.24 \text{ cystatin C}^{-1.2623} \)).[56] The levels of cystatin C which approximate the GFR levels and NKF staging using this equation are shown in table 1. Studies suggest that this marker may be better than creatinine at estimating GFR in patients with milder reductions in renal function whereas some scepticism prevails regarding cystatin C’s advantage in advanced stages of renal disease. At present there is no consensus as to whether cystatin C should replace the use of creatinine in the clinical arena.

N-terminal pro-brain natriuretic peptide, a marker of cardio-renal function

It has recently been shown that the combined use of markers of kidney function and natriuretic peptides provide very powerful predictive information on long-term mortality in patients with unstable CAD.[47] These peptides, specifically brain natriuretic peptide (BNP) and N-terminal pro-natriuretic peptide (NT-proBNP) are released from cardiac myocytes with increasing cardiac wall stress.[57] BNP, which is the biologically active peptide, works in concert with the kidneys in controlling arterial pressure and fluid volume. Hence, these peptides have come into focus as unique markers of hemodynamic balance that reflect both cardiac and renal status.[58] Few studies have to date investigated levels of NT-proBNP and the physiological determinants of these levels in healthy elderly men and women similar in age to an ACS population.
Aims

General Aim
In recent years the use of biochemical markers has received increasing attention for purposes of risk assessment and clinical management in patients suffering from unstable CAD. The general aim of this thesis was to investigate the clinical usefulness of markers of LDL oxidation and renal function in patients with unstable CAD.

Specific Aims
1. To investigate the diagnostic accuracy of OxLDL compared with traditional markers of dyslipidemia and Lp-PLA2 in identifying patients with CAD.
2. To investigate the long-term predictive value of OxLDL in patients with unstable CAD.
3. To evaluate the effect of an early invasive vs. conservative treatment strategy in patients with unstable CAD and mild to moderate renal dysfunction, using CrCl as an estimation of renal function.
4. To evaluate the effect of an invasive treatment strategy on renal function in patients with unstable CAD.
5. To examine the distributions of cystatin C and NT-proBNP and their relation to age, gender, and other physiological factors in an apparently healthy population similar in age to patients with unstable CAD.
Methods

Controls (Paper I and IV)
The healthy control subjects, referred to as the SWISCH (Sweden Women, men and ISChemic Heart disease) study population, consisted of 296 men and 146 women randomly selected from the Swedish population registry during 2000-2001 (Figure 4). Subjects were matched for age, gender and geographical residence with patients with unstable CAD included at 6 hospitals in the Fragmin and Fast Revascularisation during Instability in Coronary artery disease (FRISC) II trial.[59] An invitation letter and questionnaire (see appendix) were sent to 2500 individuals, 1504 of whom replied positively. A screening of the questionnaire respondents left 550 subjects who fulfilled the initial exclusion criteria which included manifest cardiovascular or other clinically overt diseases (e.g. malignancy, lung disease, inflammatory disease, diabetes, kidney, liver or blood disease, psychiatric illness) and/or con-

Figure 4. SWISCH study population.
continuous medication with cardiovascular drugs (i.e., aspirin, angiotensin converting enzyme [ACE] inhibitor, Angiotensin-II blocker, β-blocker, calcium antagonist, digoxin, diuretic, nitrate, antidiabetic, lipid-lowering). A total of 531 subjects participated in an outpatient visit which took place within 3 months after the questionnaires had been returned. At the outpatient visit, the questionnaires were checked for inconsistencies and subjects underwent a clinical examination which included a physical examination encompassing heart and lung auscultation, measurement of height, weight and blood pressure, an electrocardiogram (ECG) and blood sampling. Individuals were further excluded at the time of examination if chronic disease was manifest (recently diagnosed or if suspected at the time of the examination) or subjects were acutely ill. Resting 12-lead ECGs had to be without major abnormalities (atrial fibrillation, Q waves, ST-deviation, left ventricular hypertrophy, bundle branch block) for inclusion [60]. Finally, individuals were excluded if analysis of routine blood chemistry (blood glucose, hemoglobin, white blood count, platelets and creatinine) was outside normal reference levels requiring follow-up. In total, 442 subjects met the criteria and were recruited into the study.

Figure 5. FRISC-II Study Population.
Patients (Paper I, II, III)

The patient population consisted of men and women with unstable CAD recruited in the FRISC II study during 1996-1998 (Figure 5). This study was a prospective, randomized, factorial, multicenter trial designed to compare the extended treatment with low-molecular-weight (lmw) heparin, dalteparin with placebo, and an early invasive strategy with a primarily non-invasive approach in patients with unstable CAD.

In Paper IV all 2457 patients were included for analysis. The subset of patients referred to in Paper I and II were included from 6 hospitals in Sweden which were willing to participate in these substudies. Out of the 490 patients studied in Paper I, 57 were randomized only to the medical treatment (3 months treatment with dalteparin compared to placebo), leaving 433 patients evaluable in the invasive trial in Paper II.

Inclusion and exclusion criteria

Postmenopausal women and men over 40 years of age admitted to hospital with symptoms suggestive of cardiac ischemia concurrent with objective signs of cardiac ischemia were eligible for inclusion. Myocardial ischemia was verified by ECG (ST-depression ≥ 0.10mV or T-wave inversion ≥ 0.10mV) or by raised biochemical markers (creatine kinase MB isoenzyme >6 μg/L, troponin T ≥ 0.10 μg/L, qualitative TnT test positive, or catalytic activity of total creatine kinase or its B or MB isoenzyme above the local decision limit for the diagnosis of MI).

Exclusion criteria were increased risk of bleeding (cerebrovascular event within 12 months, thrombocytopenia <100 x 10⁶, uncontrolled hypertension, ongoing oral anticoagulant treatment, anemia, INR>1.4, previous open heart surgery, percutaneous coronary intervention (PCI) within the last 6 months, osteoporosis or concomitant significant heart disease. No patients with serum creatinine >150μmol/L were included.

Medical and invasive management

In patients without contraindications, treatment with aspirin and beta-blockers was initiated as soon as possible after admission. Nitrates were given as needed. ACE-inhibitors were used in patients with symptoms of heart failure or signs of left ventricular dysfunction. Use of statins was encouraged if TC levels were above 5.0 mmol/L or LDL levels above 3.5 mmol/L. Oral anti-diabetic medications and s.c. insulin therapy was initiated as needed.

All patients were treated with lmw heparin, dalteparin (120 U/kg bid, maximal 10000 U bid) for at least 5 days and always until a revascularization procedure or predischarge exercise test. Treatment with lmw heparin
was started either at admission or at the latest from randomization which took place within 48 hours of admission. Patients were randomized by a two by two factorial design to either 3 months continued treatment with s.c. dalteparin (Fragmin®) or placebo and to either an early non-invasive or an invasive strategy.

The non-invasive strategy included coronary angiography in patients in-hospital with refractory or recurrent symptoms despite maximal medical treatment or if severe ischemia was present at a pre-discharge exercise test. During long-term follow-up invasive procedures were performed as needed regardless of randomized strategy. In patients randomized to the invasive strategy coronary angiography was performed within a few days of inclusion aiming for revascularization within 7 days from start of open-label dalteparin.

Definition of endpoints
The primary endpoint of the FRISC-II study was death or myocardial infarction (MI) at 6 months. During this time period data on all events, procedures, and symptoms were collected at regular outpatient visits. The 1-year and 2-year follow-ups were performed by way of a standardized telephone interview and if needed, complemented with the information in the patients medical record.

Myocardial infarction was classified as non-procedural (spontaneous) or procedural (related to PCI or CABG). Non-procedural MI’s were diagnosed based on the presence of two out of three conventional criteria i.e. chest pain, diagnostic ECG (new Q wave) or elevation of biochemical markers of myocardial damage. For non-procedural MI’s the decision limits for biochemical markers were: 1) concentration of CK-MB mass above the local hospital’s decision level for MI at one measurement or 2) catalytic activity of CK, CK-B, or CK-MB above the decision level at 2 subsequent determinations or 3) above the double local decision level at 1 measurement. MI in relation to PCI was defined as 1) CK-MB mass 1.5 times above the local hospital’s decision level for MI at one measurement or 2) catalytic activity of CK, CK-B, CK-MB at one measurement three times above or 3) at two determinations 1.5 times above the local decision level. Only new Q waves were used for the diagnosis of MI in association with CABG.

Laboratory analyses
At the outpatient visit for controls, venous blood was collected in tubes containing EDTA or citrate and centrifuged at each site. For all patients, venous blood was collected on admission, and for some patients at a follow-up visits after 4-7 weeks and 6 months. Lipids and special markers were analyzed the
first morning after admission and after an overnight fast at follow-up. In both controls and patients analysis of routine blood chemistry and lipids (TC, HDL, TG’s) was carried out on fresh blood samples by established enzymatic methods at the local laboratories. In blood samples not analyzed immediately were frozen in aliquots and stored at –70°C until analysis. LDL levels were calculated using the Friedewald formula (LDL = TC-HDL-[0.45 x TG]) excluding individuals with triglycerides greater than 4.5 mmol/L.[61] Creatinine clearance (CrCl) was calculated according to Cockcroft-Gault.[55] High sensitive C-reactive protein (CRP), Troponin T (TnT), and OxLDL, Lp-PLA2 were analyzed at the core laboratory for the FRISC-II study in Uppsala, Sweden.

OxLDL

A commercially available sandwich enzyme-linked immunosorbent assay (ELISA) was used for measuring plasma levels of OxLDL (Mercodia AB, Uppsala, Sweden). The detection limit for the assay is <1.0 mU/L. Precision for the assay was calculated from 12 samples assayed in three replicates on 2 different occasions. The coefficient of variation within samples was 8.9% and between samples 9.2%.

C-reactive protein (CRP)

CRP serum concentrations were measured with a high-sensitivity chemiluminescent enzyme-labeled immunometric assay (Immunolite CRP, Diagnostics Products Corporation, Los Angeles, U.S.A.) with a detection limit of 0.1mg/L and a total CV of 5.6% at 2mg/l and 5% at 10mg/l.

Troponin T (TnT)

Cardiac troponin T was determined by the third generation troponin T assay on an Elecsys 2010 (Roche Diagnostics, Basel, Switzerland). The lower limit of detection is 0.01 µg/l and the upper reference level of healthy individuals is at the same level, according to the manufacturer. At this level the coefficient of variation is high. Therefore the functional sensitivity, defined as the level of CV< 20% of the assay is 0.03 µg/l, which was therefore used as the cut-off level.

Cystatin C

Plasma Cystatin C measurements were performed by a latex-enhanced reagent (N Latex Cystatin C, Dade Behring, Deerfield, IL, USA) using a Behring BN ProSpec analyser (Dade Behring). The total analytical imprecision of the method was 4.8% at 0.56 mg/L and 3.7% at 2.85 mg/L.
NT-proBNP
NT-proBNP was determined using Elecsys proBNP sandwich immunoassay on an Elecsys 2010 (Roche Diagnostics, Basel, Switzerland). The analytical range extends from 20 to 35000 ng/l. At our laboratory the total CV was 3.3% (n=21) at a level of 209 ng/l and 3.0% (n=21) at a level of 7431 ng/l.

Lp-PLA2
Plasma levels of Lp-PLA2 were determined with a commercial Lp-PLA2 kit (diaDexus Inc, San Francisco, CA. USA). The lower detection limit in this assay is 2 ng/mL and the between-run CV was 9.6%.

Statistics
Unless otherwise indicated, the characteristics of the subjects were described as means and standard deviations for normally distributed data or medians and interquartile range for skewed data or proportions (%). Tests for significance of observed mean differences were performed using the Student’s t-test. Tests for significance of observed median differences were performed using Mann-Whitney or Wilcoxon rank test as applicable. Differences in proportions were judged by chi-square analysis and Fischer’s exact test if expected values were less than 5 in any individual cell. The Kruskal-Wallis test was used to test the equality of distributions in different groups. Friedman’s test was used to compare differences in markers over time. To evaluate the correlations between the level of biomarkers and other variables the Spearman rank correlation coefficients were calculated. To identify variables independently associated to the different biomarkers, multiple linear regression analysis was used. To meet the assumptions of linear regression analysis, all skewed variables were log transformed before being entered into the equation. To identify variables independently associated with outcomes, logistic regression analyses were used. Interaction analyses were performed as needed. With the exception of Paper I, in which SAS® System for Windows®, version 8.02 was used, all data analysis was performed with the Statistical Package for Social Sciences (SPSS 11.5, SPSS Inc.) software.

Specific statistics

Paper I
Receiver operating characteristic curve analyses (ROC) were used to evaluate the diagnostic accuracy of the various markers. These were generated using Analyse-it™ version 1.67. This provided area under curve (AUC) and 95% confidence intervals (CI) for the AUC, sensitivity, and specificity. The
ROC curve is constructed by plotting sensitivity on the y-axis against the false-positive fraction (1-specificity) on the x-axis. The area under the ROC curve varies from 1.0, which corresponds to perfect discrimination (upper left corner) to 0.5 where no discrimination exists. The data from this program was utilized by KaleidaGraph™ version 3.52 to generate the overlaid ROC curves. The positive predictive value (PPV), negative predictive value (NPV), odd ratios (OR) and the 95% CIs for the ORs were calculated using Microsoft® Excel 2002.

**Paper IV**

Reference limits were calculated using a bootstrap method.[62] Bootstrapping is a statistical method used to adjust for the effect of outliers and to decrease the effect of variability in a given sample. This is done by the creation of multiple sub-samples by random selection of subjects from the original sample. The selected subject is returned to the original sample and can be selected more than once into a given sub-sample. The size of the sub-sample is the same as the original one. This procedure is then repeated a number of times to examine the relationship between the variables of interest.
Results and discussion

Results

Paper I

(Aim: To investigate the diagnostic accuracy of OxLDL compared with traditional markers of dyslipidemia and Lp-PLA2 in identifying patients with CAD).

General Findings

The baseline characteristics for healthy controls (n=442) and patients (n=490) are given in Table 2. Controls and patients were similar in age and gender distribution. Patients had a higher body-mass index (BMI), were more often current smokers and had a higher prevalence of diabetes, hypertension and hyperlipidemia.

<table>
<thead>
<tr>
<th>Table 2. Baseline characteristics for controls and patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Women, n (%)</td>
</tr>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
</tr>
<tr>
<td>Hyperlipidemia† n (%)</td>
</tr>
<tr>
<td>Hypertension† n (%)</td>
</tr>
</tbody>
</table>

The values shown are mean (standard deviation) unless otherwise stated.
† Defined as pharmacological treatment for this condition. *p < 0.05, ** p < 0.01, ***p < 0.001 (controls vs. patients).

The mean plasma levels for the various biomarkers are given in Table 3. Patients had higher levels of LDL, TGs, OxLDL and Lp-PLA2, lower levels of HDL and similar levels of TC compared to controls. The quintiles of the control population were derived for each biomarker, and the distribution of the CAD patients across these quintiles was examined. The percentages of patients in the lowest quintile (Q1) and the highest quintile (Q5) of the control group are presented in Figure 6. The greatest contrast in the percentages of patients in Q1 and Q5 was noted for OxLDL/HDL.
Table 3. Levels of lipid and lipoprotein biomarkers in the study populations

<table>
<thead>
<tr>
<th>Lipid or lipoprotein</th>
<th>Units</th>
<th>Control group Mean (SD)</th>
<th>CAD patients Mean (SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>mmol/L</td>
<td>5.71 (1.00)</td>
<td>5.81 (1.10)</td>
<td>0.179</td>
</tr>
<tr>
<td>LDL *</td>
<td>mmol/L</td>
<td>3.47 (0.90)</td>
<td>3.75 (1.06)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lp-PLA2</td>
<td>ng/mL</td>
<td>278 (97)</td>
<td>310 (191)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>mmol/L</td>
<td>1.74 (0.91)</td>
<td>2.16 (1.13)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL</td>
<td>L/mmol</td>
<td>1.37 (0.39)</td>
<td>1.03 (0.33)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TC/HDL</td>
<td>Ratio</td>
<td>4.12 (1.16)</td>
<td>5.57 (2.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OxLDL</td>
<td>U/L</td>
<td>53.82 (14.34)</td>
<td>76.21 (19.48)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OxLDL/HDL</td>
<td>Ratio (U/mmol)</td>
<td>39.70 (16.33)</td>
<td>74.07 (31.12)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Total Cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), Oxidized low-density lipoprotein (OxLDL). The values shown are mean (standard deviation). * Available in 470 patients and 417 controls.

Figure 6. The percentages of patients in the lowest quintile (Q1) and the highest quintile (Q5) of the control group for each lipid or lipoprotein biomarker. (TC: Q5 > 6.6 mmol/L, Q1 < 4.8 mmol/L; LDL: Q5 > 4.18 mmol/L, Q1 < 2.74 mmol/L; TGs: Q5 > 2.30 mmol/L, Q1 < 1.06 mmol/L; Lp-PLA2: Q5 > 340.00 pg/mL, Q1 < 206.35 pg/mL; HDL: Q5 > 0.91; Q1 < 0.56; TC/HDL: Q5 > 5.00, Q1 > 3.13; OxLDL: Q5 > 66, Q1 < 42; OxLDL/HDL: Q5 > 52.5, Q1 < 25.63.)

Table 4 presents the cutoffs, sensitivities, specificities, NPVs, PPVs and AUCs for the various markers. Table 5 shows the univariate ORs for the risk of having CAD for a positive test result versus a negative test result (i.e. above the cutoff value specified in Table 4). OxLDL/HDL was the biomarker that was associated with the most significant increase in risk.
Table 4. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the curve (AUC) for each lipid and lipoprotein biomarker.

<table>
<thead>
<tr>
<th>Optimal cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>AUC (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC 5.7 mmol/L</td>
<td>55</td>
<td>50</td>
<td>55</td>
<td>49</td>
<td>0.520 (0.482–0.557)</td>
</tr>
<tr>
<td>LDL 3.8 mmol/L</td>
<td>46</td>
<td>69</td>
<td>62</td>
<td>54</td>
<td>0.577 (0.539–0.615)</td>
</tr>
<tr>
<td>Lp-PLA2 268 pg/mL</td>
<td>60</td>
<td>58</td>
<td>64</td>
<td>53</td>
<td>0.597 (0.558–0.615)</td>
</tr>
<tr>
<td>Triglycerides 1.6 mmol/L</td>
<td>63</td>
<td>58</td>
<td>63</td>
<td>58</td>
<td>0.631 (0.594–0.667)</td>
</tr>
<tr>
<td>TC/HDL 4.8</td>
<td>66</td>
<td>76</td>
<td>75</td>
<td>67</td>
<td>0.764 (0.733–0.795)</td>
</tr>
<tr>
<td>1/HDL 1.3 mmol/L</td>
<td>76</td>
<td>68</td>
<td>72</td>
<td>72</td>
<td>0.775 (0.745–0.805)</td>
</tr>
<tr>
<td>OxLDL 63 U/L</td>
<td>74</td>
<td>75</td>
<td>77</td>
<td>71</td>
<td>0.826 (0.800–0.852)</td>
</tr>
<tr>
<td>OxLDL/HDL 53</td>
<td>76</td>
<td>82</td>
<td>82</td>
<td>76</td>
<td>0.867 (0.844–0.890)</td>
</tr>
</tbody>
</table>

Table 5. Unadjusted odds ratio (OR) and 95% confidence interval (CI) for coronary artery disease for various lipid and lipoprotein biomarkers.

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC 1.20 (0.93–1.56)</td>
</tr>
<tr>
<td>LDL 1.90 (1.44–2.51)</td>
</tr>
<tr>
<td>Lp-PLA2 2.02 (1.54–2.66)</td>
</tr>
<tr>
<td>Triglycerides 2.34 (1.79–3.05)</td>
</tr>
<tr>
<td>TC/HDL 6.12 (4.56–8.20)</td>
</tr>
<tr>
<td>1/HDL 6.61 (4.93–8.86)</td>
</tr>
<tr>
<td>OxLDL 8.26 (6.15–11.11)</td>
</tr>
<tr>
<td>OxLDL/HDL 13.92 (10.07–19.23)</td>
</tr>
</tbody>
</table>

ROC Analyses

Figure 7A illustrates the ROC curves for the traditional lipids and lipoproteins. Figure 7B gives the ROC curves for the emerging lipid and lipoprotein biomarkers. The biomarker with the highest diagnostic accuracy in distinguishing healthy controls from patients with CAD was OxLDL/HDL. At a cutoff value of 53, the sensitivity was 76% and the specificity was 82%, with a corresponding AUC (95% CI) of 0.867 (0.844–0.890). Other biomarkers with good diagnostic accuracy (AUC above 0.7) were OxLDL, TC/HDL and HDL. The levels of TC, LDL, triglycerides or Lp-PLA2 were not particularly helpful in discriminating CAD patients from matched healthy control subjects (AUC less than 0.7).
Figure 7. ROC curves for (A) traditional lipids and lipoproteins, and (B) emerging lipid and lipoprotein biomarkers.
Paper II
(Aim: To investigate the long-term predictive value of OxLDL in patients with unstable CAD).

Table 6. Baseline characteristics of patients according to level of OxLDL

<table>
<thead>
<tr>
<th></th>
<th>OxLDL &lt;76U/L (n=207)</th>
<th>OxLDL &gt;76U/L (n=226)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years*</td>
<td>64(57-70)</td>
<td>65(57-69)</td>
<td>0.937</td>
</tr>
<tr>
<td>Male gender, n(%)</td>
<td>52(25)</td>
<td>73(32)</td>
<td>0.100</td>
</tr>
<tr>
<td>Hypertension, n(%)</td>
<td>70(34)</td>
<td>65(29)</td>
<td>0.257</td>
</tr>
<tr>
<td>Hyperlipidemia, n(%)</td>
<td>33(16)</td>
<td>17(8)</td>
<td>0.006</td>
</tr>
<tr>
<td>Current smoker, n(%)</td>
<td>53(26)</td>
<td>63(28)</td>
<td>0.594</td>
</tr>
<tr>
<td>Diabetes Mellitus, n(%)</td>
<td>28(14)</td>
<td>30(13)</td>
<td>0.923</td>
</tr>
<tr>
<td>Previous MI n(%)</td>
<td>57(28)</td>
<td>52(23)</td>
<td>0.278</td>
</tr>
<tr>
<td>Heart Failure, n(%)</td>
<td>8(4)</td>
<td>10(4)</td>
<td>0.771</td>
</tr>
<tr>
<td>Angina &gt;48h, n(%)</td>
<td>154(74)</td>
<td>166(74)</td>
<td>0.823</td>
</tr>
<tr>
<td>Chest pain at rest, n(%)</td>
<td>168(81)</td>
<td>184(81)</td>
<td>0.945</td>
</tr>
<tr>
<td>ST-dep at entry, n(%)</td>
<td>75(37)</td>
<td>94(42)</td>
<td>0.240</td>
</tr>
<tr>
<td>TnT&gt; 0.01ug/L, n(%)</td>
<td>144(71)</td>
<td>158(71)</td>
<td>0.985</td>
</tr>
<tr>
<td>CRP&gt;10 mg/L, n(%)</td>
<td>55(30)</td>
<td>68(33)</td>
<td>0.424</td>
</tr>
</tbody>
</table>

* median (25th-75th percentile)

General findings
In Paper II, 433 of the patients from Paper I were studied and grouped according to below or above the median for OxLDL which was 76 U/L (Table 6). Except for the higher incidence of hyperlipidemia requiring lipid-lowering treatment at entry in patients with OxLDL below the median, no other background factors differed between the two groups. At discharge however, there were more patients being treated with lipid-lowering drugs in the group with OxLDL levels above the median compared to those below the median (55% vs. 38%, p<0.001) while the use of aspirin, β-blockers or ACE-inhibitors was similar. An equal number of patients were randomized to invasive treatment in both groups (54% vs. 55%, p=0.861). In patients randomized to the invasive strategy there was no significant association between OxLDL levels and the number of coronary vessels with significant stenosis assessed at coronary angiography. No difference was found in levels of OxLDL in patients with unstable angina compared to patients with MI (76[61-88] U/L vs. 77[65-88] U/L, p=0.725).

OxLDL and MI
During the 2-year follow-up, there were 57(13%) patients who had a MI. There was a significantly lower rate of MI in patients with an OxLDL level below as compared to above the median (9.5% vs. 16.5%, p=0.032). There was no difference in procedure-related MIs between these groups (5.5 % vs. 6.9%, p=0.534) whereas, in the patients with an OxLDL level above the
median there was a significantly higher risk of a non-procedure related MI compared to those with lower levels (10% vs. 4 %, p=0.024). In logistic regression analyses adjusted for well-known predictors of MI as well as lipids and randomized treatment, OxLDL was found to be an independent predictor of MI (Table 7). Thus, even after adjustments in patients with OxLDL above the median the risk for future spontaneous MI was significantly higher compared to those with lower levels (odds ratio [95% CI] : 3.13[1.16 to 8.44]).

Table 7. Odds Ratio (OR) and 95% Confidence Interval (CI) for OxLDL according to different multivariate models

<table>
<thead>
<tr>
<th>Model</th>
<th>OR (95% CI) for total MI</th>
<th>p</th>
<th>OR (95% CI) for non-procedure MI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>1.90(1.05-3.39)</td>
<td>0.03</td>
<td>2.67(1.17-6.11)</td>
<td>0.02</td>
</tr>
<tr>
<td>Multivariable*</td>
<td>1.90(1.03-3.49)</td>
<td>0.04</td>
<td>3.12(1.31-7.42)</td>
<td>0.01</td>
</tr>
<tr>
<td>Multivariable* + invasive treatment</td>
<td>1.87(1.02-3.45)</td>
<td>0.04</td>
<td>2.64(1.11-6.31)</td>
<td>0.03</td>
</tr>
<tr>
<td>Multivariable* + lipids</td>
<td>1.71(0.844-3.46)</td>
<td>0.14</td>
<td>3.13(1.16-8.44)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, diabetes mellitus, previous myocardial infarction, previous angina, ST-segment depression at entry, Troponin T >0.01 µg/L.

Figure 8. Rates of myocardial infarction according to levels of OxLDL and troponin T.

OxLDL and TnT
When levels of OxLDL and TnT levels were combined, the prognostic value of OxLDL appeared greatest in the group with TnT<0.01 µg/L (Figure 8). Among patients with negative TnT the 2 year MI rate was 1.7% for OxLDL <76 U/L vs. 16.9% in patients with OxLDL >76 U/L  (p=0.004). No addi-
tional prognostic information was obtained by stratification according to the OxLDL level in patients who were TnT positive.

**OxLDL and statin therapy**

In 233 of the 433 patients, OxLDL was analyzed at a follow-up visit 4-7 weeks after the index event. Although levels of OxLDL were in total lower at this timepoint (69[56-83] U/L vs. 76[63-87] U/L, p<0.001) this difference was confined to patients (n=87) who were discharged with and maintained on statin therapy through the follow-up visit (79[68-89] U/L vs. 67[53-78] U/L, p<0.001). In patients (n=124) without statin therapy at inclusion and at follow-up the OxLDL levels remained unchanged (72[61-82] U/L vs. 74[59-84] U/L, p=0.201) as was the case with patients (n=22) on statin therapy both at entry and at follow-up (72[60-86] U/L vs. 62[54-83] U/L, p=0.094).

**Paper III**

(Aim: To evaluate the effect of an early invasive vs. a conservative treatment strategy in patients with unstable CAD and mild to moderate renal dysfunction function).

**General findings**

Baseline characteristics by tertiles of estimated CrCl are shown in Table 8. At the time of entry into the study more patients with CrCl <69 ml/min were being treated with aspirin and β-blockers than patients with CrCl ≥69 ml/min (p<0.001). However during hospitalization the frequency of treatment with anti-ischemic and anti-thrombotic drugs was similar among all patients. In patients randomized to the invasive arm a similar proportion of patients underwent revascularization in the different CrCl groups. Three-vessel or left main stem disease was more common in patients with CrCl <69 ml/min CrCl >90 ml/min (42% vs. 24%, p<0.001). A larger number of patients with renal dysfunction (CrCl <69 ml/min) underwent coronary artery bypass surgery (CABG) compared to those patients with better renal function (CrCl ≥69 ml/min) in whom PCI was more common. The number of patients who received stents was similar across the different tertiles.
Table 8. Baseline characteristics according to CrCl tertiles

<table>
<thead>
<tr>
<th></th>
<th>CrCl &lt;69 (n=842)</th>
<th>69 ≤ CrCl ≤ 90 (n=781)</th>
<th>CrCl &gt;90 (n=831)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Personal factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)*</td>
<td>72(68-75)</td>
<td>66(60-71)</td>
<td>58(52-63)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (female) n/%</td>
<td>46</td>
<td>31</td>
<td>14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)*</td>
<td>24.8(22.8-26.7)</td>
<td>26.5(24.6-28.7)</td>
<td>28.0(25.8-31.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker, n/%</td>
<td>179/22</td>
<td>212/27</td>
<td>352/42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes, n/%</td>
<td>101/12</td>
<td>91/12</td>
<td>107/13</td>
<td>0.738</td>
</tr>
<tr>
<td>Hyperlipidemia, n/%</td>
<td>89/11</td>
<td>86/11</td>
<td>86/10</td>
<td>0.506</td>
</tr>
<tr>
<td>Hypertension, n/%</td>
<td>294/35</td>
<td>218/28</td>
<td>229/28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Previous cardiovascular disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angina, n/%</td>
<td>73</td>
<td>67</td>
<td>62</td>
<td>0.001</td>
</tr>
<tr>
<td>Myocardial infarction, n/%</td>
<td>246/28</td>
<td>149/19</td>
<td>160/19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart failure, n/%</td>
<td>44/5</td>
<td>22/3</td>
<td>8/1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Treatment at randomization</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE-inhibitor, n/%</td>
<td>114/14</td>
<td>98/13</td>
<td>90/11</td>
<td>0.234</td>
</tr>
<tr>
<td>Aspirin, n/%</td>
<td>362/44</td>
<td>264/34</td>
<td>240/29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B-blocker, n/%</td>
<td>323/38</td>
<td>233/30</td>
<td>228/27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin, n/%</td>
<td>84/10</td>
<td>84/11</td>
<td>80/10</td>
<td>0.745</td>
</tr>
<tr>
<td><strong>Treatment started during hospitalization</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE-inhibitor, n/%</td>
<td>87/10</td>
<td>56/7</td>
<td>46/6</td>
<td>0.001</td>
</tr>
<tr>
<td>Aspirin, n/%</td>
<td>464/55</td>
<td>494/63</td>
<td>583/70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B-blocker, n/%</td>
<td>429/51</td>
<td>474/61</td>
<td>548/66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statin, n/%</td>
<td>233/28</td>
<td>266/34</td>
<td>317/38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Risk markers at baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST-segment depression, n /%</td>
<td>451/54</td>
<td>341/44</td>
<td>321/39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Troponin T &gt; 0.01µg/L†, n/%</td>
<td>601/71</td>
<td>531/68</td>
<td>569/68</td>
<td>0.540</td>
</tr>
<tr>
<td>C-reactive protein &gt;10 mg/L, n/%</td>
<td>273/37</td>
<td>277/39</td>
<td>306/40</td>
<td>0.106</td>
</tr>
<tr>
<td>NT-proBNP , ng/L*</td>
<td>961(414-2091)</td>
<td>498(204-1272)</td>
<td>393(154-904)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Treatment randomization</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive treatment, n/%</td>
<td>426/51</td>
<td>403/52</td>
<td>392/47</td>
<td>0.172</td>
</tr>
<tr>
<td>Time to angiography, days*</td>
<td>4(2-5)</td>
<td>4(3-5)</td>
<td>4(2-5)</td>
<td>0.237</td>
</tr>
<tr>
<td><strong>Type of invasive treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No revascularization, n/%</td>
<td>88/21</td>
<td>95/24</td>
<td>84/21</td>
<td>0.867</td>
</tr>
<tr>
<td>PCI, n/%</td>
<td>148/35</td>
<td>172/34</td>
<td>209/53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PCI with stent, n/%</td>
<td>83/56</td>
<td>100/58</td>
<td>137/66</td>
<td>0.090</td>
</tr>
<tr>
<td>CABG, n/%</td>
<td>190/45</td>
<td>136/34</td>
<td>99/25</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* median (25th-75th percentile)
Figure 9A. Death/MI at 2 years according to CrCl and treatment.

Figure 9B. MI at 2 years according to CrCl and treatment.

Figure 9C. Mortality at 2 years according to CrCl and treatment.
**CrCl and the composite endpoint of death or MI**

During follow-up there were 345 (14.1%) patients who reached the combined endpoint of death or MI. In the total patient population, reduced CrCl was associated with a higher rate of the combined endpoint (CrCl $<69$ ml/min 18.4%, CrCl $69-90$ ml/min 12.2%, CrCl $>90$ ml/min 11.4%, p<0.001). Figure 9A shows the rates of death/MI according to tertiles of CrCl and randomized treatment. When comparing treatment groups within the CrCl tertiles, significantly lower rates of the combined endpoint were observed in patients managed invasively compared to non-invasively in CrCl categories $<69$ ml/min and 69-90 ml/min but not in the group with CrCl $>90$ ml/min. When adjusted for other covariables CrCl $<69$ ml/min remained independently associated with the risk of the composite of death or MI in the non-invasively treated group but not in the group treated invasively (Table 9). When the interaction term for treatment strategy and CrCl group was added in a logistic regression analysis including both invasively and non-invasively treated patients, the interaction between treatment strategy and CrCl $<90$ ml/min did not reach statistical significance concerning the composite endpoint (p=0.11).

**Table 9. Univariate and Multivariate Logistic Regression Analysis**

<table>
<thead>
<tr>
<th></th>
<th>Non-invasive</th>
<th>Multivariate</th>
<th>Invasive</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Death or MI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrCl $&gt;90$ ml/min</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CrCl 69-90 ml/min</td>
<td>1.30(0.86-1.94)</td>
<td>1.30(0.76-2.22)</td>
<td>0.87(0.56-1.38)</td>
<td>0.72(0.39-1.30)</td>
</tr>
<tr>
<td>CrCl $&lt;69$ ml/min</td>
<td>2.19(1.51-3.18)</td>
<td>1.96(1.12-3.42)</td>
<td>1.35(0.89-2.04)</td>
<td>1.09(0.56-2.14)</td>
</tr>
<tr>
<td><strong>Death</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrCl $&gt;90$ ml/min</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CrCl 69-90 ml/min</td>
<td>0.60(0.27-1.38)</td>
<td>0.43(0.14-1.38)</td>
<td>2.17(0.75-6.31)</td>
<td>1.98(0.45-8.70)</td>
</tr>
<tr>
<td>CrCl $&lt;69$ ml/min</td>
<td>2.64(1.47-4.74)</td>
<td>1.65(0.64-4.28)</td>
<td>5.65(2.17-14.75)</td>
<td>3.2(0.68-15.2)</td>
</tr>
<tr>
<td><strong>MI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrCl $&gt;90$ ml/min</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CrCl 69-90 ml/min</td>
<td>1.68(1.10-2.63)</td>
<td>1.64(0.93-2.97)</td>
<td>0.75(0.46-1.24)</td>
<td>0.62(0.33-1.17)</td>
</tr>
<tr>
<td>CrCl $&lt;69$ ml/min</td>
<td>2.31(1.51-3.53)</td>
<td>1.94(1.06-3.57)</td>
<td>0.96(0.75-2.08)</td>
<td>0.948(0.46-1.96)</td>
</tr>
</tbody>
</table>

**CrCl and MI**

During follow-up, there were 267(10.9%) patients who had a MI. In the total patient population reduced CrCl was associated with a higher rate of MI (13.3% in patients with CrCl $<69$ ml/min, 10.2% in patients with CrCl $69-90$ ml/min and 9% in patients with CrCl $>90$ ml/min, p=0.015). Figure 9B shows the rates of MI according CrCl tertiles and randomized treatment. When comparing treatment groups within the CrCl tertiles, significantly lower rates of MI were observed in patients managed invasively compared to non-invasively in CrCl categories $<69$ ml/min and 69-90 ml/min but not in
the group with CrCl >90 ml/min. When adjusted for other covariables, CrCl <69 ml/min remained independently associated with the risk of MI in the non-invasively treated group but not in the group treated invasively (Table 9). When the interaction term for treatment strategy and CrCl group was added in a logistic regression analysis including both invasively and non-invasively treated patients, the interaction between treatment strategy and CrCl <90 ml/min was independently associated with the risk of future MI (p=0.006).

**Figure 10A.** Trends in creatinine clearance (CrCl) during 6 months of follow-up in patients treated non-invasively.

**Figure 10B.** Trends in creatinine clearance (CrCl) during 6 months of follow-up in patients treated invasively.

**CrCl and mortality**

During follow-up there were 111(4.5%) patients who died. In the total patient population reduced CrCl was associated with a higher mortality (CrCl <69 ml/min 8.2 %, CrCl 69-90 ml/min 2.6%, CrCl >90 ml/min 2.6%, p<0.001). When adjusted for other covariables CrCl <69 ml/min remained independently associated with mortality, relative risk 2.12 (95% CI: 1.17-3.84). Although there was a trend towards a higher mortality in patients with
lower CrCl, when patients were divided according to treatment strategy, the adjusted association between CrCl and mortality was not statistically significant (Table 9). Mortality rates were numerically lower in the invasive vs. non-invasive groups across the CrCl tertiles, but significant only at CrCl >90 ml/min. When the interaction term for treatment strategy and CrCl group was added in a logistic regression analysis including both invasively and non-invasively treated patients, the interaction between treatment strategy and CrCl ≤90 ml/min tended to be associated with mortality (p=0.08).

**CrCl at follow-up**
At a follow-up visit 6 months after the index event, levels of CrCl declined significantly in patients, treated both non-invasively and invasively, with CrCl ≥ 69 ml/min whereas no change was noted in patients with CrCl <69 ml/min (Figure 10A and 10B).

**Paper IV**
(Aim: To examine the distributions of cystatin C and NT-proBNP and their relation to age, gender, and other physiological factors in an apparently healthy population similar in age to patients with unstable CAD.)

**General findings**
The baseline characteristics according to gender are given in Table 10. When individuals were stratified according to quartiles of cystatin C, individuals in the highest quartiles were older, had a higher BMI, higher systolic and diastolic blood pressure and levels of CRP and lower CrCl (Table 11). In a multiple linear regression analysis, including all variables significantly associated to the cystatin C level in the univariate analyses (age, BMI, blood pressure, CRP) only three variables remained positively associated to the level of cystatin C; age (p<0.001), BMI (p=0.02) and CRP (p=0.005). When individuals were stratified according to quartiles of NT-proBNP, individuals in the highest quartiles were older, had higher systolic blood pressure, higher levels of CRP, cystatin C and lower CrCl. (Table 12) In multiple linear regression analysis including variables significantly associated to NT-proBNP in univariate analyses (age, systolic blood pressure, cystatin C, CrCl) only age (p<0.001), gender (p<0.001) and CRP (p=0.04) remained significantly associated to NT-proBNP after adjustments. When adjusted for age, women had a 1.44-fold higher median level of NT-proBNP than men (Figure 11). When adjusted for gender, an increase of 10 years of age resulted in a 1.32-fold higher median level of NT-proBNP.
Table 10. Baseline characteristics of control population

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Women (n=146)</th>
<th>Men (n=296)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>65 (59-71)</td>
<td>68 (63-72)</td>
<td>64 (58-70)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoker, n, (%)</td>
<td>63 (14)</td>
<td>16 (11)</td>
<td>47 (16)</td>
<td>0.164</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.74 (1.67-1.80)</td>
<td>1.63 (1.60-1.68)</td>
<td>1.78 (1.73-1.82)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76 (68-84)</td>
<td>68 (61-75)</td>
<td>80 (73-86)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 (23-27)</td>
<td>26 (23-28)</td>
<td>25 (23-27)</td>
<td>0.311</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>140 (130-150)</td>
<td>140 (130-150)</td>
<td>140 (130-150)</td>
<td>0.313</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>80 (75-85)</td>
<td>80 (75-85)</td>
<td>80 (75-85)</td>
<td>0.337</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>1.40 (0.79-2.40)</td>
<td>1.60 (0.87-2.68)</td>
<td>1.30 (0.78-2.20)</td>
<td>0.011</td>
</tr>
<tr>
<td>Creatinine, μmol/l†</td>
<td>91 (83-99)</td>
<td>80 (73-89)</td>
<td>96 (89-103)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CrCl, ml/min†</td>
<td>74 (62-87)</td>
<td>64 (56-74)</td>
<td>78 (66-91)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

‡ Evaluable in 408 / 135 / 273 of the cases.

Table 11. Relation between cystatin C quartiles and baseline characteristics

<table>
<thead>
<tr>
<th>Cystatin C</th>
<th>Cystatin C</th>
<th>Cystatin C</th>
<th>Cystatin C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.75 mg/l</td>
<td>0.75-0.84 mg/l</td>
<td>0.84-0.93 mg/l</td>
<td>≥0.93 mg/l</td>
<td></td>
</tr>
<tr>
<td>n=95</td>
<td>n=105</td>
<td>n=106</td>
<td>n=102</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>62 (56-67)</td>
<td>65 (56-70)</td>
<td>66 (60-71)</td>
<td>67 (64-72)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>33 (35)</td>
<td>31 (30)</td>
<td>33 (31)</td>
<td>38 (37)</td>
</tr>
<tr>
<td>Smoker, n (%)</td>
<td>15 (16)</td>
<td>15 (14)</td>
<td>13 (12)</td>
<td>15 (15)</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.74 (1.66-1.82)</td>
<td>1.74 (1.68-1.78)</td>
<td>1.74 (1.68-1.80)</td>
<td>1.72 (1.64-1.77)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>75 (66-84)</td>
<td>75 (65-81)</td>
<td>77 (71-84)</td>
<td>76 (70-84)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24 (23-26)</td>
<td>25 (23-26)</td>
<td>26 (24-27)</td>
<td>26 (24-28)</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>130 (130-140)</td>
<td>140 (130-150)</td>
<td>140 (130-150)</td>
<td>140 (130-150)</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>80 (75-85)</td>
<td>80 (75-85)</td>
<td>80 (75-85)</td>
<td>85 (80-90)</td>
</tr>
<tr>
<td>NT-proBNP, ng/l‡</td>
<td>65 (41-0.90)</td>
<td>79 (47-123)</td>
<td>72 (44-104)</td>
<td>84 (50-122)</td>
</tr>
<tr>
<td>Creatinine, μmol/l</td>
<td>86 (77-93)</td>
<td>91 (83-98)</td>
<td>92 (82-99)</td>
<td>97 (88-106)</td>
</tr>
<tr>
<td>CrCl, ml/min</td>
<td>80 (70-93)</td>
<td>73 (62-84)</td>
<td>73 (63-86)</td>
<td>65 (57-81)</td>
</tr>
<tr>
<td>CRP, mg/l†</td>
<td>1.00 (0.53-1.63)</td>
<td>1.35 (0.85-2.18)</td>
<td>1.40 (0.78-2.70)</td>
<td>1.80 (1.00-3.43)</td>
</tr>
</tbody>
</table>

If not stated otherwise, the values shown are median-values (25th-75th percentile). Abbreviations see Table 10. * Evaluable in 89 / 101 / 92 / 91 of the cases. † Evaluable in 94 / 104 / 103 / 98 of the cases.
Table 12. Relation between NT-proBNP quartiles and baseline characteristics.

<table>
<thead>
<tr>
<th>NT-proBNP</th>
<th>Age, years</th>
<th>Smoker, n, (%)</th>
<th>Height, m</th>
<th>Weight, kg</th>
<th>BMI, kg/m²</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>P-Creatinine, μmol/l*</th>
<th>CrCl, ml/min*</th>
<th>Cystatin C, mg/l†</th>
<th>CRP, mg/l‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men &lt;41.2 ng/l</td>
<td>61 (56-66)</td>
<td>18 (18)</td>
<td>1.75 (1.66-1.79)</td>
<td>77 (70-83)</td>
<td>22 (23-27)</td>
<td>132 (124-140)</td>
<td>92 (85-99)</td>
<td>68 (55-77)</td>
<td>0.81 (0.72-0.91)</td>
<td>1.10 (0.73-2.00)</td>
<td></td>
</tr>
<tr>
<td>Women &lt;68 ng/l</td>
<td>64 (60-69)</td>
<td>16 (16)</td>
<td>1.74 (1.67-1.80)</td>
<td>75 (66-85)</td>
<td>25 (23-27)</td>
<td>140 (124-150)</td>
<td>92 (83-103)</td>
<td>64 (55-73)</td>
<td>0.94 (0.75-0.94)</td>
<td>1.35 (0.77-2.48)</td>
<td></td>
</tr>
<tr>
<td>Men 41.2-64 ng/l</td>
<td>67 (58-72)</td>
<td>13 (13)</td>
<td>1.74 (1.66-1.80)</td>
<td>77 (68-84)</td>
<td>26 (23-28)</td>
<td>140 (124-150)</td>
<td>92 (85-99)</td>
<td>60 (53-69)</td>
<td>0.83 (0.75-0.92)</td>
<td>0.85 (0.80-1.00)</td>
<td></td>
</tr>
<tr>
<td>Women 68-100ng/l</td>
<td>68 (64-72)</td>
<td>14 (14)</td>
<td>1.74 (1.66-1.80)</td>
<td>77 (67-85)</td>
<td>25 (23-27)</td>
<td>145 (124-158)</td>
<td>91 (84-99)</td>
<td>60 (51-68)</td>
<td>0.85 (0.80-1.00)</td>
<td>0.85 (0.80-1.00)</td>
<td></td>
</tr>
<tr>
<td>Men 65-91 ng/l</td>
<td>68 (64-72)</td>
<td>14 (14)</td>
<td>1.74 (1.66-1.80)</td>
<td>77 (67-85)</td>
<td>25 (23-27)</td>
<td>145 (124-158)</td>
<td>91 (84-99)</td>
<td>60 (51-68)</td>
<td>0.85 (0.80-1.00)</td>
<td>0.85 (0.80-1.00)</td>
<td></td>
</tr>
<tr>
<td>Women 101-158 ng/l</td>
<td>68 (64-72)</td>
<td>14 (14)</td>
<td>1.74 (1.66-1.80)</td>
<td>77 (67-85)</td>
<td>25 (23-27)</td>
<td>145 (124-158)</td>
<td>91 (84-99)</td>
<td>60 (51-68)</td>
<td>0.85 (0.80-1.00)</td>
<td>0.85 (0.80-1.00)</td>
<td></td>
</tr>
<tr>
<td>Men ≥92 ng/l</td>
<td>68 (64-72)</td>
<td>14 (14)</td>
<td>1.74 (1.66-1.80)</td>
<td>77 (67-85)</td>
<td>25 (23-27)</td>
<td>145 (124-158)</td>
<td>91 (84-99)</td>
<td>60 (51-68)</td>
<td>0.85 (0.80-1.00)</td>
<td>0.85 (0.80-1.00)</td>
<td></td>
</tr>
<tr>
<td>Women ≥159 ng/l</td>
<td>68 (64-72)</td>
<td>14 (14)</td>
<td>1.74 (1.66-1.80)</td>
<td>77 (67-85)</td>
<td>25 (23-27)</td>
<td>145 (124-158)</td>
<td>91 (84-99)</td>
<td>60 (51-68)</td>
<td>0.85 (0.80-1.00)</td>
<td>0.85 (0.80-1.00)</td>
<td></td>
</tr>
</tbody>
</table>

If not stated otherwise, the values shown are median-values (25th-75th percentile). Abbreviations see table 10. * Evaluable in 102 / 102 / 102 / 100 of the cases. † Evaluable in 96 / 92 / 98 / 87 of the cases. ‡ Evaluable in 99 / 100 / 101 / 98 of the cases.

Figure 11. Level of NT-proBNP in relation to age and gender. (Black boxes represent males and white boxes females. Box-plot represent median, interquartile range and whiskers 10th and 90th percentiles).
Reference limits

When reference limits for cystatin C were calculated, cases were divided according to age. The calculated URL was 1.12 mg/l (95% CI: 1.08-1.14) for those ≤65 years (n=212), and 1.21 mg/l (95% CI: 1.16-1.24) for those >65 years (n=196). When calculating GFR using cystatin C according to Larsson, the URL’s approximated to the following GFR ranges: 67 ml/min (95% CI 66-70) for those ≤65 years (n=212), and 61 ml/min (95% CI 59-64) for those >65 years (n=196).

When reference limits for NT-proBNP were calculated, cases were stratified by gender and age. Women had a 1.5 fold higher URL than men, whereas those older than 65 years of age had a 1.5 fold higher URL than those below 65 years of age. The upper reference limits for the different age groups, defined as the 97.5th percentile, are listed in Table 13.

| Table 13. Estimates of median and upper 97.5 percentile of NT-proBNP in controls |
|-----------------|-----------------|--------|-----------------|-----------------|
|                | Men             | Women  |                 |                 |
| Age            | N Median q0.975 | 95 % C.I. | N Median q0.975 | 95 % C.I.       |
| 40-65          | 166 54 184 (162 to 206) | 43 79 268 (228 to 314) |
| 66-76          | 112 79 269 (233 to 306) | 86 115 391 (339 to 446) |

Within parentheses are 95 % confidence intervals (C.I.) for the percentiles. N represents the number of subjects.

Discussion

Patients with unstable CAD represent a major healthcare problem. In spite of modern treatment these patients are at high-risk for subsequent cardiac events. This population is however very heterogeneous and clinicians are therefore faced with the task of risk stratification to optimize individual patient care and avoid risk-filled therapies and procedures.

Biological markers are easily available tools, providing clinicians with a window into the diverse pathophysiological mechanisms at work in disease in the individual patient. Measurement of novel biomarkers in the diseased state however, requires knowledge of this marker in the healthy state taking into account variables which may affect levels such as age and gender. Hence the need for a matched control population when evaluating new markers.

In this thesis we have investigated markers reflecting LDL oxidation and renal function both physiological processes associated with accelerated atherosclerosis. Our studies have primarily focused on the clinical usefulness of OxLDL and creatinine in the risk evaluation and management of patients...
with unstable CAD (Paper I, II, III). In our control population we also investi-
gated levels and physiological determinants of OxLDL, and creatinine as well as cystatin C, a novel marker of renal function and NT-proBNP, a novel marker of myocardial function closely associated with renal function (Paper I, Paper IV). Both cystatin C and NTproBNP have recently been shown to provide powerful prognostic information in a broad range of patients with ACS whereas few studies have investigated these markers in healthy elderly individuals.

OxLDL as a diagnostic marker (Paper I)

In Paper I, we compared the diagnostic accuracies of 8 lipid or lipoprotein biomarkers that have been suggested to identify patients with a raised risk and occurrence of CAD. Diagnostic accuracy was determined by measuring the AUC of the ROC curve, which is a common method for quantifying and comparing the accuracy of different diagnostic tests. [63, 64] Findings from this study have demonstrated that OxLDL especially when used in combination with HDL is a better biomarker than standard lipid measurements for discriminating between patients with CAD and healthy subjects. In addition we showed that the levels of another new lipoprotein biomarker Lp-PLA2 provided no information to separate CAD patients from healthy subjects. Furthermore, the levels of TC, LDL, and TGs were found to discriminate poorly between CAD patients and healthy subjects.

There are currently only a handful of studies in which OxLDL and traditional lipid or lipoprotein biomarkers were measured in CAD patients and compared with the levels in control subjects without CAD.[24-26] Although there are substantial differences between these studies and ours with respect to the clinical characteristics of the study populations and the type of assay and monoclonal antibody used, findings support the superiority of OxLDL to other lipid/lipoprotein markers in identifying patients with CAD.

The greater diagnostic accuracy of OxLDL versus native LDL in identifying CAD patients could be due to an increased atherogenicity of OxLDL compared to native LDL. Oxidative modification of LDL greatly enhances the conversion of macrophages in the arterial wall into cholesterol-laden foam cells, which are an essential component of the atherosclerotic plaque. [65] Furthermore, OxLDL has other biological properties that native LDL does not in the promotion of inflammation, thrombosis and stimulation of the immune system.[21] The diagnostic accuracy of OxLDL was further improved with the combined use of HDL. This finding is in line with other studies in which lipid and apolipoprotein ratios that include both an athero-
genic and an antiatherogenic lipid component have been shown to be strongly associated with CAD.[66, 67]

Also, in agreement with other studies, we found that both TC and LDL have poor diagnostic characteristics. [24-26, 68] TC may not accurately dis-
OxLDL as a prognostic marker (Paper II)

In Paper II, we evaluated the usefulness of OxLDL as a prognostic marker in patients with unstable CAD. We found that patients with OxLDL above the median (76 U/L) had a significantly heightened risk of suffering from an MI during the two years following an acute coronary event compared to those with lower levels of OxLDL. The association seemed confined to that of OxLDL and non-procedure related MI’s as no significant association was found between the level of OxLDL and procedure related MI’s. When the level of OxLDL was tested in multivariable analyses that included well-known predictors of adverse outcome, lipid variables and randomized treatments, OxLDL was still an important independent predictor of MI but not mortality. We also found that OxLDL may identify unstable CAD patients at risk for future MI particularly in the absence of myocardial necrosis. Our study also seems to suggest that circulating levels of OxLDL are not elevated in patients suffering from an acute coronary event compared to patients with stable CAD. Furthermore, our results suggest that statin therapy is associated with lower levels of plasma OxLDL.

Findings from our study add to the limited yet growing number of publications in recent years citing the utility of OxLDL as a prognostic marker in individuals both with and without CAD. In a study by Nordin Fredrikson et al., higher levels of OxLDL were reported in 26 cases, who subsequently suffered an acute MI compared to an equal number of controls. [27] In the study of Meisinger et al. increased plasma levels of OxLDL were found to be predictive of future coronary events in apparently healthy middle-aged men. [28] In a large cohort of elderly individuals from the general population, Holvoet et al. demonstrated an association between the OxLDL level and the risk of future MI. [29] In a prospective study on 238 patients with stable CAD, Shimada et al. found the OxLDL level to be independently associated with the risk of subsequent cardiac death, MI, or revascularization. [30] The only study to the contrary is that of Tsimikas et al. in which baseline levels of OxLDL in patients experiencing an ACS were not predictive of risk at 4 months of follow-up. [31] Our study is the first to suggest that OxLDL has a prognostic value in unstable CAD patients in the long-term.
Several mechanisms may explain this increased risk associated with raised levels of OxLDL. Vulnerable plaques which are prone to rupture and lead to clinical events are characterized by extensive macrophage infiltration, an increasing lipid pool and fibrous cap thinning.[71] OxLDL is an important determinant of plaque morphology and may contribute to lesion activation and instability through a plethora of mechanisms that include the enhancement of inflammation, the upregulation of metalloproteinases and thrombus formation. [65, 72-74]

These findings have provoked discussions as to whether OxLDL is a marker of an acute or chronic process. Our study sheds some light on this issue with evidence suggesting that OxLDL as measured by the mAb-4E6 antibody is a marker of a chronic process. We found levels of OxLDL to be similar in patients not treated with statins during an acute event and when stable, 6 weeks after the index event. In line with our findings are also those of Holvoet et al. who, using an antibody similar to ours, found no difference in plasma levels of OxLDL between patients with stable CAD and unstable CAD. [75] Our findings do contrast with those of Anselmi et al., however, in which lower levels of OxLDL were measured in stable compared to unstable CAD patients using the same antibody as ours.[76] In this study however, a comparison was made between different patients whereas we have investigated within patient differences over time. In a single study by Ehara et al. using the FOH1a/DLH3 antibody, levels of OxLDL were higher in ACS compared to stable CAD patients suggesting that this epitope, on the other hand, may be a marker of an acute process.[77]

An important aspect related to this question is the effect of statins on levels of OxLDL. In our study, we found that OxLDL levels were lower in patients treated with statins than in those not receiving treatment. These findings have been confirmed in another larger recent study by Ndrepepa et al. [78]. Statins may reduce levels of OxLDL by reducing levels of LDL. In addition, they also possess antioxidative and anti-inflammatory, and endothelial protective properties, which may play a part in reducing oxidative stress.[79]

When OxLDL was combined with TnT, the greatest prognostic value was noted in patients without evidence of myocardial damage whereas no additional predictive value was demonstrated in the TnT positive group. Our interpretation of this finding is that elevation of TnT in itself is such a strong marker of a raised risk of future MI that it might outweigh any contribution from OxLDL in the TnT positive group. The mechanisms behind the prognostic value of TnT can be attributed to a combination of severe coronary artery stenosis and lesion related thrombosis.[80] In the TnT negative group the OxLDL level may be seen as an indicator of a progressing vulnerable lesion that is unrelated to an ongoing thrombotic process.
Renal function as a prognostic marker (Paper III, IV)

In Paper III, we investigated the prognostic importance of mild to moderate reductions in renal function in patients with unstable CAD. The study population consisted of patients from the FRISC-II interventional trial which was the first study to show that an early invasive treatment strategy reduced mortality and the occurrence of MI in patients with unstable CAD and as such provided a unique opportunity to investigate our question regarding the use of an early invasive strategy in this sub-group of patients. In stratifying patients we estimated CrCl from the Cockcroft-Gault equation which is the most validated and therefore one of the most commonly used prediction equations.

In our study there was an incremental increase in mortality with decreasing CrCl. When adjusted for well-known markers of increased risk, CrCl was still independently associated with mortality. These findings are in accordance with several previous studies and confirm the importance of renal function as an early marker of increased risk.[46-48] Creatinine clearance was also independently associated with the risk of future MI in patients treated non-invasively. This is in line with findings from the GUSTO-IV trial, whereas other studies have failed to demonstrate an independent association between renal function and risk of (re)infarction.[46, 47, 81]

Up until now only one other study has reported on the effects of early revascularization in relation to renal function in patients with unstable CAD in a randomized trial. Using patients with ACS and renal dysfunction included in the TACTICS-TIMI 18 trial, Januzzi et al. found that invasive management was associated with a statistically significant reduction of the primary composite endpoint of death/MI/rehospitalization for ACS and the secondary endpoint of death/MI in both patients with mild to moderate renal dysfunction and those without.[48] However, this trial was not powered to examine the effect on the harder endpoints, death or MI alone, and follow-up was performed for only 6 months.

Our study extends upon these findings by examining the long-term benefit of an early invasive strategy concerning the primary endpoint of death or MI as well as evaluating death and MI as separate endpoints. In this study we demonstrated a reduction in the raised risk of the composite of death and MI seen in patients with CrCl <69 ml/min and 69-90 ml/min while there was no influence in those with CrCl >90 ml/min who also had a lower risk of these events. Thus, an early intervention was most beneficial in patients with mild to moderate renal dysfunction. This interaction between renal function and treatment strategy was more evident when using MI as a separate endpoint. The increased risk of subsequent MI seen in patients with CrCl <69 ml/min and 69-90 ml/min was lowered to about the same level as those with CrCl <90 ml/min by an early invasive strategy. The reduction in mortality by early intervention seen in the main trial was observed to occur both in pa-
tients with normal and reduced renal function, although the number of events was too low to allow any definite conclusions.

Another important finding in this study is the lack of a detrimental effect, at least in the short term, of an invasive procedure on kidney function. Although the rates of contrast-induced nephropathy or acute renal failure associated with surgical revascularization were not documented in this study, we found that there was no difference in kidney function in patients with CrCl <69 ml/min between the invasive and non-invasive groups. Hence, an early invasive treatment strategy can be safely recommended in most cases of patients with renal dysfunction of this magnitude without further compromise.

Up until now only one study has investigated the prognostic significance of cystatin C in patients with unstable CAD. [46] In this study by Jernberg et al., the cystatin C level was shown to be independently associated with mortality. In Paper IV we investigated levels of cystatin C in healthy elderly individuals similar in age and gender distribution to patients with unstable CAD. We found in accordance with other studies that cystatin C was related to age but not gender probably reflecting a physiological decline in renal function. [50-54, 82] When our reference levels (age <65 years, 1.12 mg/L, GFR= 67 mL/min; age>65 years, 1.21 mg/L, GFR=61 ml/min) were applied as cut-off values in Jernberg et al.’s study, the 35 month mortality was 44% in the group with elevated cystatin C compared with 10% (p<0.001) in the group with lower cystatin C levels (sensitivity 64%, specificity 80%) suggesting that this may be a suitable decision limit in this patient population. An additional interesting finding in this study was that cystatin C levels even below our URL were associated with increased mortality. This finding is in line with a recent study in ambulatory elderly men and women in which even mild to moderate levels of renal dysfunction, as assessed by cystatin C but not creatinine, have been associated with an increased risk of cardiovascular disease.[83] This seems to suggest that detection of even mild renal function is important in defining cardiovascular risk and that cystatin C may play an important role here.

The increased risk for cardiovascular events in general in patients with renal dysfunction can partly be explained by an increased frequency of classical risk factors such as old age, hypertension, dyslipidemia, and diabetes. However, in our study renal dysfunction remained prognostically important even after adjustment for all of these risk factors. Several novel risk factors such as homocysteinemia, raised Lp(a), endothelial dysfunction, vascular calcification, platelet aggregation, chronic inflammation and oxidative stress have been identified in patients with renal dysfunction which could contribute to the high incidence of subsequent cardiac events.[84]

In Paper IV we found a novel association between cystatin C and CRP. This finding relates to discussions concerning mechanistic links between kidney function and atherosclerosis, which is associated with increased in-
Inflammatory activity. In addition it has been suggested that cystatin C may be more than a proxy of renal function. One of the biological activities of cystatin C is to inhibit the activity of proteolytic enzymes involved in tissue remodeling which may occur with vascular injury. Our finding on cystatin C has since been corroborated by another larger study in an elderly population.[83] In this study CRP was related to cystatin C but not to creatinine suggesting that either cystatin C is a more sensitive marker of renal function or that this marker represents some other physiological process.

In Paper IV we also studied NT-proBNP a marker of myocardial function which is closely associated with renal function. The interplay between the natriuretic peptides and renal physiology are critical in maintaining hemodynamic balance. In a study by James et al. in patients with unstable CAD, NT-proBNP was shown to be a powerful prognostic marker associated with mortality, especially when used together with kidney function. [47] In this study it was found that levels of NT-proBNP above our estimated URL (290 ng/L) for healthy controls was associated with an increased mortality suggesting that this cut-off level may be useful in risk prediction.

Further studies on the impact of gender on decision limits in risk stratification are though needed as we and others have shown that levels of NT-proBNP are higher in women in all age groups. [85-87] The reasons for this sex related difference are still unclear although an estrogen-mediated activation of the renin-angiotensin-aldosterone system has been proposed. [88] Also, the presence of diastolic dysfunction which is more common in women than men could possibly contribute to this gender difference especially in older women.[89] Another important aspect related to the use of decision limits in risk stratification is the rise of NT-proBNP with increasing age. [85-87] One reason for this is probably a higher prevalence of subclinical cardiac conditions in the elderly. The prevalence of asymptomatic left ventricular dysfunction in the general population is estimated to about 2%, with a higher prevalence in the elderly. [90] It has also been discussed whether clearance of NT-proBNP is affected by age.[91] We investigated therefore the relation of NT-proBNP levels to renal function, as reflected by both creatinine clearance and cystatin-C, and found that in otherwise healthy individuals without overt renal dysfunction, a slight decline seems to have little effect on the NT-proBNP levels. This is important as NT-proBNP unlike BNP is cleared predominantly by the kidneys.
Clinical implications and future directions

Oxidized low-density lipoprotein

One of the cornerstones in both primary and secondary prevention of CAD is the measurement of lipids and lipoproteins. By tradition physicians measure TC, LDL, HDL and TG’s after which lipid-lowering treatment is prescribed. Despite evidence to suggest that there is “room for improvement” in the use of lipid markers in cardiovascular risk prediction and management this tradition continues.[66, 67]

In our studies, OxLDL was found to be superior to traditional lipids and lipoprotein markers in separating healthy individuals from CAD patients. Our findings together with those from recent studies on OxLDL in healthy individuals suggest that OxLDL may be useful as a marker capable of detecting preclinical atherosclerosis.[92, 93] This could have major implications in the early stages of CAD risk management.

Another area of application may be in patients in whom cardiovascular events occur despite “normal to low levels” of LDL such as in diabetes, the metabolic syndrome and renal dysfunction. In these subsets of patients small LDL particles have been implicated in accelerating atherogenesis. The level of LDL in these cases may be misleading. The measurement of OxLDL, which better reflects LDLs atherogenicity, may prove to be useful in this situation. However, future studies comparing OxLDL to apolipoprotein B and apolipoprotein A, for which standardized assays are available, are though needed.

In understanding the pathophysiology of OxLDL in CAD patients we have contributed with an important prognostic study in which we found OxLDL to be an independent predictor in unstable CAD patients. One of the strengths of our study is that our analyses have included major risk factors, including LDL, and other important biomarkers such as troponins, CRP and NT-proBNP which other prognostic studies on OxLDL have not. A most interesting finding from our studies worth additional comment was that OxLDL predicted future MI in patients without positive troponins. This is a patient group which we as cardiologists consider low-risk yet are aware of the fact that some patients within this group do not fare as well. Hence, the use of OxLDL in prognostication in these patients may contribute with significant information. Prospective studies with a large number of events are however needed to validate our findings.
Biochemical markers of renal function

In the acute care of unstable CAD patients one of the most common questions asked at the coronary care unit is “what is the patients level of creatinine” as a raised level, without doubt, negatively affects the decision for referral for cardiac catheterization. Our study sheds new light on this predicament. We have shown that even slight renal dysfunction is associated with a higher risk of subsequent cardiac events such as MI in patients with unstable CAD and that these patients in fact benefit from an early interventional strategy without further compromise of renal function. Given the growing number of elderly patients with kidney disease our findings have important and widespread clinical impact in the management of unstable CAD patients.

Central to this issue is the adequate determination of renal function using a sensitive marker. In our study on healthy controls we investigated cystatin C, a novel marker of renal function which appears to have several advantages over creatinine. This may be especially true in the unstable CAD patient population which consists mainly of elderly, with several co-morbidities in which declining muscle mass may mask increases in serum creatinine due to renal dysfunction. As we have shown cystatin C is related to age but not gender or anthropometric measures like creatinine simplifying therefore the calculation of GFR. However, before there is a general acceptance of this marker as a replacement for creatinine, validation studies are needed. Of further interest are also studies investigating the biological activities of this new marker as it relates to atherosclerosis.

Finally NT-proBNP, a marker physiologically associated with renal function and of contemporary prognostic value in ACS patients, was investigated regarding reference levels in healthy elderly men and women. Our study is one of the first studies performing such an investigation. Findings from our study serve to emphasize the need for stratification of normal levels by age and gender and that mild renal renal dysfunction does not appear to significantly affect levels. Further large studies are needed to establish algorithms for calculating cutoffs of clinical use in risk prediction with regard to these parameters.
Limitations

Paper I
The control population consisted of apparently healthy individuals and the patient population of individuals suffering from unstable CAD. Although this may not be an optimal matching of cases and controls to evaluate the importance of the different biomarkers in identifying CAD patients there are studies that suggest that the levels of these markers are not significantly affected by an acute coronary event. Concerning traditional lipids there is some evidence to suggest that levels of TC, LDL and HDL fall, and levels of TGs rise in the post-infarction period, with maximal changes occurring 4–7 days after infarction.[94] We obtained blood samples within 2 days of an ischemic event, and our results imply that the levels of lipids and lipoproteins at this time give a relatively accurate assessment. Therefore, we also use these values in clinical practice. Little is known, on the other hand about the kinetics of OxLDL during an acute coronary event. However, as discussed above, several findings suggest that the mAb-4E6 antibody detects a marker of a chronic process. In Brilakis et al.’s study no differences were found between the levels of Lp-PLA2 in stable and unstable CAD patients.[34]

An additional limitation of this study is that we have not adjusted for major clinical risk factors. This was due to the design of the two studies and definitions used to classify risk factors. We do however feel that our study findings hold despite this lack of correction as whatever bias may be introduced by the case group having more high-risk factors applies to all of the biomarkers investigated. Our study serves to emphasize the need for a definitive prospective study on these markers.

Paper II
Aside from the limitations discussed for Paper I the major limitation of this study was that it was a small study, exploratory in nature with findings which may be seen as hypothesis generating.
Paper III
In this study patients were divided according to tertiles of creatinine clearance to achieve equally large groups for statistical comparisons. This may be seen as a limitation when there is a question of the clinical application of our results and comparison with other studies. A more adequate stratification may have been to use the NKF staging. Another limitation is the low number of fatal events in the study which does not allow for any conclusions to be drawn concerning the relation of kidney function to mortality alone. Finally, we have not reported the results of other complications relevant to invasive treatment and renal dysfunction such as bleedings as these variables were not available to us during the whole follow-up period. In the FRISC-II trial in general, patients were low-risk for bleeding events as this was a major exclusion criteria.

Paper IV
Although the control population consisted of an apparently healthy elderly population, subjects were not examined with echocardiography and GFR was not determined by any “gold standard” method. Thus, asymptomatic cases of left ventricular or renal dysfunction might not have been excluded especially in elderly subjects. Also our control population did not include individuals with BMI >30 kg/m² which has been shown to affect the levels of NT-proBNP.
Conclusions

Based on the present work, the following conclusions can be made:

- OxLDL and especially the ratio between OxLDL/HDL appear to be a better diagnostic marker of CAD than traditionally measured lipids and Lp-PLA2.
- Raised levels of OxLDL appear to predict the risk of future MI in patients with unstable CAD and especially in those without evidence of myocardial damage.
- Mild to moderate renal dysfunction is independently related to outcome in unstable CAD and these patients derive the largest benefit from early revascularization without further compromise of renal function.
- Reference limits for OxLDL, cystatin C and NT-proBNP were established in a healthy elderly population showing that adjustments for age and gender may be needed when evaluating the diagnostic and prognostic importance of these different markers in diseased populations.
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References

Appendix


(Instructions: In this questionnaire there are 2 different types of questions. Some of the questions are to be answered by encircling the most appropriate answer. Other questions require a short answer.)

Allmän information (General Information)

<table>
<thead>
<tr>
<th>1. Civilstånd:</th>
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<th>fräskild</th>
<th>ensamstående</th>
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<td>(divorced)</td>
<td>(single)</td>
</tr>
<tr>
<td>2. Yrke:</td>
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<td>arbetar, deltid</td>
<td>sjukskriven, heltid</td>
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<td>(part time)</td>
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<td>(sick leave, fulltime)</td>
<td>(sick leave, part time)</td>
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<td>delpensionerad</td>
</tr>
<tr>
<td></td>
<td>(unemployed)</td>
<td>(retired)</td>
<td>(partial retirement)</td>
</tr>
</tbody>
</table>

3. Rökvanor: (smoking habits)

- aldrig rökt (never smoked)
- röker eller har slutat röka senaste månaden (stoppe smoking more than 1 month ago)
- har slutat röka för mer än en månad sedan (curren smoker or stopped smoking less than 1 month ago)

Frågor 4-7 besvaras endast om Du är kvinna. Är Du man fortsätt direkt till fråga 8.

(Questions 4-7 are to be answered only if you are a women, otherwise proceed to question 8.)

4. Antal tidigare graviditeter (number of previous pregnancies)

5. Ålder vid menstruationsdebut (age for start of menstruation)

6. Har Du slutat menstruera? (Have you reached menopause?)

- ja ; när var senaste mensen
- nej (no)
- vet ej (unknown)

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6. Behandlas Du med någon form av östrogenpreparat? (Are you being treated with estrogens?)

- Om ja, vilken typ (if yes which type?)
  - □ plåster (patch) ________________
  - □ tabletter (tablets) ________________

- Om ja, sedan hur länge _______? (if yes since how long?)

---

**Hälsohistoria** *(Health History)*

8. Har Du eller har Du haft ? *Do you have or have you had ?*

<table>
<thead>
<tr>
<th>Condition</th>
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<tr>
<td>diabetes</td>
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<td>□</td>
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<tr>
<td>blodpropp(ben, lunga, hjärna)</td>
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______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

10. Vilken/ vilka sjukdomar har Du som kräver läkarkontroller? *Do you have any diseases which require regular physician check-up?*

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
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Editor: The Dean of the Faculty of Medicine

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