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Importance of peripheral arterial disease as a risk marker in patients with myocardial infarction

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Abstract
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The purpose of this thesis was to describe the true prevalence of widespread arterial disease in a cohort with patients with a recent myocardial infarction (MI) to find valuable clinical methods to detect these patients. Our aim was also to investigate biomarker relationships with peripheral artery disease (PAD) and the importance of PAD in patients' long-term outcomes.

We studied patients with a recent MI in a prospective observational study, the REBUS ((Relevance of Biomarkers for Future Risk of Thromboembolic Events in Unselected Post-myocardial Infarction Patients) trial. A total of 421 patients were included in the study, 390 of whom had their ankle-brachial index (ABI) measured and a mean-time follow up of 5.5 years. Atherosclerotic changes were assessed in three arterial beds by coronary angiography, measuring the ABI and carotid ultrasound. Ninety-two biochemical biomarkers were assessed at baseline by a proximity extension assay (PEA) chip. 263 out of 421 filled in a self-administered Walking Impairment Questionnaire (WIQ). Polyvascular (PvD) disease was defined as pathological findings in all three arterial beds.

We found that PAD and PvD are underdiagnosed in patients who suffered a recent MI. We also found the ABI to be a strong and useful method to identify patients with PAD as well as patients with more widespread arterial disease, such as PvD (paper I).

The results of the scoring system, the WIQ, showed it is useful for finding patients with PAD and PvD, even when completed soon after an acute MI event (paper II).

We also found that biochemical biomarkers associated with the inflammatory pathway – tumour necrosis factor receptor 1 (TNFR-1), tumor necrosis factor receptor 2 (TNFR-2) and growth differentiation factor 15 (GDF-15) – were able to predict pathological ABI, i.e. PAD, in these MI patients. These results could also be validated in another observational study and cohort of MI patients, the VaMIS cohort (paper III). Pathological ABI was also found to be a strong predictor for cardiovascular events of all-cause mortality, new ACS, and a composite endpoint of all-cause mortality, new ACS, new stroke/TIA or new PAD event. When evaluating the three inflammatory biomarkers as a surrogate marker for ABI, they showed a similar association with all-cause death and the composite endpoint (paper IV).

Keywords: Coronary heart disease, Peripheral artery disease, Polyvascular disease, Inflammatory biomarkers, TNFR-1, TNFR-2, GDF-15, Cardiovascular events.

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To my beloved family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Jönelid B, Johnston N, Berglund L, Andrén B, Kragsterman B, Christersson C. (2016) Ankle brachial index most important to identify polyvascular disease in patients with non-ST elevation or ST-elevation myocardial infarction. *European Journal of Internal Medicine* 30:55-60.

II Jönelid B, Kragsterman B, Berglund L, Andrén B, Johnston N, Lindahl B, Oldgren J, Christersson C. (2019) Low Walking Impairment Questionnaire score after a recent myocardial infarction identifies patients with polyvascular disease. *JRSM Cardiovascular Disease* 8:1-9.

III Jönelid B, Christersson C, Hedberg P, Leppert J, Lindahl B, Lindhagen L, Oldgren J, Siegbahn A. Biomarkers in addition to clinical characteristics for prediction of peripheral artery disease in patients with recent myocardial infarction. *Submitted Manuscript*

IV Jönelid B, Lindahl B, Johnston N, Lindhagen L, Oldgren J, Siegbahn A, Christersson C. Biochemical biomarkers associated with peripheral artery disease contribute to prediction of outcome during long-term follow up after myocardial infarction. *Manuscript.*

Contents

Abbreviations

ACS	Acute Coronary Syndrome
CAD	Coronary artery disease
PAD	Peripheral artery disease
PvD	Polyvascular disease
TIA	Transient ischemic attack
NSTEMI	Non-ST-elevation myocardial infarction
STEMI	ST-elevation myocardial infarction
MI	Myocardial infarction
UAP	Unstable angina pectoris
CHF	Congestive heart failure
PCI	Percutaneous coronary intervention
WIQ	Walking Impairment Questionnaire
REBUS	Relevance of Biomarkers for Future Risk of Thromboembolic Events in Unselected Post-myocardial Infarction Patients Study
VaMIS	Västmanland Myocardial Infarction Study
IC	Intermittent claudication
CLI	Critical limb ischemia
BP	Blood pressure
TNFSF	Tumor necrosis factor superfamily
TNF α	Tumor necrosis factor alpha
TNFR-1	Tumor necrosis factor receptor 1
TNFR-2	Tumor necrosis factor receptor 2
GDF-15	Growth differentiation factor 15
CRP	C-reactive protein
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
LDL	Low-density lipoprotein
CI	Confidence interval

Introduction

Atherosclerosis and polyvascular disease

Atherosclerosis is a systemic disease that often occurs in more than one location and should, in the clinical setting, be considered an integral disease.
Polyvascular disease (PvD) or multisite artery disease as referred to in recent guidelines[1, 2] is associated with an increased risk for cardiovascular (CV) events. [3] A limited number of studies support this association in patients suffering from acute coronary syndrome and PvD.[3-11] Most of these studies are based on selected myocardial infarction (MI) populations derived from registries.[4-9, 12] When defining PvD there is a lack of objective criteria and the prevelance of PvD is often based on a combination of a previous history of peripheral artery disease (PAD) combined with stroke and/or coronary artery disease.[4, 5] The true prevalence of PvD is therefore largely unknown, especially given the asymptomatic nature of CV disease prior to clinical manifestation.[12]

Peripheral artery disease

PAD, with manifestation of atherosclerotic disease predominately in the legs, is diagnosed as an ankle-brachial index (ABI) < 0.9 (or >1.4).[13] Symptomatic patients, with intermittent claudication or critical limb ischemia, run an increased risk for CV events. Recent population-based data has demonstrated high risk for those diagnosed with PAD in a hospital setting, with one in six experiencing a major CV event and one in five dying within 1 year.[14] Even the patients with clinically asymptomatic PAD are at high risk of CV events and benefit from most CV prevention strategies, where strict control of risk factors is the cornerstone of care.[1, 2, 15] Areas that remain understudied include the true prevalence of widespread arterial disease, their clinical characteristics and long-term outcome to improve the understanding of widespread arterial disease as a risk factor in the setting of coronary artery disease (CAD).

Background

Atherosclerosis – pathophysiology

Atherosclerosis is a disease in which the inside of the artery narrows due to the buildup of plaque in a complex mechanism and interaction between genetic factors and metabolic mechanisms.[16] The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries, especially at sites of hemodynamic strain, and can lead to ischemia of the heart, brain or extremities.[17]

Atherosclerosis, a lipid-driven immune and inflammatory condition begins in early life with the formation of fatty streaks, a relatively benign manifestation resulting from low-density lipoproteins (LDL) cholesterol infiltrating a dysfunctional endothelium into the vessel wall where it may be retained[18]. As a condition, atherosclerosis develops over the course of several years and results in atherosclerotic lesions, called "atheroma", in the innermost layer of the artery, the intima.[18] Within the fatty streaks, LDL particles begin to oxidize and trigger endothelial cells to express leukocyte adhesion molecules and other chemoattractants on their surface. This stimulates the entrance of monocytes into the atheroma.[19] The monocytes in the atheroma begin to differentiate into macrophages, producing reactive oxygen species, pro-inflammatory cytokines, such as MCP-1 (monocyte chemoattractant protein-1), IL-1β (interleukin 1 beta) and TNF-α (tumor necrosis factor alpha), and these cytokines initiate the inflammatory process followed by further infiltration of additional inflammatory cells.

In the arterial intima, macrophages undergo morphological changes and upregulate scavenger receptors to internalize the modified lipoproteins, thereby forming foam cells. This, in combination with increased smooth muscle cell proliferation, results in thickening of the intima.[18, 20]

T cells are also recruited and activated in the atheroma and, together, the foam cells and T cells produce cytokines, proteases and other inflammatory molecules that promote recruitment of additional macrophages and smooth muscle cells into the intima, causing the atheroma to grow. During this process, a necrotic core within the atheroma begins to form in some, but not all lesions,

while some of the macrophages in the atheroma undergo apoptosis or necrosis.[21] As the necrosis proceeds, its lipid-rich content is released into the tissue, where it incites further inflammation.[20]

The increasing number of smooth muscle cells within the atheroma intensifies the synthesis of collagen, creating a fibrous cap overlying the lipid-rich core of the atheromatous plaque. This cap stands between the blood compartment, with its latent coagulation factors, and the lipid core, and may be filled with thrombogenic material.[18] The activated macrophages also produce proteolytic proteins that leads to the degradation of extracellular matrix proteins and weakens the protective fibrous cap, increasing the risk for plaque rupture.[18, 21] Over time, some fibrous caps develop into thin and vulnerable plaque, a mechanism which is not fully understood, but microbes, autoantigens and various inflammatory molecules activate T cells, macrophages and mast cells, leading to secretion of inflammatory cytokines, e.g. interferon gamma and tumor necrosis factor, proteases (e.g. matrix metalloproteinases), in turn leading to weakening of the protective fibrous cap and reduced stability of the plaque. Fracture of the cap exposes blood coagulant components to tissue factor in the plaque, triggering occlusive thrombus formation that limits blood flow.[16-18] The formation of a necrotic and calcified core, fibrous cap, hemorrhage and micro-thrombi are the characteristics of advanced, symptomatic lesions.

Previous studies have identified the characteristics of plaques that have ruptured and caused fatal MI, which include thin fibrous caps, large lipid cores, and abundant inflammatory cells, as well as spotty calcifications and few smooth muscle cells.[18, 20] The mechanism behind plaque rupture is poorly understood, however, and in some instances plaque rupture is not necessary for thrombus formation and can occur through mechanisms such as plaque erosion.[20]

Acute coronary syndrome (ACS) is nearly always caused by a plaque rupture followed by luminal thrombus, or by a sudden plaque hemorrhage imposed on an atherosclerotic plaque with or without concomitant vasospasm.[20] In ST-elevation myocardial infarction (STEMI), the thrombus is mostly occlusive and sustained, whereas in non-ST-elevation myocardial infarction (NSTEMI) and unstable angina pectoris (UAP), the thrombus is usually incomplete and more dynamic.[20]

Platelets play a central role in the process of thrombosis, their main physiological role being to contribute to primary hemostasis. When endothelial damage or rupture of an atherosclerotic plaque occurs, exposing the innermost core of the atheroma to the blood circulation, a chain of events is triggered, ultimately leading to platelet-rich clot formation.[19] Platelets are activated in four steps: adhesion, activation, secretion and aggregation.[22] The platelets adhere

to the site of the vascular injury and express receptors, such as glycoprotein IIb/IIIa, that crosslink with von Willebrand factor and fibrinogen, causing platelets to bind to each other, and the ruptured plaque. Adhesion of platelets to collagen, von Willebrand factor and other components of the subendothelial matrix activates them, and the platelets change in shape to a spherical form and aggregate.[19, 22] The activated platelets release granules containing cytokines and growth factors, together with thrombin, and contribute to the migration and proliferation of smooth-muscle cells and monocytes.[19] The activation of platelets leads to the formation of free arachidonic acid, which can be transformed into prostaglandin, such as thromboxane A_2, a potent vasoconstricting and platelet-aggregating substance, or into leukotrienes, which can amplify the inflammatory response.[19, 20]

Coronary artery disease

According to the World Health Organization, 15.2 million of 56.9 million deaths worldwide in 2016, and 45% (3.9 million) of deaths in Europe, were caused by cardiovascular disease, i.e. coronary artery disease (CAD) and stroke, which share the common cause of atherosclerosis. Although age-standardized mortality rates for ischemic heart disease have decreased,[23] these diseases have remained the leading causes of death globally in the last 15 years and the number of deaths continues to increase, mainly due to population growth and population aging.[23, 24] Despite improvement in mortality, cerebrovascular disease and CAD also make a very substantial contribution to morbidity,[25, 26] with more than 85 million people living with cardiovascular disease in Europe.

In Sweden, 26,400 patients suffered from an acute MI 2017, whereof 5900 patients died, with an increasing incidence with higher age. Although differences in incidence between men and women have decreased over the years, both morbidity and mortality remains higher in men.[27, 28]

The decline in age-related mortality has been attributed to improved treatment of established coronary heart disease (e.g. revascularization), secondary preventive treatment (e.g. treatment with lipid-lowering statins, heart failure treatment) and control of risk factors (e.g. smoking cessation, lowering blood pressure, increasing physical activity).[29] But despite these actions, there is still a substantial risk for CV events in patients with risk factors and to an even higher degree in patients with established cardiovascular disease, with an increased event rate as the number of arterial disease locations increases.[30]

Myocardial ischemia can most often be identified from a patient's history, from ECG, together with measurement of cardiac biomarkers. Symptoms often include various combinations of chest-, upper extremity-, mandibular- or epigastric discomfort during exercise or at rest.[31] There can also be symptoms of dyspnea or fatigue or, in some patients, no symptoms at all. The onset of myocardial ischemia is the initial step[32] in the development of MI and results from an imbalance between oxygen supply and demand. Coronary artery disease is a collective term for stable angina pectoris and acute coronary syndrome, where the latter is the acute phase of CAD comprising ST-elevation MI, non-ST-elevation MI and unstable angina pectoris. The presence of cardiac symptoms and either abnormal ECG or a dynamic rise in cardiac biomarkers (Troponins) is necessary for ACS diagnosis.[31] ACS is nearly always caused by a luminal thrombus or a sudden hemorrhage imposed on an atherosclerotic plaque with or without concomitant vasospasm.[20]

Carotid artery disease

Atherosclerosis in the carotid arteries can result in an ischemic stroke or a transient ischemic attack (TIA), and accounts for approximately 85% of all stroke cases.[33] The diagnosis of stroke also includes hemorrhagic stroke. In Sweden, in 2017 approximately 26,500 patients suffered from a stroke (all types), whereof 6900 died.[34] As discussed above, together with CAD, stroke is the leading cause of mortality worldwide and, over the past 20 years, there has been a decrease in the total cardiovascular disease burden, as well as in stroke incidence and mortality, with an age-adjusted decrease of almost 34% in the United States[33] and almost 40% in Sweden,[27, 34] although no such decrease in incidence has been seen in developing countries.[35] 75% of patients suffering from stroke in Sweden are over 70 years of age.[34] While stroke is more common in men than in women, in middle-aged and younger age groups, women have a higher lifetime risk of stroke than men.[34, 35] The earliest visible manifestation of carotid artery disease is the thickening of the intima-media, detectable with carotid ultrasonography, and IMT thickness has been shown to have predictive value for the occurrence of CV events.[36] Plaque rupture is the predominant etiology of carotid artery thrombosis and is a major contributor to embolization in ischemic stroke and highly reminiscent of CAD.[37] In contrast to coronary plaques, carotid plaques seem to undergo a slow rupture over many years and often remain asymptomatic.[38] These ulcerations increase the risk of focal microthrombi formation and thrombosis with silent infarction, or slow formation of distal and, later, occlusive thrombi.[38] Another difference between CAD and atherosclerosis in carotid arteries is that large coronary plaques tend to be fibrotic and stable while carotid arteries with severe stenosis are usually unstable.[38] The greatest atherosclerotic plaque accumulation occurs on the outer wall of the proximal segment of the sinus of the internal

carotid artery, in the region of the lowest wall shear stress.[37] The effects of a stroke depend on which part of the brain is injured and how severely it is affected. A very severe stroke can cause sudden death. The most common symptom of a stroke is sudden weakness or numbness of the face, arm or leg, most often on one side of the body. Other symptoms can include confusion, difficulty speaking or understanding speech, dizziness, and loss of balance or coordination.[39]

Peripheral artery disease

PAD is a manifestation of the atherosclerotic disease predominately in the legs.[40] It is estimated that over 200 million people have PAD worldwide, with a spectrum of symptoms ranging from none too severe.[41] Disability and mortality associated with PAD has increased over the past 20 years all over the world, in part due to increased exposure to chronic disease risk factors, and this increase in burden has been greater among women than among men.[12, 40, 42] Non-white ethnicity is a risk factor for PAD, with an over two-fold higher increase in risk for black ethnicity.[40, 43] The relative increase in the burden of PAD in developing regions now exceeds the increase in developed nations.[40, 42]

The burden of PAD is largely age-related, with a prevalence that rises sharply with age, though it can also affect younger adults.[40, 42] The definition of the disease has evolved over time. Earlier the focus was more on intermittent claudication, whereas now, with later studies, there is a greater focus on the widely used ankle-brachial index (ABI) < 0.9 or >1.4,[13, 40] with a specificity of the ABI < 0.9 in the range of 86%, and a somewhat lower sensitivity, of about 75%.[2, 44] ABI, is according to guidelines the first test for screening and diagnosis of PAD and duplex ultrasound is the first imaging method.[1, 2]

PAD is divided into three main categories: asymptomatic PAD; and symptomatic PAD, subdivided into intermittent claudication (IC) and critical limb ischemia (CLI). Most patients are predominantly asymptomatic, but should be carefully assessed to detect clinically masked PAD.[1, 2] The classic symptoms of IC are pain or discomfort in the calves, thighs, hips or feet (depending on where the artery is narrowed) during exercise, sometimes with progressive symptoms.[45] Importantly, many patients with PAD report no symptoms, which may be related to factors such as atypical symptoms (more common in women)[12] or symptoms associated with the patient's comorbidity. When the more acute condition, CLI, occurs, as a result of a severe blockage of an artery, the patient's main symptom is pain in the feet and toes. Patients with this condition may also experience other CLI symptoms, such as coldness, non-heal-

ing wounds on their feet and legs and/or pain or numbness. Symptomatic patients, with IC or CLI, have an increased risk for CV events.[36, 43] Recent population-based data has demonstrated a high risk for people diagnosed with PAD in a hospital setting, with one in six experiencing a major CV event and one in five dying within 1 year.[14] Even the patients with clinically asymptomatic PAD are at high risk of having a CV event, emphasizing the importance of measuring the ABI.[15, 43] In a meta-analysis of 16 population-based studies, a low ABI (≤ 0.90) was associated with approximately two times the 10-year total mortality, cardiovascular mortality and major coronary event rates compared with the overall rates for each of the Framingham Risk Score categories.[15] Population studies also suggest that a high ABI (>1.40), indicating a hardened, often calcified, ankle artery, which could mask underlying PAD, is also associated with a higher mortality.[46] Patients with PAD benefit from most CV preventive strategies and, as for other patients with atherosclerosis in other vascular beds, strict control of risk factors is the cornerstone of care.[1, 15, 46]

Polyvascular disease

PvD, or multisite artery disease, is defined as the presence of atherosclerosis in more than one vascular bed, i.e. PAD, CAD and cerebrovascular disease[1, 2]. PvD appears to pose a graduated risk for cardiovascular events that increases with the number of arterial beds involved, with the worst short- and long-term outcomes in cases where there is involvement in all three vascular beds.[3, 5, 7, 30, 47]

Previous studies show that patients with acute coronary events and PvD have both worse in-hospital and worse long-term outcomes following the acute MI.[3-5] A number of studies support this association in patients suffering from ACS and PvD.[3-11] Most of these studies are based on selected MI populations derived from registries[4-9, 12] and an additional limitation is the lack of objective criteria used to define PvD. The true prevalence of PvD is therefore largely unknown, especially given the asymptomatic nature of cardiovascular disease prior to clinical manifestation.[12]

Sex

Incidence of CAD is still higher in men than in women, although the difference has decreased over time. On average, women develop CAD about 10 years later than men, the reasons for this still being unclear.[48] In Sweden, both the age-standard incidence and mortality is twice as high in men as it is in women.[28] One of the persisting controversies is whether or not women and

men with ACS present with different symptoms. For both groups, chest pain is the most predominant symptom, although women have more atypical symptoms like jaw pain, back pain and nausea.[49, 50] This may be a contributing cause of the delay in seeking medical attention, which is more common among women.[51] While the incidence of ACS and survival post MI has improved over the past decades, there are sex-based differences in outcomes post ACS, with younger women having a higher risk of death, as well as in short-term mortality after STEMI.[50, 52]

Sex differences in the prevalence of PAD are less clear, depending on whether the ABI has been measured or whether prevalence rates are based more on symptoms.[40] ABI-based PAD studies have found a more equal distribution between men and women, in some cases even with a higher prevalence among women,[53] although it seems that intermittent claudication is often more common in men.[40, 53] At the global level, there seems to be an increase in the worldwide burden of PAD, with a greater increase among women than among men.[42] It is well known that PAD can exist without symptoms but, in patients with symptoms, men present more classic IC symptoms, whereas women more frequently describe symptoms of tiredness, unsteadiness and numbness.[40, 54] Women's comorbid diseases may contribute to these symptoms.[55] The association between total mortality, cardiovascular mortality and coronary events seems to be similar in men and women, with a higher risk for an event in patients with lower ABI values and or values ≥ 1.40, especially in women.[12]

Established modifiable risk factors and their management

A number of groundbreaking studies, including the Framingham Heart Study and the INTERHEART study, have identified a number of major risk factors associated with myocardial infarction and the development and progression of cardiovascular disease.[56, 57] Together, these studies have identified nine modifiable risk factors, in addition to age and sex, as major cardiac factors: smoking, high blood cholesterol, high blood pressure, diabetes, abdominal obesity, unhealthy diet, psychosocial factors, lack of physical activity, and high alcohol consumption.[56] In studies of PAD risk factors, it is more difficult to study patients with onset of PAD, defined by ABI, because PAD is often asymptomatic and evidence for risk factors is therefore often evidence of association, with the exceptions of smoking cessation and treatment of dyslipidemia.[40, 43]

Smoking

Smoking is a key risk factor for development of cardiovascular disease and its harmful effects, with a worse prognosis and a graded relation between the number of cigarettes smoked and risk of both CAD and PAD.[40, 43, 56, 58] In patients with PAD the association with smoking may even be stronger than in patients with CAD, and the diagnosis of PAD is generally made about 10 years earlier in smokers than in non-smokers.[59, 43] Smoking cessation seems to have beneficial effects in all patients with varying degrees of CAD and PAD.[60, 61]

Hyperlipidemia

Numerous studies have documented the pathological role of dyslipidemia, especially hypercholesterolemia, in the development of atherosclerosis, CVD and their outcomes, with the main carrier of cholesterol being LDL.[56, 62-64] Reduction of hyperlipidemia has shown to improve outcome in patients with both CAD and PAD,[1, 43, 63, 64] and treatment with lipid-lowering statins is a cornerstone of care in these patients.[1, 65]

Hypertension

Elevated blood pressure (BP) is a risk factor for CAD, PAD and cerebrovascular disease, among other diseases.[56, 62] The risk of death from either CAD or stroke increases progressively and linearly from BP levels as low as 115 mmHg systolic (SBP) and 75 mmHg diastolic upwards,[66] although the curves for absolute risk flatten in the lower BP ranges. The optimal target for BP in hypertensive patients with overt CAD is an issue of discussion. A J-curve phenomenon for achieved SBP < 130 mmHg cannot be excluded,[67] mainly in patients with advanced atherosclerotic diseases and/or frailty, suggesting that an overly aggressive blood pressure-lowering strategy can be negative for the patient.[68] The recent guideline recommendation (2018) is that patients with CAD be treated to an office target of 130/80.[68] The association of hypertension with PAD has been demonstrated in many studies[69-71] and a higher prevalence is seen among older patients, making it a significant contributor to the total burden of PAD in the population.[69, 70]

Diabetes

Diabetes mellitus confers about a two-fold increased risk for CAD[72, 73] and, consequently, it is of great importance to control for diabetes mellitus in prevention of cardiovascular disease. Diabetes is about a third more strongly associated to fatal MI than to non-fatal MI,[73] and patients with previous MI and diabetes mellitus have a poorer prognosis compared to non-diabetics.[74-76] Many studies have also shown an association between diabetes mellitus and

the development of PAD.[40, 77] PAD has shown to be more aggressive in patients with diabetes mellitus compared to non-diabetics, with early large vessel involvement coupled with distal symmetrical neuropathy.[43] The need for a major amputation has been reported to be up to five times higher in diabetic patients, where infections play an important and aggravating role.[40, 43]

Inflammation

There are several biochemical biomarkers used in current clinical practice for risk stratification and for guiding different therapies as the biomarkers add important information, often independently of other risk factors. With increasing evidence that the immune system participates in atherosclerosis,[21, 78] , with inflammation as an emerging risk factor, many biochemical biomarkers are under investigation. At present, there is one biomarker for inflammation in clinical use, C-reactive protein (CRP), which, when measured with a highly sensitive assay (hsCRP), has been able to prospectively predict cardiovascular risk and rises in tandem with many established cardiovascular risk factors.[21, 78, 79] CRP is an acute phase protein predominately produced in the liver. CRP has several advantages as a marker: its stability, its reliability, and the ease with which it can be measured.[78] Examples of other pro-inflammatory biomarkers used include interleukin 6 (IL-6) and the tumor necrosis factor alpha (TNF-α). IL-6 are mainly secreted from immune-competent cells produced in various tissues in response to infection, but also in the atherosclerotic plaques and adipose tissue [80]and stimulates the production of large amounts of acute-phase reactants, including CRP.[17] Levels of CRP and IL-6 are elevated in patients with UAP and MI, where high levels predicting worse prognosis.[17, 80, 81] TNF-α is a prototypic member of the tumor necrosis factor superfamily (TNFSF) and acts through two transmembrane receptors: tumor necrosis factor receptor 1 (TNFR-1) and tumor necrosis factor receptor (TNFR-2). TNFR-1 stimulates apoptosis and is responsible for most cellular responses to TNF-α, including cytotoxicity, cell growth, upregulation of adhesion and cytokine genes,[82, 83] whereas TNFR-2 signaling and consequent biological functions are less well characterized.[82, 84] TNF-α augments the expression of chemokines and adhesion molecules. These are necessary to recruit pro-inflammatory monocytes and induce them to migrate to the vessel intima, stimulate differentiation of monocytes into foam cell macrophages, and increase oxidized LDL uptake by macrophages with increased scavenger receptor expression,[82, 83] processes important in development of atherosclerosis. TNF-α is also associated with cell apoptosis, resulting in vascular damage and vascular calcification with an unstable atherosclerotic plaque phenotype,[83] as well as with findings of bone formation in PAD.[85] In the clinical setting, elevation of TNF-α above baseline is associated with a higher risk of CAD, acute MI and heart

failure, by enhancing inflammation in initially healthy patients, as well as in patients with CAD.[83, 86 87]

Growth differentiation factor 15 (GDF-15) is a protein that belongs to the transforming growth factor beta (TGF-β) cytokine superfamily. The function of GDF-15 is not fully understood, but one of its roles seems to involve the regulation of inflammatory pathways and apoptosis,[88-90] processes observed in cardiovascular disorders. Little is known about the tissues that produce GDF-15 in patients with CV disease, but GDF-15 is expressed in atherosclerotic plaque in the carotid and coronary arteries.[89, 90] Higher levels of GDF-15 have been used to predict CV events and also been associated with cardiovascular comorbidities and is a strong prognostic protein in patients with CAD and their association with future CV events,[91] but also with all-cause mortality and cancer.[89, 92] Several biomarkers have been associated with PAD in population-based studies and with different pathways,[93] although many of these are also elevated in CAD and other vascular disorders. Data supporting an association between inflammation and thrombosis in patients with PAD exists and is robust,[94] but few studies have evaluated biomarkers in patients with both CAD and PAD.[95, 96]

Table 3 Additional predictive value of the biomarkers compared to the performance of the model with clinical characteristics, as measured by the c-statistic.

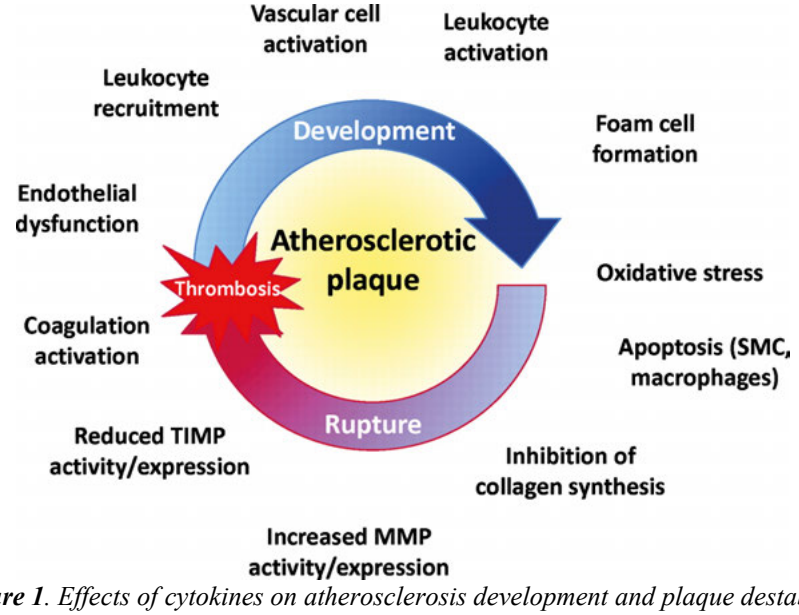


Figure 1. *Effects of cytokines on atherosclerosis development and plaque destabilization. SMC=smooth muscle cell, MMP=matrix metallopeptidase, TIMP=tissue inhibitor of metalloproteinase*

Aims

The overall aim of this thesis was to describe the true prevalence of widespread arterial disease in a cohort with patients with a recent MI and try to find valuable clinical methods to detect these patients.

Our aim was also to investigate biomarker relationships with PAD and the importance of PAD in patients' long-term outcomes, in order to improve cardiovascular risk stratification and our understanding of patients with ACS and PAD.

The **main aims** of the individual papers of the thesis were to answer the following questions:

I Is prevalence of PvD and PAD higher in an unselected MI population than previously known? (Paper I)

II Is ABI a useful clinical tool to identify patients with PvD in patients with recent MI? (Paper I)

III Can WIQ be used to identify patients with PvD and to predict future CV events in patients with recent MI? (Paper II)

IV Which specific biomarkers are associated with the occurrence of PAD in a cohort of patients with a recent MI? And can those biomarkers be used, in addition to clinical characteristics, to identify PAD in patients with recent MI? (Paper III)

V Are pathological ABI and biomarkers analyzed early after an MI associated with mortality and the risk of new cardiovascular events during long-term follow-up? (Paper IV)

Methods

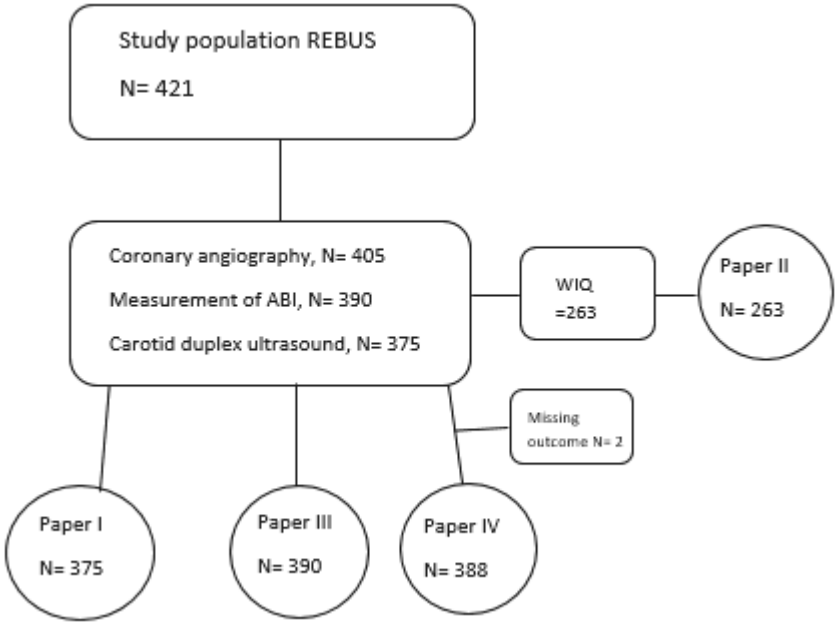


Figure 2 *My cohorts in this thesis*

Study population

The REBUS study

The REBUS (Relevance of Biomarkers for Future Risk of Thromboembolic Events in Unselected Post-myocardial Infarction Patients) study was a prospective observational study of patients with a recent MI (NCT01102933, ClinicalTrials.gov).[97] Patients with NSTEMI or STEMI admitted to the acute coronary care unit at the Cardiology Department at Uppsala University Hospital from 2010-2012 were included. The inclusion criterion was MI diagnosed based on the universal definition of biomarker increase or decrease along with either symptoms of cardiac ischemia or ECG changes, including

new left bundle branch block[15] The only exclusion criteria were death within 5 days of MI or inability to participate in follow-up visits. The patients were to be included 3-5 days after the index MI, before discharge from the hospital, and then followed serially until the last visit at 2 years. The first visit was performed at 2-3 weeks after inclusion in the study. Information regarding medical treatment was collected at each visit. All participants signed an informed consent form, and the study was approved by the local ethics committee and conducted in accordance with the ethical principles of the Declaration of Helsinki (Dnr 2009/210). A total of 421 patients were included in the study, 390 of whom had their ankle-brachial index (ABI) measured.

The VaMIS study (Paper III)

The cohort for validation of the biomarker data in the REBUS study was 766 of 1008 participants in the VaMIS (Västmanland Myocardial Infarction Study).[98] All patients ≥ 18 years of age diagnosed with an acute MI and admitted to the coronary care unit of Västmanland County Hospital, Västerås, Sweden, were eligible for inclusion. MI was diagnosed according to the ECG criteria and using a troponin I level as the biomarker criterion.[31] The patient's medical history and lifestyle were assessed through a standard patient questionnaire during the index hospitalization. Self-reported data was confirmed from medical records. The study complied with the Declaration of Helsinki and was approved by the local ethics committee (Dnr 2005/169).

Evaluation and definition of atherosclerosis

Categorization of atherosclerosis in the vascular beds was determined according to the following:

I Coronary angiogram was performed during hospitalization for the index MI when clinically indicated. The patients were categorized into two groups: 1) normal findings (0-29%), or 2) abnormal findings including all stenosis (>30%) or occlusions.

The only difference between the REBUS and the VaMIS cohort was in classification of coronary findings, were irregularities between 0-29% was classified as normal in the REBUS cohort, and between 0-20% in the VaMIS cohort.

II The carotid arteries were examined with duplex ultrasonography 3 months after the index MI. The intima-media thickness was measured using the leading edge to leading edge method in a region free of overt plaque in the far wall of the common carotid artery, approximately 1 cm proximal to the carotid bulb. Color duplex and flow velocities were

used to grade stenosis. The patients were divided into two groups after examination: 1) normal findings, and 2) abnormal findings, including all atherosclerotic lesions (plaques, stenosis or occlusions) and patients with a previous history of carotid endarterectomy.[16]

III PAD was evaluated in all patients by measuring the ABI at rest, first 2–3 weeks after the index event and then again after 24 months. PAD was defined as an abnormal ABI score (<0.9 or >1.4) on at least one leg.

Polyvascular disease (Paper I and II)

In this cohort, PvD was defined as dysfunction of all three examined arterial beds (i.e. coronary, carotid and lower extremity). The arterial bed was considered dysfunctional if there were abnormal findings in the coronary arteries, carotid arteries or ABI, as described above.

Walking Impairment Questionnaire (Paper II)

WIQ was developed as a self-administered tool to assess patients with PAD and by measuring their self-perceived ability with respect to walking distance, walking speed and ability to climb stairs in the outpatient setting. WIQ is a validated correlate of objective walking ability[99-101] and is associated with the risk of future CV events,[102-105] but there is no published study that evaluating WIQ in a population with a recent MI.

In this study we used a revised version of the WIQ, adapted and validated for the metric system (see below), and translated to Swedish.[54, 101] (Figure 3). Participants were given the WIQ forms at the follow-up visit 2-3 weeks after inclusion, and completed the forms in privacy at the hospital.

WIQ scores contain three domains that measure important factors of walking impairment: walking distance, walking speed and the ability to climb stairs. Each subdomain is scored on a scale of 0 (worst/inability) to 4 (best/without limitations): *walking distance* reflects the patient's perceived difficulty to walk a specific distance, ranging from 15 to 500 meters (or approx. 5 blocks), in the last week; *walking speed* is the patient's assessment of his/her degree of difficulty to walk a block at specific speeds (from a slow walk to jogging speed); and the *stair climbing* score is the patient's reported difficulty to climb a specific number of flights of stairs (1 to 3).

Individual scores are calculated stepwise: First, the graded scale is multiplied by a pre-specified weight for each question. Second, the products are summed

up and then divided by the maximum possible score, ranging from 0 (the patient is unable to perform any of the tasks) to a maximum of 100 for all of the questions (the patient reports no limitations). In our study, the resulting individual scores were then divided into quartiles and our main analysis involved comparing the lowest (worst) quartile vs. the highest (best) quartile.

1. Distance subscale of the WIQ

	No difficulty	Slight difficulty	Some difficulty	Much difficulty	Did not do
A.Walk 15 meters					
B. Walk 50 meters? (½ block)					
C. Walk 100 meters? (1 block)					
D. Walk 200 meters? (2 blocks)					
E. Walk 300 meters? (3 blocks)					
F. Walk 500 meters? (5 blocks)					

2. Speed subscale of the WIQ

	No difficulty	Slight difficulty	Some difficulty	Much difficulty	Did not do
A.Walk 1 block slowly?					
B. Walk 1 block at average speed?					
C. Walk 1 block quickly?					
D. Run or jog 1 block					

3. Stair subscale of the WIQ

	No difficulty	Slight difficulty	Some difficulty	Much difficulty	Did not do
A.Climb 1 flights of stairs					
B. Climb 2 flight of stairs					
C. Climb 3 flight of stairs					

Figure 3 *The Walking Impairment Questionnaire*

Outcome events REBUS (Paper II and IV)

Paper II

The endpoint consisted of a composite endpoint with all- cause mortality, new MI, new stroke or congestive heart failure after two-year follow up comparing the patients scoring the lowest and highest WIQ scores. The definition used for new MI was the same as for the index MI. Stroke was diagnosed as an abrupt onset of focal neurological deficit persisting more than 24 hours and assessed by computed tomography or magnetic resonance imaging scan. Congestive heart failure was defined as hospitalization due to symptoms suggestive of heart failure, verified by positive findings on lung x-ray, echocardiography or increased levels of N-terminal pro b-type natriuretic peptide (NT-pro BNP).

Paper IV

Information from the National Patient Register (NPR), which includes the diagnosis codes of all hospital admissions in Sweden since 1987, was used to identify outcome events during follow-up.[106] Data linkage was based on the patient's unique 10-digit personal identification number assigned to all Swedish residents at birth or immigration. All patients were followed up by computerized linkage of the database with the most recent national census registry, the Swedish Cause of Death Registry and the NPR, all managed by the National Board of Health and Welfare. The start date was the day of inclusion in the study. The study cohort was followed until death or the end of follow-up (31 December 2017), whichever occurred first. The outcome events were: all-cause mortality, new hospitalization for acute coronary syndrome (ACS), or a composite outcome event including all-cause mortality, new hospitalization for ACS, ischemic stroke/TIA and PAD. The International Classification of Diseases, Tenth Revision (ICD-10) was applied to identify outcome events.

Table 1 ICD codes

Outcome Event	**ICD-10 Code**
Acute coronary syndrome (ACS)	I20.0, I21, I22
Ischemic stroke and transient ischemic attack (TIA)	I63, I64, G45
Peripheral artery disease (PAD)	I70, I71, I72, I73

Blood sampling

REBUS: Blood samples were collected in EDTA plasma tubes at inclusion of the study 3-5 days after the index MI. The tubes were then centrifuged at 2000 g for 10 minutes and the plasma stored within 2 hours at -80°C until further analysis.

VaMIS: Blood samples were collected in 5 ml lithium heparin-coated vacuum tubes at admission to the hospital. The tubes were then centrifuged at 2000 g for 10 minutes, and the plasma frozen and stored at -70°C within 2 hours until further analysis.

Proteomics (Paper III-IV)

In the REBUS cohort, measurement of protein biomarkers in plasma was performed using the Proseek Multiplex CVD III $^{96\times96}$ proximity extension assay, and in VaMIS the Proseek Multiplex CVD I $^{96\times96}$ kit was used (both from Olink Bioscience, Uppsala, Sweden; www.olink.com/products/cvd-i and iii-panel). Both assays have been described earlier,[107-109] by the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, Uppsala, Sweden. Together, the CVD III and CVD I assays measure 184 proteins related to cardiovascular disease, though there is some overlap between the two panels (for the exact content of each panel, see the Olink Bioscience website given above). The proximity extension assay (PEA) technology uses pairs of antibodies that are equipped with DNA reporter molecules. Target binding of both antibodies in pairs generates double-stranded DNA amplicons, which are quantified using a Fluidigm Biomark HD real-time PCR platform. The PEA technique has a major advantage in that only correctly matched antibody pairs

give rise to a signal, yielding an exceptionally high specificity and sensitivity.[110, 111] This platform provides normalized protein expression data, where a high protein value corresponds to a high protein concentration, but not an absolute quantification.

Statistical methods

In all four papers, the data are presented as means and standard deviations for continuous variables and as numbers and percentages for categorical variables.

In Paper I, univariate and adjusted associations between outcomes and risk factors were assessed with logistic regression models. Results from the logistic regression models are presented as estimated odds ratios with 95% confidence intervals (CIs) and p-values per 1 standard deviation (SD) increase for continuous risk factors, and by comparison to a reference category for categorical variables. To evaluate associations between atherosclerosis in three arterial beds and the locations (coronary arteries, abnormal common carotid artery and ABI), specificity (i.e. the rate of patients with normal values in a location in patients with atherosclerosis with involvement of <3 beds) and positive predictive values (i.e. the rate of patients with atherosclerosis in 3 arterial beds in patients with abnormal values in a location) were calculated and 95% CIs were assessed with a normal approximation. In this descriptive paper, no adjustment was made for multiple tests.

In Paper II, scores for each WIQ subdomain were determined and divided into quartiles. Given the large number of patients with a WIQ distance score of 100, we grouped participants scoring in both the third and fourth quartiles into one group (third/fourth quartile). The correlations between all three WIQ subscales were examined with the Spearman rank correlation coefficient. A linear regression model was used for comparisons of age and BMI at baseline between WIQ quartiles. For other baseline variables Fisher's Exact Test was used for comparison of characteristics between WIQ quartiles.

Univariate associations and adjusted associations between atherosclerotic burden and WIQ score quartiles were assessed with logistic regression models. In Model 1, the adjusted variables was age and gender and, in Model 2 – age, gender, congestive heart failure, atrial fibrillation and diabetes mellitus. Results from logistic regression models are presented as estimated odds ratio, comparing the lowest quartile to the highest, with 95% CI and p-values.

Proportional hazards Cox regression models were used to compare differences in rates of the CV composite endpoint, occurring between 2-3 weeks after the

index MI and 2-year follow-up, across WIQ score quartiles. Results from Cox regression models are presented as estimated hazard ratios, comparing the lowest quartile to the highest, with 95% CIs and p-values.

In Paper III, for the primary analysis, the REBUS study population was used as the discovery sample, and the VaMIS cohort was used only for the validation analysis. A total of 92 biomarkers (CVD III panel) were included in the statistical analysis. The very few missing values (two for elafin and cathepsin D) were filled by simple imputation[112] using chained equations, with age, gender and all biomarkers as predictors. Biomarkers were tested for univariate association with PAD using Mann-Whitney tests, correcting for multiplicity using permutation[113] methods.

In the prediction model, we used all biomarkers simultaneously, to identify patients with PAD. Considering that there are few patients for the number of predictors, standard logistic regression is likely to lead to severe overfitting. We therefore used random forest instead, a flexible machine learning technique that builds prediction models by averaging over a large number of classification trees, built by recursive binary splitting of the biomarkers. Heterogeneity between trees is induced by a combination of bootstrap and random selection of biomarker split candidates.[114] The random forest also gives a variable importance plot, ranking the predictors according to how valuable they have been in predicting the outcome, i.e. patients with PAD. As a performance measure of the random forest, we computed the C-statistics. From the plot of variable importance, a number of biomarkers with a higher discriminatory accuracy (i.e. the ones nearest 100%) were chosen and validated in the VaMIS cohort. In CVD I (VaMIS cohort), the values of the biomarkers were standardized within the Olink panels. The purpose of the validation was to assess whether the predictive ability, adding the selected biomarker to the clinical characteristics, as measured by the C-statistic, showed a similar increase in both cohorts. In the validation step, three levels of adjustment were calculated using logistic regression: Model I included age, sex, smoking, hypertension, diabetes mellitus as clinical characteristics; Model II included the above-mentioned characteristics adding a biomarker one by one; and Model III included the risk factors and the group of three biomarkers (TNFR-1, TNFR-2 and GDF-15). Odds ratios for clinical characteristics from these validation models were compared to assess potential differences between the two cohorts.

In Paper IV continuous variables were compared between patients with and without pathological ABI, with the Mann-Whitney test with 95 % confidence intervals. For other baseline demographic and clinical characteristics variables and outcome events, Pearson Chi-Square test was used for comparison.

Time to outcomes of mortality or ACS was measured from the date of inclusion and for the composite outcome events (all-cause mortality, ACS, ischemic stroke/TIA or PAD) time to the occurrence of the first event to the end of FU (31 December 2017).

The biomarkers; TNFR-1, TNFR-2 and GDF-15, analyzed early after the MI, were used as a group to identify patients with the highest probability of having a pathological ABI. The biomarker levels were log-transformed to reduce skewness before regression analysis. Patients were classified as "probable pathological ABI" or not, based on these three biomarkers, using a logistic regression model with actual pathological ABI as outcome and the biomarkers as predictors. Since 20 % of the cohort had pathological ABI[115], the 77 patients with the highest probability of pathological ABI based on this model (20 % of the study cohort, comparable with the proportion of pathological ABI) were classified as probable pathological ABI, and compares to the other group. Since pathological ABI was associated with elevated values of the biomarkers, we shall refer to this group as the group with high biomarker values.

All-cause mortality in patients with and without pathological ABI and patients with high compared to low values of the group of biomarkers were compared using Kaplan-Meier survival curves.

Cox regression models were fitted for each outcome; all-cause mortality, new ACS and the for the composite outcome, in patients with and without pathological ABI, as univariate models and with adjustment for established CV risk factors measured at baseline including: age, sex, smoking (smoker and non-smoker), revascularization (PCI or CABG), diabetes mellitus, hypertension, renal failure, previous MI, ischemic stroke and PAD. In the Cox regression analysis for the group of biomarkers, patients with the highest biomarker value were compared to the lower values with a univariate model as well as in adjusted model with established CV risk factors listed above. To evaluate whether the pathological ABI and the group of biomarkers added prognostic information above the established CV risk factors, Receiver operating characteristics (ROC) curves were constructed. The area under the ROC curve (AUC) was studied for pathological ABI and the group of biomarkers with and without established CV risk factors.

In all of the papers, all statistical tests and CIs were two-sided, and statistical significance was declared at $p < 0.05$. In papers I and II the statistical analyses were performed with the SAS 9.4 program package (SAS Institute, Inc, Cary, NC), and in papers II and IV with SPSS Statistics software. In Paper III, the statistical analyses were performed in R, cf. Section 13.

Results

Table 2 Baseline characteristics for the REBUS cohort

N (%)	**Total** (N=390)	**Pathological ABI** (N=78)	**Normal ABI** (N=312)	**p-value***
Age, mean (SD)	66.9 (10.2)	71.1 (11.0)	65.9 (9.8)	<0.001
Sex, male female	303 (77.7) 87 (22.3)	53 (67.9) 25 (32.1)	250 (80.1) 62 (19.9)	0.019
BMI	27.3 (4.1)	27.2 (4.3)	27.3 (4.0)	0.407
Smoking, yes	102 (26.3)	20 (25.6)	82 (26.4)	0.884
NSTEMI	205 (52.6)	44 (56.4)	161 (51.6)	0.479
STEMI	185 (47.4)	34 (43.6)	151 (48.4)	
Revascularization PCI CABG	 312 (80.4) 5 (1.2)	 60 (76.9) 1 (1.3)	 252 (81.2) 4 (1.3)	 0.616
3-vessel CAD	109 (28.1)	30 (38.5)	79 (25.5)	0.007
Diabetes mellitus	61 (15.6)	18 (23.1)	43 (11.1)	0.046
Hypertension	209 (53.6)	50 (64.1)	159 (51.0)	0.038
Renal failure	16 (4.1)	8 (10.3)	8 (2.6)	0.002
Previous MI	72 (18.6)	16 (20.5)	56 (18.1)	0.619
Previous stroke	17 (4.4)	7 (9.0)	10 (3.2)	0.027
Previous PAD	9 (2.3)	7 (9.0)	2 (0.6)	<0.001
Medical treatment at discharge				
ASA	382 (98.5)	77 (98.7)	305 (98.4)	0.832
Statins	367 (94.6)	71 (91.0)	296 (95.5)	0.120
Beta-blockers	361 (93.0)	73 (93.6)	288 (92.9)	0.831
ACEI or ARB	318 (81.6)	65 (83.3)	253 (81.6)	0.428
Oral anticoagulants	20 (5.2)	2 (2.6)	18 (5.8)	0.247

*p-value comparing patients with and without pathological ABI.

NSTEMI – Non-ST-elevation myocardial infarction, STEMI – ST-elevation myocardial infarction, PCI - Percutaneous coronary intervention, CABG – Coronary artery bypass surgery, MI – myocardial infarction, , ASA- acetylsalicylic acid, ACEI – angiotensin converting enzyme inhibitor, ARB – angiotensin receptor blocker

Paper I – Peripheral artery disease and myocardial infarction

The total study population consisted of 375 patients with recent MI, whereof 52.5% (n=197) with NSTEMI and 47.5% (n=178) with STEMI.

Atherosclerosis in one coronary vessel (i.e. non-significant/significant stenosis) was more common in the STEMI group than in the NSTEMI group (47.9% vs. 34.6%, $p<0.001$), whereas no significant difference was noted, between NSTEMI and STEMI patients in the involvement all three coronary arteries after adjustment (adjusted $p=0.0588$). In the multivariate analyses, only age was associated with three-vessel disease (Table 3).

In total, 56.9% of the NSTEMI patients and 52.8% of the STEMI patients had atherosclerotic changes in their carotid arteries. Of these patients, 8.8% of NSTEMI vs. 4.9% of STEMI patients had a stenosis with blood flow disturbance. Multivariate analyses indicated that atherosclerosis in the carotid arteries was associated with age and hypertension (Table 3).

Abnormal ABI was seen in 21.8% of the NSTEMI patients and 18.5% of the STEMI patients. In multivariate analyses, age, renal failure and previous PAD were associated with abnormal ABI (Table 3). Of the patients with NSTEMI, 16.2% had atherosclerosis in all three arterial beds, compared to 11.2% of the STEMI patients. In the multivariate analyses, age, diabetes mellitus and female gender were independently associated with atherosclerosis in three arterial beds (Figure 4).

The most important parameter for identifying patients with atherosclerosis in three arterial beds in this data, in comparison with coronary angiography and duplex ultrasound was ABI – with a positive predictive value (PPV) of 68.4% (95% CI, 57.7–79.2%) and specificity of 92.4% (95% CI, 89.5–92.4%).

Table 3 Multivariate analyses with clinical characteristics associated with widespread atherosclerosis

Outcome	Risk factor[1]	OR (CI)	*p-value
Coronary arteries, 3-vessel disease vs. < 3 vessels	Age[2]	1.22 (1.06–1.40)	0.0058
	Female gender	0.65 (0.34–1.24)	0.196
	Smoking	0.67 (0.37–1.23)	0.202
	Diabetes	1.73 (0.89–3.38)	0.108
	Hypertension	1.11 (0.65–1.88)	0.696
	Previous MI	0.77 (0.40–1.49)	0.447
	Previous stroke	2.42 (0.69–8.47)	0.165
	Previous PAD	0.20 (0.02–1.87)	0.159
	Renal failure	2.62 (0.72 –9.50)	0.143
Carotid arteries, abnormal	Age[2]	1.32 (1.15–1.52)	<.0001
	Female gender	1.74 (0.95–3.19)	0.070
	Smoking	0.89 (0.51 –1.53)	0.669
	Diabetes	1.37 (0.70–2.71)	0.359
	Hypertension	2.30 (1.41–3.76)	0.0008
	Previous MI	1.26 (0.67 –2.36)	0.478
	Previous stroke	8.55 (1.04 –70.62)	0.050
	Previous PAD	0.59 (0.10 –3.56)	0.563
	Renal failure	2.01 (0.49 –8.23)	0.331
PAD, abnormal ABI	Age[2]	1.26 (1.07–1.48)	0.005
	Female gender	1.51 (0.78–2.90)	0.220
	Smoking	1.48 (0.75–2.92)	0.252
	Diabetes	1.65 (0.81–3.75)	0.169
	Hypertension	1.35 (0.73–2.52)	0.339
	Previous MI	0.79 (0.38–1.65)	0.528
	Previous stroke	2.26 (0.64–7.97)	0.206
	Previous PAD	6.95 (1.25–38.71)	0.027
	Renal failure	3.17 (1.02–9.87)	0.046

[1]Risk factor: Yes vs. No.

[2]Age, OR per 5-year increase

MI=Myocardial infarction. PAD=Peripheral artery disease. OR=Odds ratio. CI=Confidence interval.

*p-value for comparison between abnormal and normal findings in arterial beds.

Adjusted for age, gender, smoking, waist circumference, BMI, type 2 diabetes mellitus, previous MI, previous stroke, hypertension, atrial fibrillation, peripheral arterial occlusive disease, congestive heart failure, and renal failure

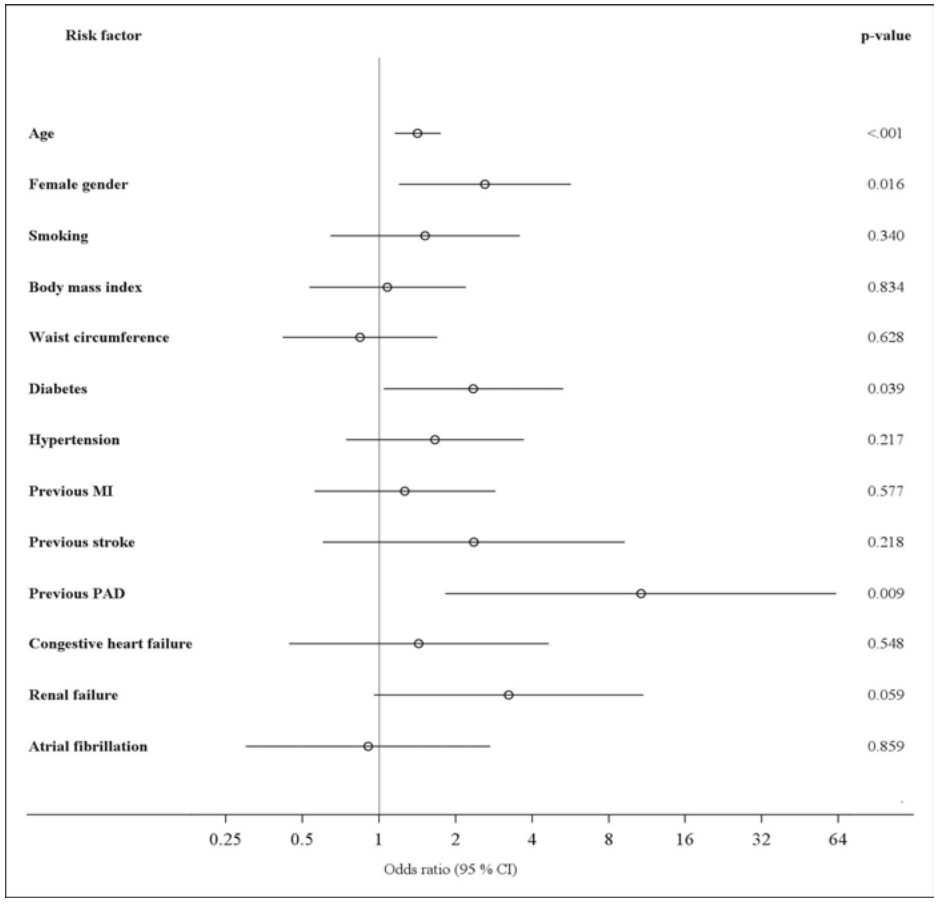


Figure 4 *Multivariate analysis of clinical characteristics associated with polyvascular disease*

OR=Odds ratio. CI=Confidence interval.

MI=Myocardial infarction. PAD=peripheral artery disease.

[1]Risk factor: Yes vs. No.

[2]Age, OR per 1 SD increase.

*p-value for comparison between risk factor and risk for atherosclerotic involvement in three arterial beds.

Adjusted for age, gender, smoking, waist circumference, BMI, type 2 diabetes mellitus, previous MI, previous stroke, hypertension, atrial fibrillation, peripheral arterial occlusive disease, congestive heart failure, and renal failure

Paper II – Associations between WIQ score and patients with PAD and their outcomes.

The study included 263 of 421 patients in the REBUS study who had completed the WIQ questionnaire soon after the index MI, on visit 1 after 2-3 weeks, and who had undergone evaluation of all arterial beds: coronary, carotid and peripheral arteries.

Atherosclerotic burden and WIQ score

257 (97.7%) of the 263 patients had an abnormal coronary angiogram, 52 (19.8%) had an abnormal ABI, and 136 (51.7%) had an abnormal carotid duplex. PvD, with three affected arterial beds, was found in 34 (12.9%) patients. The proportion of patients with abnormal ABI and PvD was higher in the lowest quartile for all WIQ score categories (Figure 5A-B).

Distance score*:* In patients with abnormal ABI, there was an association between the lowest scoring quartile relative to the highest quartile, after adjustment, OR 3.9 (95% CI, 1.6-9.2, p=0.002) (Figure 6A). A similar result was found in patients with PvD, with an association between those scoring in the lowest quartile relative to highest score after adjustment, OR 5.4 (95% CI, 1.8-16.1, p=0.002) (Figure 6B).

Speed score: The lowest quartile was associated with abnormal ABI relative to the highest quartile after adjustment OR 3.2 (95% CI, 1.2-8.6, p=0.022) (Figure 6A). In patients with PvD, the association with the lowest quartile relative to the highest remained after adjustment OR 7.4 (95% CI, 1.5-36.5, p=0.015) (Figure 6B).

Stair-climbing score: In contrast to the scores for distance and speed, for stair climbing the association between abnormal ABI and the lowest quartile relative to highest quartile did not persist after full adjustment (Figure 6A). A similar result was found in patients with PvD (Figure 6B).

WIQ score and risk of cardiovascular events

43 (16.3%) of 263 patients reached a composite cardiovascular endpoint during the 2-year follow-up. 6 (2.3%) of these patients died, 21 (8.0%) had a new MI, 17 (6.5%) were hospitalized for CHF, and 6 (2.3%) had a stroke.

Distance (Figure 7*)*: Patients scoring in the lowest quartile for walking distance had a higher risk of the composite cardiovascular endpoint compared to those with the highest scores after adjustment for age and sex, HR 3.0 (95%

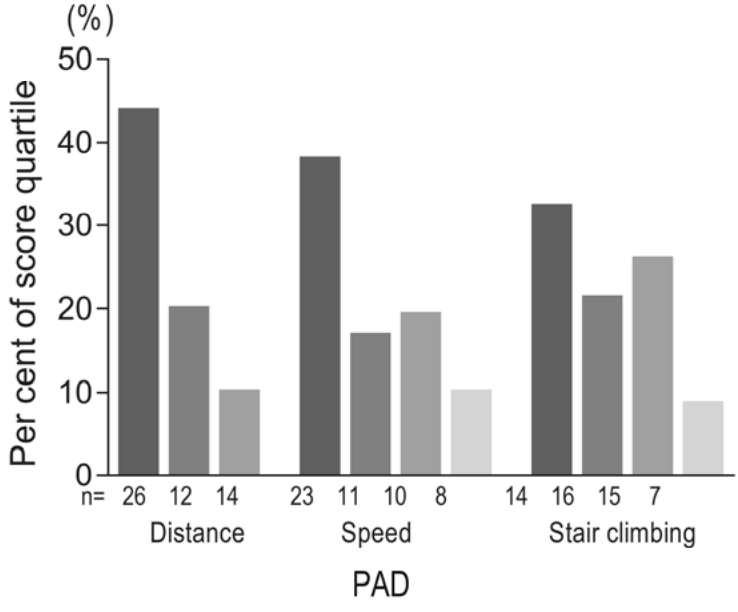
CI, 1.4-6.4, p=0.005), but the association did not remain significant in the fully adjusted model.

Speed (Figure 7): After adjustment for age and sex, there was an association in patients scoring in the lowest quartile for walking speed relative to those with the highest scores and the composite endpoint, HR 2.5 (95% CI, 1.1-5.8, p=0.039). The association was attenuated in the fully adjusted model.

Stair climbing (Figure 7): Patients scoring in the lowest quartile for stair climbing had a higher risk for the composite cardiovascular endpoint compared to those with the highest scores in all models, with an HR of 5.3 (95% CI, 1.5-19.0, p=0.011) in the fully adjusted model .

Medical treatment and WIQ score

A high proportion of the patients adhered to guideline-recommended medical treatment for secondary prevention. At visit 1, 99.2% were treated with antiplatelet drugs, 92.8% with statins, 91.6% with beta blockers, and 81.7% with angiotensin converter enzyme inhibitor (ACEI) or angiotensin II receptor blocker (ARB). The adherence to medical treatment persisted during follow-up and at 2 years.



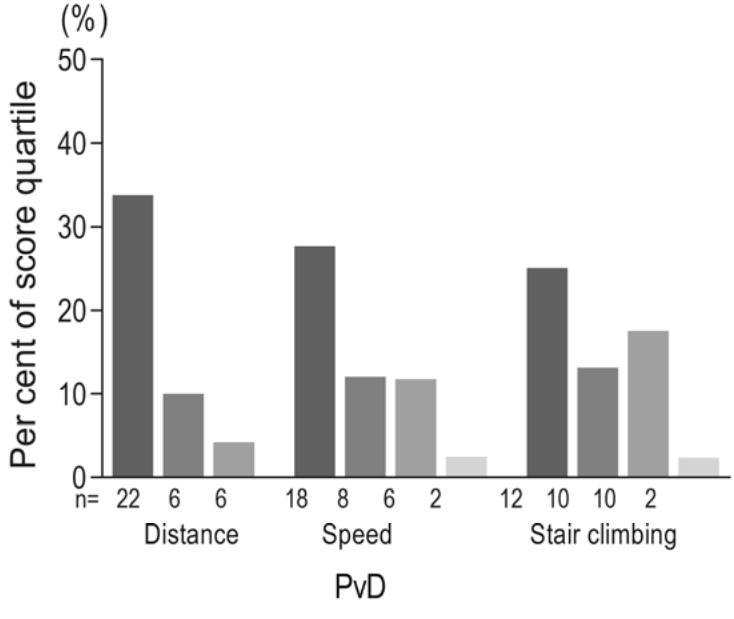
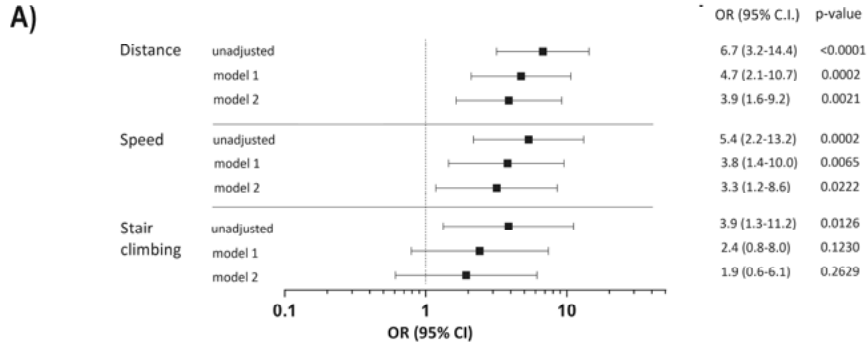


Figure 5 *shows the distribution of WIQ scores, with the lowest score on the left and the highest score on the right for each WIQ subscale in patients with A) Abnormal ABI, and B) Polyvascular disease (PvD).*



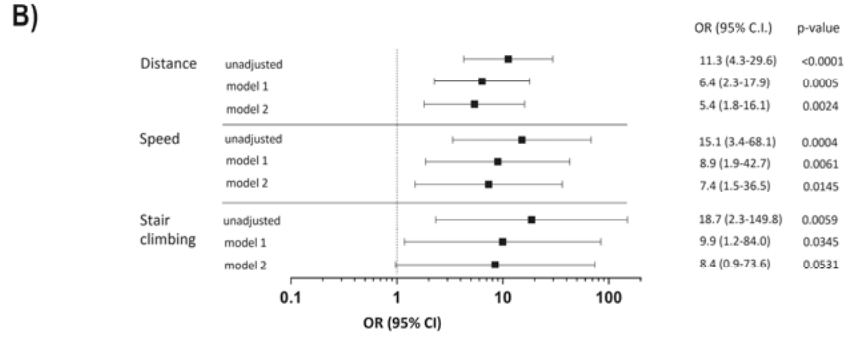


Figure 6 *shows the risk of atherosclerotic disease for patients scoring in the lowest group/quartile in patients with A) Abnormal ABI, and B) Polyvascular disease.*

Model 1 = adjusted for age and gender. Model 2 = adjusted for age, gender, congestive heart failure, atrial fibrillation and diabetes mellitus.

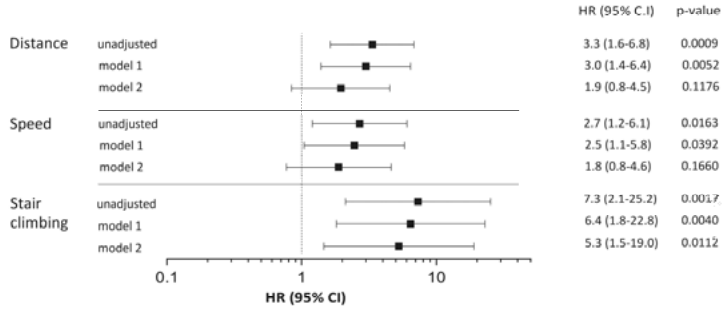


Figure 7 *shows the risk of composite cardiovascular endpoint at 24 months for patients scoring in the lowest WIQ group/quartile.*

Model 1 = adjusted for age and gender. Model 2 = adjusted for age, gender, congestive heart failure, atrial fibrillation and diabetes mellitus.

Paper III – Biomarkers and association with PAD

Baseline characteristics of the patient cohorts

Of the 390 patients in the REBUS cohort, there were 87 (22.3 %) women and the median age of the whole cohort was 66.8 years with no difference between the sexes. 27 (31.0%) of the women were smokers and 25 (28.7%) had an abnormal ABI compared to 72 (23.8%) and 53 (17.5%), respectively, of the men. Of the 766 patients in the VaMIS cohort, 227 (29.9 %) were women and the patients in the cohort were older, especially the women, than those in the REBUS cohort, and there were fewer with hypertension and fewer smokers and a larger proportion of NSTEMI. 56 (24.7 %) of the women compared to 119 (22.1 %) of the men in the VaMIS cohort had a pathological ABI.

Biomarkers associated with peripheral artery disease

The biomarkers associated with PAD were identified in the REBUS cohort. In the univariate analyses comparing the profile of all the 92 biomarkers in the CVD III chip in the REBUS cohort, there was a significant difference overall ($p < 0.001$) between the patients with PAD and those without PAD. Nine biomarkers were highly significant and the estimator of the Cumulative Distribution Function (ECDF) plots are presented in Figure 8.

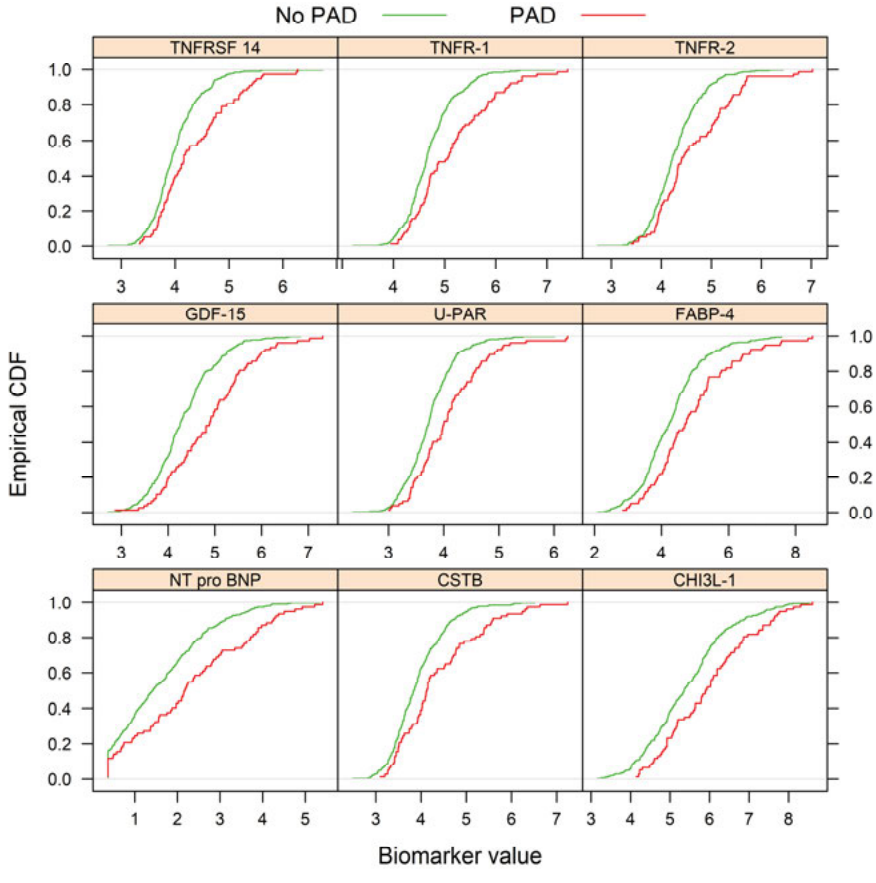


Figure 8 *Empirical cumulative density function (CDF) plots for the significant biomarkers: TNFRSF-14 (tumor necrosis factor receptor superfamily member 14), TNFR-1 (tumor necrosis factor receptor 1), TNFR-2 (tumor necrosis factor receptor 2), GDF-15 (growth differentiation factor 15), UPAR (urokinase plasminogen activator surface receptor), FABP-4 (fatty acid-binding protein adipocyte), Nt-pro BNP (N-terminal pro b-type natriuretic peptide), CSTB (cystatin B) and CHI3L1 (chitinase-3-like protein 1), in the univariate analysis in patients with and without PAD. Red line = patients with PAD; green line = patients without PAD.*

Clinical characteristics for prediction of PAD

The clinical characteristics, without adding the biomarkers, showed an accuracy in prediction of patients with PAD in the REBUS cohort with a C-statistics of 0.683 (95 % CI, 0.610, 0.756) (Table 4). In the VaMIS cohort the C-statistics was 0.729 (95% CI, 0.687-0.770) (see Table 4). Although there were fewer patients with hypertension and fewer smokers in the VaMIS cohort, both conditions seemed more closely associated to outcome in VaMIS patients compared to the REBUS cohort (Table 4).

Table 4 Clinical characteristics for prediction of PAD before adding the biomarkers, as analyzed by the C-statistics in the REBUS cohort and the cohort for validation, VaMIS.

REBUS				**VaMIS**		
Variable	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Age	1.06	(1.03,1.09)	< 0.001	1.08	(1.06, 1.10)	<0.001
Sex, male	0.58	(0.32,1.03)	0.064	1.21	(0.82, 1.80)	0.33
Diabetes mellitus	1.63	(0.84, 3.15)	0.15	1.52	(0.96, 2.39)	0.072
Hypertension	1.73	(0.79, 2.38)	0.26	1.84	(1.26, 2.67)	<.0.01
Smoking	1.41	(0.75, 2.64)	0.28	2.14	(1.37, 3.36)	<0.001
C-statistics	0.683	(0.610, 0.756)		0.729	(0.687, 0.770)	

Biomarkers for prediction of PAD

In the prediction model we identified six biomarkers in the REBUS cohort associated with a higher ability to predict the outcome (i.e. PAD) and they were also later available for validation in the VaMIS cohort: TNFRSF-14, TNFR-1, TNFR-2, GDF-15, IL-6 RA (interleukin 6 receptor subunit alpha), and (CTSD) (cathepsin D). Part of the results of the random forest analysis of the biomarkers are shown as an enlargement in Figure 9. The discriminatory accuracy of adding one biomarker at a time to the clinical characteristics, as analyzed by the C-statistics, is shown in Table 5. TNFRSF-14, TNFR-1, TNFR-2 and GDF-15 appeared to increase the predictive value, with an increase in C-statistics (Table 5). When the three biomarkers, TNFR-1, TNFR-2 and GDF-15, were added to the clinical characteristics, the C-statistic increased from 0.683 to 0.715 (Table 5).

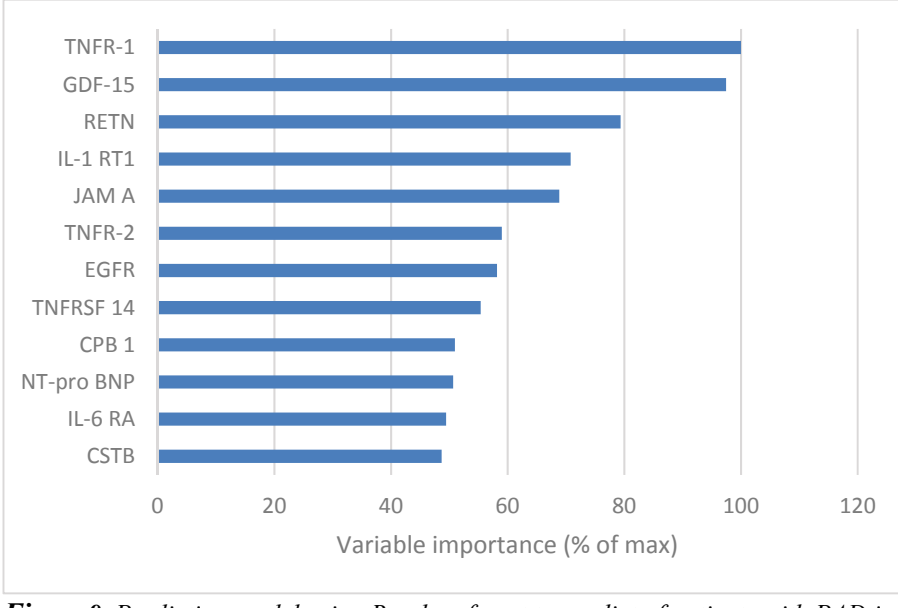


Figure 9. *Prediction model using Random forest to predict of patients with PAD in the REBUS cohort. The biomarkers presented with the highest variable importance: TNFR-1 (tumor necrosis factor receptor 1), GDF-15 (growth differentiation factor 15), RETN (resistin), IL-1 RT1 (interleukin 1 receptor type 1), JAM A (junctional adhesion molecule a), TNFR-2 (tumor necrosis factor receptor 2), EGFR (epidermal growth factor receptor), TNFRSF-14 (tumor necrosis factor receptor superfamily member 14), CPB 1 (carboxypeptidase B), Nt-pro BNP (N-terminal pro b-type natriuretic peptide), IL-6 RA (interleukin 6 receptor subunit alpha), and CSTB (cystatin B).*

Additional predictive value for PAD with biomarkers and comparison with VaMIS

The discriminatory accuracy of adding one biomarker (selected as described earlier) at a time to the clinical characteristics in the VaMIS cohort, as analyzed by the C-statistics, is shown in Table 5. TNFR-1, TNFR-2 and GDF-15 appeared to increase the predictive value, with an increase in C-statistic (Table 5). Of the six biomarkers identified in the REBUS cohort, three could be validated in the VaMIS cohort, with a similar pattern of an increase in C-statistics in both the REBUS and the VaMIS cohorts: TNFR-1, TNFR-2 and GDF 15 (Table 5). When these three biomarkers were added, the C-statistic increased from 0.729 to 0.752, with a similar pattern in VaMIS patients as in the REBUS cohort (Table 5).

Table 5 Additional predictive value of the biomarkers compared to the performance of the model with clinical characteristics, as measured by the C-statistic.

	Clinical characteristics (CC)	**CC+ TNFRSF-14**	**CC+ TNFR-1**	**CC+ TNFR-2**	**CC+ GDF-15**	**CC+ IL-6 RA**	**CC+ CTSD**	**CC+ TNFR-1, TNFR-2, GDF-15**
REBUS (n=390)	0.683 (0.610, 0.756)	0.702 (0.632, 0.772)	0.709 (0.640, 0.779)	0.703 (0.633, 0.773)	0.710 (0.640, 0.781)	0.682 (0.608, 0.755)	0.683 (0.610, 0.757)	0.715 (0.645, 0.784)
VaMIS (n=766)	0.729 (0.687, 0.770)	0.732 (0.691, 0.773)	0.746 (0.706, 0.787)	0.745 (0.704, 0.785)	0.752 (0.711, 0.792)	0.729 (0.688, 0.770)	0.736 (0.695, 0.777)	0.752 (0.711, 0.792)

Paper IV – Biomarkers and their association with patients with PAD and CV outcomes

Clinical characteristics

The clinical characteristics of the REBUS cohort are shown in Table 2. In this cohort of 388 patients, 20.1% (n=78) had a pathological ABI. Patients with pathological ABI were older, with a mean age of 71.1 years vs. 66.9 years and more were women than patients with normal ABI, 32.1 % vs. 19.7 %. Compared to patients with normal ABI, patients with pathological ABI at inclusion had more comorbidities at inclusion (Table 2). Similar results were found for the TNFR-1, TNFR-2 and GDF-15 group of biomarkers, where the patients with high biomarkers values had a higher mean age and more comorbidities at inclusion compared to the group with low biomarker values, though there was no difference between men and women (Table 6).

Table 6 Clinical characteristics of patients with high and low values of the group of biomarkers.

N (%)	**High values of bi-omarkers** (N=77)	**Low values of bi-omarkers** (N=311)	**P-value***
Age, mean (SD)	75.0 (8.75)	64.9 (9.61)	<0.001
Sex, Male Female	 61 (79.2) 16 (20.8)	 241 (77.5) 70 (22.5)	 0.744
BMI	27.1 (4.35)	27.3 (4.02)	0.384
Smoking, Yes	17 (22.1)	85 (27.3)	0.348
NSTEMI	41 (53.2)	164 (52.7)	
STEMI	36 (46.8)	147 (47.3	0.936
Revascularization PCI CABG	 56 (72.7) 2 (1.3)	 256 (82.3) 3 (1.0)	 0.240
3-vessel disease, coronary arteries	29 (37.7)	80 (25.7)	0.006
Pathological ABI	33 (42.9)	45 (14.5)	<0.001
Diabetes mellitus	29 (37.7)	32 (10.3)	<0.001
Hypertension	56(72.7)	152(48.9)	<0.001
Renal failure	13 (16.9)	3 (1.0)	<0.001
Previous MI	21 (27.3)	51 (16.4)	0.028
Previous stroke	7 (9.1)	10 3.2)	0.024
Previous PAD	4 (5.2)	5 (1.6)	0.061
Medical treatment			
ASA	75 (97.4)	306 (98.4)	0.844
Statins	70 (90.9)	297 (95.5)	0.111
Beta-blockers	72 (93.5)	289 (92.9)	0.858
ACEI or ARB	67 (87.0)	244 (78.5)	0.085
Oral anticoagulants	8 (10.4)	12 (3.9)	0.020

*p-value for comparison between patients with high vs. low values of biomarkers. NSTEMI = non-ST-elevation myocardial infarction; STEMI = ST-elevation myocardial infarction; PCI = percutaneous coronary intervention; CABG = coronary artery bypass surgery; MI = myocardial infarction; ASA = acetylsalicylic acid; ACEI = angiotensin converting enzyme inhibitor; and ARB = angiotensin receptor blocker.

Outcome events

The mean follow-up time was 5.5 years (min 0- max 7 years). Table 7 shows the total number of events. During follow-up a higher proportion of patients with pathological ABI died compared to patients with normal ABI, 30.8 % vs. 12.6 %. Similar results for all-cause mortality were found in the biomarker

group with higher values. A total of 108 ACS occurred with significantly more events in patients with pathological ABI, 64.1 % compared to those with normal ABI, 18.7 %. In the group with high biomarker values the similar results were seen with 71.4 % compared to 17.0 % in patients with lower values. Patients with pathological ABI also suffered a much higher proportion of the composite endpoint (all-cause mortality, new ACS, new ischemic stroke/TIA and new PAD), 51.3 % vs 26.8%, with a similar result in the group with high levels of biomarkers.

Table 7 Total outcome events, long-term follow up

	Total	Pathological ABI (N=78)	Normal ABI (N=310)	p-value*	High values of biomarkers (N=77)	Low values of biomarkers (N=311)	p-value*
All-cause Mortality	63	24	39	<0.001	30	33	<0.001
New ACS	108	50	58	<0.001	55	53	<0.001
Composite endpoint	123	40	83	<0.001	48	75	<0.001

*p- value for comparison between pathological and normal ABI and for high compared to low values of biomarkers. ACS; Acute coronary syndrome. Composite outcome; mortality, new acute coronary syndrome, ischemic stroke/TIA, peripheral artery disease.

Pathological ABI and outcome events

In the univariate analysis, the Kaplan-Meier survival curve in Figure 10 shows all-cause mortality in patients with or without pathological ABI. In the univariate Cox regression analysis, pathological ABI was associated with all-cause mortality which attenuated after adjustment, HR 1.71 (95% CI 0.97-3.04, p=0.071) (Figure 11). Pathological ABI was associated with a higher risk of new ACS after adjustment, HR 1.97 (95% CI 1.12-3.49, p=0.019). A similar result was found for the composite outcome event, HR 1.97 (95 % CI 1.29-3.01, p=0.002), Figure 11.

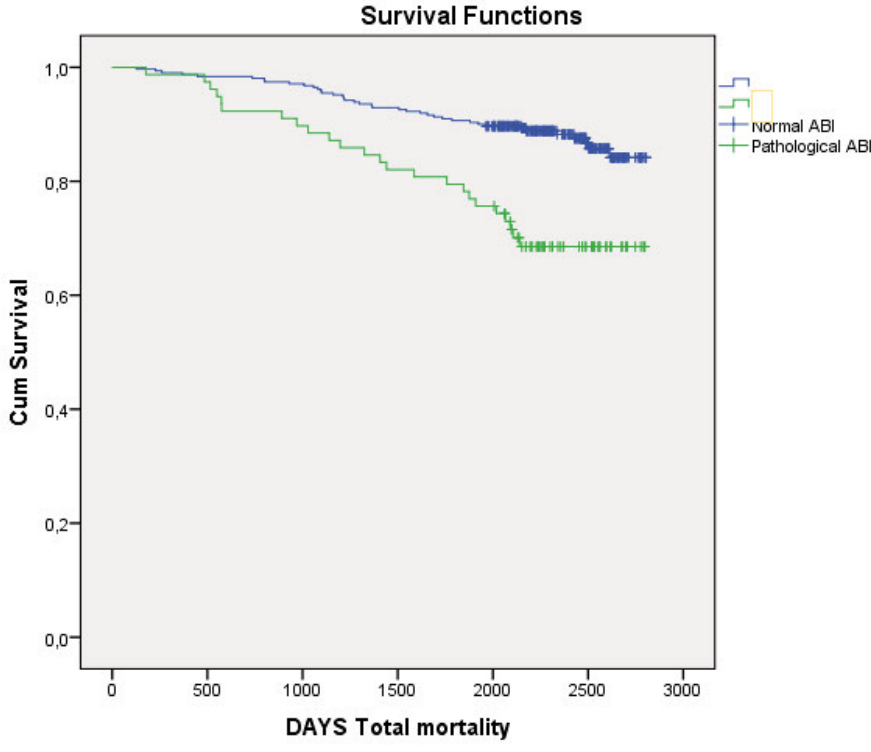


Figure 10 *Kaplan-Meier survival curve for all-cause mortality in a long-term follow-up in patients with and without pathological ABI.*

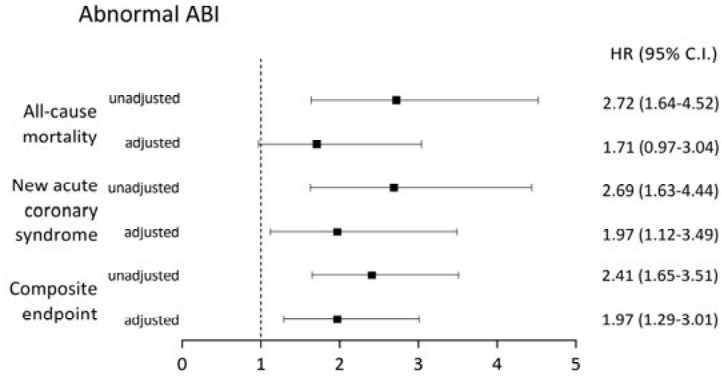


Figure 11 *Cox regression analyses showing the hazard ratios for patients with pathological ABI compared to normal ABI in predicting long-term outcome. Composite endpoint = all-cause death, new MI, new ischemic stroke or new PAD event. Adjusted for age, sex, smoking, revascularization at index MI, diabetes mellitus, hypertension, previous MI, previous stroke, previous PAD, and renal failure.*

Biomarkers TNFR-1, TNFR-2 and GDF-15, and outcome events

The Kaplan-Meier survival curve in Figure 12 shows the difference in all-cause mortality comparing patients in the group with the higher biomarker values with those with lower values. In the univariate Cox regression analysis higher composite biomarker values were associated with all-cause mortality, new ACS and the composite outcome event (Figure 13). After adjustment for established risk factors the biomarker group with the higher values was associated with all-cause mortality (HR 1.84, 95 % CI 1.00-3.38, p=0.049) and the composite outcome event (HR 1.62, 95 % CI 1.03-2.53, p=0.035). The risk for a new ACS in the group with higher biomarker values attenuated after adjustment.

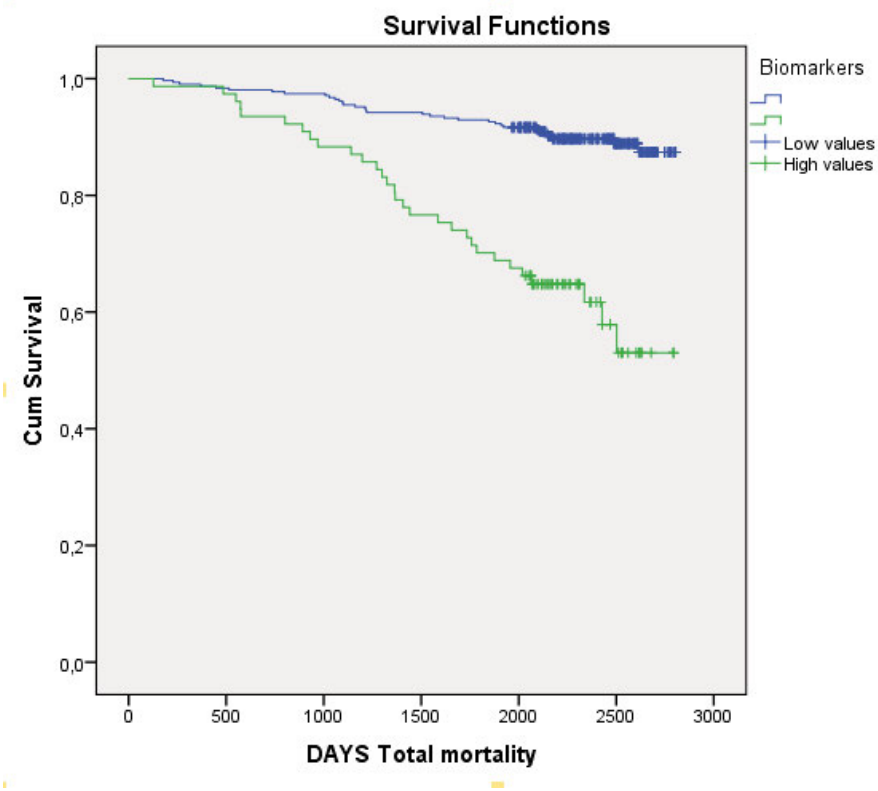


Figure 12 *Kaplan-Meier survival curve for all-cause mortality in a long-term follow-up in patients with recent MI and high compared to low values of a composite biomarker of TNFR-1, TNFR-2 and GDF-15.*

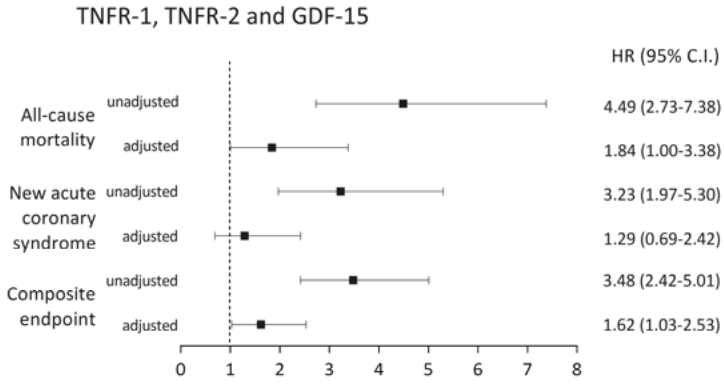


Figure 13 *Cox regression analyses showing the hazard ratios for patients with higher values for the composite biomarker (TNFR-1, TNFR-2 and GDF-15) in predicting long-term outcome. Composite endpoint = all-cause death, new MI, new ischemic stroke or new PAD event.*

Adjusted for age, sex, smoking, revascularization at index MI, diabetes mellitus, hypertension, previous MI, previous stroke, previous PAD, and renal failure.

Prediction of outcome events using pathological ABI and biomarkers

In the ROC analyses, pathological ABI showed accuracy in predicting all-cause mortality, with an ROC AUC of 0.607 (95% CI, 0.526-0.689, p=0.007), new ACS an AUC of 0.595 (95% CI, 0.516-0.674, p=0.014) and the composite endpoint an AUC of 0.591 (95% CI, 0.528-0.654, p=0.004). Adding the established CV risk factors to the analyses increased the predictive value of pathological ABI for all outcome events (Table 8). For new ACS the AUC increased from 0.753 (95 % CI 0.684-0.822, p<0.001) to 0.763, (95% CI, 0.697-0.830, p=<0.001) (Table 8).

Regarding the biomarker group, in the ROC analyses, the group with higher values showed accuracy in predicting all-cause mortality, with an AUC of 0.666 (95% CI, 0.586-0.746, p=<0.001), new ACS an AUC of 0.615 (95% CI, 0.535-0.694, p=0.003) and the composite endpoint an AUC of 0.640 (95% CI, 0.578-0.703, p=<0.001). Adding the same established CV risk factors produced a similar pattern, with an increase in AUC for all outcome events (Table 8). For all-cause mortality the AUC increased from 0.789 (95 % CI 0.729-0.849, p<0.001) to 0.805 (95% CI, 0.746-0.863, p=<0.001) (Table 8).

When pathological ABI, the group with higher biomarker values and established CV risk factors were combined no significant change in the AUC for any of the events was noted.

Table 8 Prediction of outcome events after recent myocardial infarction

	Clinical characteristics** AUC (95% C.I.)	Clinical characteristics** and pathological ABI[§] AUC (95% C.I.)	Clinical characteristics** and biomarkers[β] AUC (95% C.I.)	Clinical characteristics**, pathological ABI[§] and biomarkers[β] AUC (95% C.I.)
All-cause mortality	0.789 (0.729-0.849)	0.797 (0.736-0.857)	0.805 (0.750-0.863)	0.809 (0.750-0.868)
New acute coronary syndrome	0.753 (0.684-0.822)	0.763 (0.697-0.830)	0.758 (0.691-0.826)	0.763 (0.696-0.829)
Composite outcome event*	0.750 (0.696-0.804)	0.761 (0.708-0.813)	0.759 (0.705-0.813)	0.765 (0.712-0.819)

*Composite outcome; mortality, new acute coronary syndrome, ischemic stroke/TIA, peripheral artery disease. ** Clinical characteristics; age group, sex, smoking, revascularization (PCI or CABG). [§]ABI; ankle brachial index, [β]biomarkers; high values of the biomarker group TNFR-1, TNFR-2 and GDF-15.

Discussion

In this thesis, several aspects of patients with both CAD and PAD were studied. The overall aim was to assess the prevalence of PAD and PvD in patients with recent MI and to find valuable clinical methods to detect these patients. In order to better understand and potentially improve the risk stratification of a cardiovascular event and the patient's long-term outcome, we also wanted to investigate the relationship between biochemical biomarkers and PAD.

Main findings

In Paper I, we found that PAD and PvD are underdiagnosed in patients who suffered a recent MI. We also found the ABI to be a strong and useful method to identify patients with PAD as well as patients with more widespread arterial disease, such as PvD.

The results in Paper II showed that a scoring system, the Walking Impairment Questionnaire, is useful for finding patients with PAD and PvD, even when completed soon after an acute MI event.

In Paper III, we found that biochemical biomarkers associated with the inflammatory pathway – TNFR-1, TNFR-2 and GDF-15 – were able to predict pathological ABI, i.e. PAD, in patients. These results could also be validated in another observational study and cohort of MI patients, the VaMIS cohort.

In the results in Paper IV we found pathological ABI to be a strong predictor for cardiovascular events of all-cause mortality, new ACS, and a composite endpoint of all-cause mortality, new ACS, new stroke/TIA or new PAD event. When we evaluated the three inflammatory biomarkers (TNFR-1, TNFR-2 and GDF-15) as a surrogate marker for ABI, they showed a similar association with all-cause death and the composite endpoint.

Prevalence of PAD and PvD in patients with myocardial infarction and the importance of the ABI (Paper I)

The true prevalence of both PAD and PvD is largely unknown, often due to prevalence being based on a combination of previous clinical diagnosis of CV disease, i.e. PAD and/or CAD or earlier stroke/TIA,[4, 5 3, 7, 8, 116-118] with no measurement of ABI and/or investigation of the arterial beds. This is unfortunate, especially given that PAD and PvD, in both symptomatic- and asymptomatic patients, are associated with worse outcome than CAD alone.[3-11, 30] In the present study, there was a significant difference, among the MI patients, between the reported frequency of clinically diagnosed PvD at admission and the proportion of patients with arteriosclerotic findings after investigation.

Compared to other studies in ACS patients, PvD was more common in our MI cohort.[9-11, 119] This could be explained in part by our definition of abnormal/normal findings in coronary and carotid arteries, where we wanted to identify all atherosclerotic changes in the arterial beds. In the present study, few patients had a history of PAD, which the very broad confidence interval indicates, but there was consistency between the history and measurement of ABI. Studies that use ABI and carotid ultrasound, as ours did, report higher frequencies of PvD.[7, 117, 120]

In Paper I, we found no difference in prevalence of PvD between NSTEMI and STEMI patients, when we adjusted for background risk factors. Patients with PAD and PvD share many of the risk factors with patients with CAD, such as hypertension, smoking and diabetes mellitus.[3, 7-9] In this aspect, our results are in line with previous studies, and our findings regarding the importance of age and diabetes mellitus as predictors of widespread atherosclerosis concur with other studies.[3, 7-9] Of particular interest is our finding of female gender as an independent predictor of PvD. Previous studies have reported a higher prevalence of asymptomatic PAD in women, when diagnosed using ABI, and that women with symptomatic PAD often have more frequent cardiovascular comorbidities.[53, 121, 122] The women included in our study represent an unselected group of MI patients, in contrast to many other studies in which exclusion criteria such as age and other comorbidities are frequently applied, which may explain the higher burden of atherosclerosis.[13, 123-125] We could not see any differences between sex in the prevalence of NSTEMI or STEMI, distribution of cardiovascular risk factors, occurrence of three-vessel CAD, or frequency of revascularization.

In our overall aim to find valuable clinical methods to identify the patients with widespread arterial disease, the ABI stands out as the most important predictor of atherosclerosis in all three of the arterial beds studied, with a high specificity compared to both carotid ultrasound and coronary angiography. It

is essential to emphasize the importance of measuring ABI in our CAD patients, a simple method in clinical practice to find these patients with a poorer prognosis.[3, 9, 30, 126, 127]

Associations between WIQ score and patients with PAD and their outcomes (Paper II)

In Paper II, when using, the Walking Impairment Questionnaire (WIQ), we found that PvD, as well as PAD, was associated with the lowest scoring groups of both walking distance and walking speed, even after adjustment. The majority of patients with PvD were also found in the group that scored the lowest in stair climbing. The patients with the lowest scores had a higher mean age and more comorbidities and, not surprisingly, there was also an association with higher age. There was, however, a higher proportion of women in all domains with the highest age, and a larger proportion of women in the lowest scoring quartile compared to the highest. No differences were seen in the proportion of current smokers or the distribution of type of index MI in any of the subdomains.

Since the questionnaire concerns matters of physical activity, it is of importance to evaluate conditions that deteriorate walking ability and could influence the results, such as heart failure.

In this study, 20 (7.6%) patients suffered from congestive heart failure at inclusion, with significantly more patients in the lowest score group in all three WIQ domains. In patients with a higher mean age, as in this cohort, and more comorbidities, there are multiple pathways linking PAD and heart failure, with several shared risk factors, such as diabetes and hypertension,[128, 129] where elevated afterload due to hypertension and elevated aortic stiffness can ultimately lead to heart failure.[128, 129] PAD associated with overt atherosclerosis involving coronary atherosclerosis also increases the risk for heart failure.[130]

The echocardiography after the index event also showed a larger proportion of impaired left ventricular ejection fraction, which was reflected in their walking distance and speed scores, with significantly more of these patients in the lowest score group, but surprisingly not in the stair-climbing score, although the stair score has also been suggested as a surrogate marker of a patient's cardiopulmonary capacity and, consequently, prognosis.[103, 131]

In patients with CV disease, it is important to encourage high adherence to secondary prevention, such as drugs. In post-MI patients the prevalence of

these drugs is generally higher[132] than in many PAD populations where secondary prevention is less prominent,[14, 45, 54] although the use of proven medical therapies has improved over the past 10 years.[133] The patients represented in the data collected had a high proportion of guideline-recommended secondary prevention drug therapy, with no major differences between the lowest or highest quartiles, and adherence remained high after 2 years of follow-up.

A few studies have examined the usefulness of WIQ scores for predicting CV outcomes.[104, 105] A limited number of studies have also evaluated WIQ in patients without PAD, with varying results.[102, 103, 105] In the population with a recent MI studied here, the patients in the lowest WIQ score group had an increased risk for a CV outcome, and the stair-climbing score was more closely associated and had a greater association than the scores for walking distance and walking speed, while the distance and speed scores seemed better at identifying patients with PAD and those with PvD. This differences remained after adjustment.

PAD and the association to biomarkers and long-term outcome (Paper III-IV)

Overall, the patients in the REBUS cohort in papers III and IV had a high proportion of guideline-recommended secondary prevention drug therapy, with no differences between patients with and without PAD.

Despite this, the results from Paper IV nevertheless show that, after long-term follow-up of a mean of 5.5 years, patients with a recent MI and PAD have a higher risk of suffering a new CV event including ACS, ischemic stroke/TIA or death.

Together with other secondary prevention interventions, e.g. smoking cessation, this higher risk is important to evaluate in patients in order to prevent or delay a new CV event.

In our cohort, the rate of a new ACS was significantly higher in patients with PAD (64 %) compared to those without. In many of the patients, their events occurred early during follow-up, and a small number of patients experienced more than one event. These results are in accordance with the results from other cohorts with an ACS population, both in prospective studies[9, 10, 119] as well as in registries.[3, 30]

Revascularization is the first line of treatment in patients with ACS and was performed in the majority of the patients in this study, including the patients

with PAD, yet the association between PAD and new ACS persisted after adjustment for revascularization.

Suggested explanations for this include that there may be a difference in pathophysiology in different locations of atherosclerosis, which may be of importance for the development of new CV events.[134] Moreover, biomarkers reflecting different pathophysiologic mechanisms may be associated with PAD.[94, 95] Some data suggest, for example, that inflammatory activity may be higher in patients with PAD compared to patients with CAD,[96, 135] and there is some evidence that, even when well-treated, the risk for new CV events in patients with PAD is not attenuated, which may be explained in part by the increased inflammatory burden.[95, 96]

The overall importance of biomarkers is, however, not well-described, and several biomarkers have been associated with PAD in population-based studies and with different pathways,[93] although many of these biomarkers are also elevated in CAD and other vascular disorders.

In Paper III, we investigated associations with 92 biochemical biomarkers and found a significant difference in the profile of biomarkers in MI patients with and without PAD. In the prediction of PAD in these MI patients, six biomarkers linked to atherosclerosis and inflammation were identified and three of these – TNFR-1, TNFR-2, GDF-15 – had an additional value to clinical characteristics to better predict PAD as an outcome. These biomarkers were also able to be validated in another MI cohort from an observational study similar to ours with recent MI, the VaMIS study. Adding the three biomarkers, as a group, to clinical characteristics further increased the C-statistic compared to a single biomarker.

The findings in Paper III, an exploratory study, suggested a possible pathological role of these biomarkers (TNFR-1, TNFR-2 and GDF-15) to consider, and in Paper IV we studied the long-term outcome of the REBUS patients with and without PAD and also, based on the work and results of Paper III, the group of patients with high levels of this biomarkers compared to low values and their association to long-term outcomes. And, additionally, to investigate whether the group of biomarkers for inflammation could further improve prediction of recurrence of CV events after an MI.

Our findings in Paper IV were similar to the results when using pathological ABI, with a significantly higher event rate with higher values of these biomarkers. When outcome events from peripheral vascular beds and ischemic stroke were included, the predictive value of the group of biomarkers was stronger than ACS alone.

Not surprisingly, the group of patients with higher biomarker values were older than patients with pathological ABI, and had worse outcomes with respect to all-cause mortality. These findings persisted after adjustment for clinical characteristics in the all-cause mortality and the composite endpoint. Findings in earlier studies have shown that higher values of GDF-15 and TNF-α (which acts through the two transmembrane receptors: TNFR-1 and TNFR-2) increases the risk for new CV events,[86, 89, 90, 92, 95, 136] as well as all-cause mortality in GDF-15,[137] and may help to explain the worse outcomes associated to the biomarker group.

It should also be noted that the outcomes in patients with higher biomarker values do not seem to be associated with sex, as in the case in patients with pathological ABI.

To assess the possibility of using pathological ABI and the group of the three biomarkers as diagnostic tools for prediction of outcome events, the ROC analysis showed a slightly higher AUC for the biomarker group than for pathological ABI. Both exhibited a low discrimination value for prediction of outcome events themselves. When clinical characteristics were added, the AUC clearly improved for both, and remained high for the group of biomarkers even after adjustment for ABI. In this cohort, the findings suggest that the group of biomarkers (TNFR-1, TNFR-2 and GDF-15) may provide additional information to the prediction of CV outcomes in patients with recent MI.

Limitations

The studies of the present thesis have limitations to be considered when interpreting the results. A general limitation concerns a possible lack of generalizability to other populations due to the REBUS study being a single-center observational study, and the sample size of the study's patient cohort suggests a need for larger prospective studies for evaluating patients with PAD and PvD to elucidate and their associated risks.

In all of the studies cited in the papers, rather than using a single method we used a combination of morphological and functional methods to identify atherosclerosis in the different arterial beds, which may have influenced the results. Another limitation is that the study sample was limited to Caucasian patients who had had an acute MI, and care should be exercised when drawing conclusions concerning other age groups and ethnicities. Despite this, our findings could be verified in the VaMIS cohort, another MI cohort from a different region of Sweden (Paper III). However, larger prospective studies with control groups with biomarkers as a primary endpoint are needed to confirm our findings. There are also some limitations concerning the PEA technique, which does not permit an absolute quantification of the target proteins, so translation into clinically relevant cut-off values is not possible. Collection of the blood samples occurred 3-5 days after admission for the REBUS cohort but at admission for the VaMIS cohort, and we cannot rule out a more acute phase expression, compared to the more stable patient with CAD and PAD.

Finally, although multivariable adjustment was performed, given the size of the cohort and the events, residual confounding from unknown or unmeasured factors cannot be ruled out.

General Conclusions

The aim of this thesis has been to find valuable clinical methods to identify patients with PAD and PvD, given that these patients are underdiagnosed partly because of their asymptomatic nature.

Based on the findings of the thesis, we can conclude that:

I PAD and PvD are more frequent among patients admitted for NSTEMI or STEMI than reported at admission, and there seems to be little difference in distribution between the two MI groups. The clinical characteristics for widespread atherosclerosis in PvD in this cohort are higher age, female gender, and diabetes mellitus.

II ABI is a simple and useful measurement that appears to be predictive of PvD in these patients in need of more aggressive secondary preventive measures and follow-up.

III WIQ scores can be a useful tool for identifying patients with additional atherosclerotic burden with PAD and PvD; patients with PAD and PvD had the lowest scores for the WIQ subscales of walking distance and walking speed, and the majority of patients with PvD were found in the lowest scoring category with respect to stair climbing. Patients with the lowest scores had an increased risk for new CV events compared to patients in the highest scores.

IV The results indicate that, although our patients were in recovery from an MI, the WIQ score was able to provide valuable information to assess patients early after an MI for a more widespread atherosclerotic burden associated with a higher risk.

V Three out of a panel of 92 biomarkers – TNFR-1, TNFR-2 and GDF-15 – were identified as being associated with PAD in patients with recent MI and, when added to clinical characteristics, were able to improve prediction of PAD.

VI Despite a high proportion of revascularization and guideline-recommended secondary medical treatment, patients with a recent MI and PAD have a higher risk of suffering a new CV event including ACS, ischemic stroke/TIA or death.

VII Higher values of TNFR-1, TNFR-2 and GDF-15, as a composite, seems to better predict all-cause mortality than pathological ABI, suggesting that biomarkers may provide additional information in the prediction of CV outcome in patients with recent MI.

Clinical implications and future perspectives

Patients with CAD and PAD share many conventional risk factors and, besides improving secondary prevention, it is important in the evaluation of patients' future risk of new CV events to emphasize the importance of measuring ABI in our CAD patients, especially given that so many of these patients with a poorer prognosis are also without symptoms or experience atypical ones. Measuring ABI, a simple and highly available method in everyday clinical life, would be beneficial for the patient, to detect asymptomatic PAD and begin or improve secondary prevention treatment and interventions we know are beneficial for patients with atherosclerotic diseases. Another aspect of detecting a more widespread atherosclerotic disease is that it also gives us more information about the patient's future risk for new CV events, a risk that is higher in this group compared to those with MI alone. We need to pay attention to symptoms that could be an indication for intervention. Although we do not have answers to all aspects of the role of inflammation in atherosclerotic disease, the area of inflammation is likely to be further investigated and its markers may come to play a larger role in the evaluation and treatment of the individual patients. Indeed, studies of drugs targeting inflammatory mechanisms involving CV outcomes are already under way. Canakinumab, a monoclonal antibody that targets IL-1β, has already been investigated in a larger cohort of patients with previous MI[138] and the findings showed that treatment with the two higher doses led to a significantly lower rate of recurrent CV events compared to placebo. Other studies targeting inflammation involving lipoprotein-associated phospholipase A2 (LP-PLA2),[139, 140] treatment with colchichine,[141] methotrexate[142] and other anti-atherosclerotic therapies,[143] have produced varying results.

Summary in Swedish (sammanfattning på svenska)

Kranskärlssjukdom som uppstår genom s.k. åderförkalkning är egentligen en process med inlagring av blodfetter i kärlväggarna, där det sedan uppstår en process som gör att ytan i kärlet blir skört och att blodkärlet blir trängre och stelare. Denna åderförfettning kan leda till hjärtinfarkt, kärlkramp, stroke och förträngning i benens kärl.

Vi vet av mycket forskning att det är flera saker vi kan göra för att försöka förebygga att åderförfettningen blir så uttalad att vi får symptom och kärlhändelser. T.ex är det viktigt att inte röka, att förebygga högt blodtryck, höga halter av blodfetter, undvika övervikt och motionera regelbundet.

Det vanligaste är att man inte har några symptom av sin åderförfettning fram till en kärlhändelse, då antingen en blodpropp lossnat eller blodkärlet är tillräckligt trångt för att inte kunna försörja vävnaden tillräckligt med syre och näringsämnen och man får t.ex en hjärtinfarkt. Åderförfettningen kan också ge symptom från andra kärlområden och ibland från flera samtidigt. Vi vet att de patienter som har t.ex en hjärtinfarkt också kan ha åderförfettning på andra ställen i den arteriella kärlbädden och att dessa patienter har en större risk att få nya kärlhändelser, men har också en ökad risk för död. Eftersom många inte har symtom är det viktigt att försöka hitta dessa patienter för att kunna förebygga nya kärlhändelser.

I denna avhandling har vi studerat en grupp av patienter i studien REBUS (421 st) som lagts in på sjukhus för en hjärtinfarkt, och försökt hitta bra kliniska metoder att använda för att hitta de patienter med åderförfettning även i kärlen på halsen och/eller i benartärerna. Vi undersökte patienterna med kranskärlsröntgen, mätte ett ankel-brachial-index (ABI) som speglar åderförfettning i benen och gjorde ultraljud av kärlen på halsen. Vi tog också ett antal blodprover på patienterna som analyserades, bl.a. med s.k. biomarkörer -en biologisk markör som är mätbar och speglar olika biologiska och patogena processer i kroppen som kan följas.

I arbete I, undersökte vi hur vanligt det var med åderförfettning i andra kärl än kranskärlen i hjärtat och såg att det var betydligt vanligare än vad som var

känt när patienten skrevs in på sjukhus. 20.3 % hade åderförfettning även i benen mot 2.1 % kända (där mätt ABI var patologiskt) och 13.8 % hade åderförfettning i alla tre kärlbäddar. De som var mer kärlsjuka hade flera sjukdomar som vi vet påverkar utvecklingen av åderförfettning, såsom diabetes mellitus, högt blodtryck, tidigare hjärt-kärlsjukdom mm, jämfört med de patienter som hade endast en hjärtinfarkt. Bästa sätt att hitta dessa patienter med mest utbredd åderförfettning var i denna grupp att mäta ABI.

I arbete II, undersökte vi om en enkät som patienten fyllde i 2-3 veckor efter hjärtinfarkten kunde identifiera de patienter som hade en mer utbredd kärlsjukdom. Vi fann att de patienter som hade lägsta (=sämst resultat) poäng i enkäten också hade den mest utbredda åderförfettningen och att de patienter som hade låga poäng också hade fler kärlhändelser 2 år efter sin hjärtinfarkt.

I arbete III, undersökte vi om det var några biomarkörer av de 92 st vi analyserade som kunde kopplas ihop med kärlsjukdom i benen och om några av dessa kunde prediktera (förutsäga) vilka patienter som har kärlsjukdom i benen genom att mäta dessa blodprover.

Vi kunde se att några biomarkörer fanns i högre mängd hos de patienter med kärlsjukdom och hjärtinfarkt jämfört med de som endast hade hjärtinfarkt och tre av biomarkörerna som speglar inflammation (TNFR-1, TNFR-2, GDF-15) kunde predicera vilka patienter som hade kärlsjukdom i benen. För att jämföra om vi kunde få en bättre prediktion att hitta dessa patienter med både de kända riskfaktorer för åderförfettning vi känner till och dessa biomarkörer analyserade vi detta. Vi såg då att de kunde förbättra prediktionen att hitta de patienter som har kärlsjukdom i benen. När vi jämförde med en annan studie med en liknande grupp av patienter med hjärtinfarkt (VaMIS) kunde vi se samma mönster.

I arbete IV, undersökte vi vad som hänt med patienterna efter en lång tids uppföljning, i medeltal 5.5 år. Trots att patienterna hade bra medicinering mot riskfaktorer (blodfettssänkande, blodtryckssänkande, proppförebyggande etc.) så hade de patienter som hade haft hjärtinfarkt och som har kärlsjukdom i benen fler hjärtinfarkter och andra kärlhändelser samt även fler dödsfall.

I detta arbete undersökte vi också de tre biomarkörer som vi funnit (arbete III) kunna predicera kärlsjukdom i benen, om dessa kunde förutspå dessa kärlhändelser hos våra patienter med hjärtinfarkt. Vi såg att höga värden av dessa biomarkörer, analyserade som en grupp, bättre kunde förutspå död än när man mätte ABI, vilket skulle kunna innebära att dessa biomarkörer kan ha en tilläggsinformation för att kunna prediktera nya kärlhändelser i denna grupp.

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