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ANKLE-BRACHIAL INDEX, HIGH-SENSITIVITY C-REACTIVE PROTEIN AND ENDOTHELIAL FUNCTION IN A CARDIOVASCULAR RISK POPULATION

by

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ABSTRACT

Kari Syvänen. Ankle-brachial index, high-sensitivity C-reactive protein and endothelial function in a cardiovascular risk population.

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Atherosclerotic vascular disease is the leading cause of death in the Western world. Its main three manifestations are coronary heart disease, cerebrovascular disease, and peripheral arterial disease. Asymptomatic peripheral arterial disease is usually diagnosed using the ankle brachial index, and values ≤ 0.90 are used to determine the diagnosis. The classical risk factors of peripheral arterial disease, such as smoking and diabetes, are well known and early interventions are mandatory to improve the prognosis. What is not well known is the role of inflammation as a risk factor. Yet, a novel approach to cardiovascular diseases is the measurement of endothelial function.

In this thesis, we studied the ankle-brachial index, C-reactive protein and endothelial function in a cardiovascular risk population. A total of 2856 subjects were invited to the study and 2085 (73%) responded. From these subjects, a cohort of 1756 risk persons was screened. We excluded the subjects with previously known cardiovascular disease or diabetes, because they were already under systematic follow-up. Out of the study subjects, 983 (56%) were women and 773 (44%) men. The ankle brachial index and high-sensitivity C-reactive protein were measured from 1047 subjects. Endothelial function was assessed by measuring reactive hyperemia pulse amplitude tonometry from 66 subjects with borderline peripheral arterial disease.

In this study, smoking was a crucial risk factor for peripheral arterial disease. Subclinical peripheral arterial disease seems to be more common in hypertensive patients even without comorbidities. The measurement of the ankle brachial index is an efficient method to identify patients at an increased cardiovascular risk. High-sensitivity C-reactive protein did not correlate with the ankle brachial index or peripheral arterial disease. Instead, it correlated with measures of obesity. In a cardiovascular risk population with borderline peripheral arterial disease, nearly every fourth subject had endothelial dysfunction. This might point out a subgroup of individuals in need of more intensive treatment for their risk factors.

Key words: ankle brachial index, high-sensitivity C-reactive protein, endothelial function, peripheral arterial disease, reactive hyperemia pulse amplitude tonometry

TIIVISTELMÄ

Kari Syvänen. Nilkka olkavarsipaine, herkkä C-reaktiivinen proteiini ja endoteelin toiminta valtimotaudin riskiväestössä.

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Valtimotauti on ollut jo vuosikymmeniä länsimaiden johtava kuolinsyy. Sen keskeisimmät ilmentymät ovat sepelvaltimotauti, aivovaltimotauti sekä alaraajavaltimotauti. Oireettoman alaraajavaltimotaudin diagnoosi on suhteellisen helppo tehdä käyttäen nilkka olkavarsipaineen mittausta. Diagnostisena raja-arvona käytetään yleensä tulosta ≤ 0.90. Valtimotaudin perinteiset riskitekijät, kuten tupakointi ja diabetes, ovat varsin hyvin tunnettuja. Viime vuosina mielenkiinto onkin kohdistunut esimerkiksi tulehdusreaktion tutkimiseen valtimotaudissa. Lisäksi on selvitelty verisuonen sisäpinnan solukon, endoteelin, merkitystä valtimotaudin synnyssä.

Tässä väitöskirjatyössä selvitettiin valtimotaudin riskiväestössä nilkka olkavarsipaineen antamaa informaatiota, sekä tutkittiin, antavatko herkän C-reaktiivisen proteiinin ja endoteelin toiminnan tutkimukset lisätietoa potilaiden valtimotautiriskiä arvioitaessa. Tutkimukseen kutsuttiin 2856 henkilöä, joista 2085 (73 %) oli kiinnostunut osallistumaan. Heistä seulottiin 1756 valtimotaudin riskihenkilöä. Henkilöt joilla oli ennestään diagnosoitu diabetes tai valtimotauti pois suljettiin tutkimuksesta. Tutkimusväestöstä 983 (56 %) oli naisia ja vastaavasti 773 (44 %) miehiä. Nilkka olkavarsipaine ja herkkä C-reaktiivinen proteiini määritettiin 1047 henkilöltä. Endoteelin toimintaa tutkittiin 66 henkilöltä, joilla oli raja-arvoinen perifeerinen valtimotauti, eli heidän nilkka olkavarsipaineensa oli välillä 0.91-1.00.

Tupakointi osoittautui keskeiseksi alaraajavaltimotaudin riskitekijäksi. Lisäksi todettiin että verenpainetautia sairastavilla henkilöillä, joilla ei ole todettuja liitännäissairauksia, oireeton alaraajavaltimotauti on yleisempää kuin normaalin verenpaineen omaavilla henkilöillä. Nilkka olkavarsipaineen mittaus osoittautui toimivaksi valtimotautiriskin mittariksi. Herkkä C-reaktiivinen proteiini ei korreloinut nilkka olkavarsipaineeseen tai alaraajavaltimotautiin. Sen sijaan yhteys sen ja lihavuuden välillä todettiin. Tämä todennäköisesti johtuu rasvakudoksen osallistumisesta tulehdusreaktion syntyyn. Joka neljännellä raja-arvoista valtimotautia sairastavista henkilöistä todettiin häiriö endoteelin toiminnassa. Nämä henkilöt saattavat tarvita muita voimakkaampia toimenpiteitä valtimotaudin riskitekijöiden kontrolloimiseksi.

Avainsanat: Nilkka olkavarsipaine, herkkä C-reaktiivinen proteiini, endoteelin toiminta, alaraajavaltimotauti

TABLE OF CONTENTS

ABBREVIATIONS

ABI	Ankle brachial index
ADP	Arteria dorsalis pedis
ATP	Arteria tibialis posterior
BMI	Body mass index
CHD	Coronary heart disease
CLI	Critical leg ischemia
CRP	C-reactive protein
CVD	Cerebrovascular disease
DBP	Diastolic blood pressure
DM	Diabetes
FMD	Flow-mediated dilatation
HDL	High-density lipoprotein
HsCRP	High-sensitivity C-reactive protein
IFG	Impaired fasting glucose
IC	Intermittent claudication
IGT	Impaired glucose tolerance
LDL	Low-density lipoprotein
MBO	Metabolic syndrome
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PAD	Peripheral arterial disease
RH-PAT	Pulse amplitude tonometry after reactive hyperemia
RHI	Reactive hyperemia index
SBP	Systolic blood pressure
SCORE	Systemic Coronary Risk Evaluation
SD	Standard deviation
TASC II	Inter-Society Consensus for the Management of Peripheral Arterial Disease
WC	Waist circumference

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

I Syvänen K, Aarnio P, Jaatinen P, Korhonen P. Effects of age, sex and smoking on ankle-brachial index in a Finnish population at risk for cardiovascular disease. *Int J Angiol*. 2007; 16:128-130

II Korhonen PE, Syvänen KT, Vesalainen RK, Kantola IM, Kautiainen H, Järvenpää S, Aarnio PT. Ankle-brachial index is lower in hypertensive than in normotensive individuals in a cardiovascular risk population. *J Hypertens*. 2009; 27:2036-2043

III Syvänen K, Korhonen P, Jaatinen P, Vahlberg T, Aarnio P. High-sensitivity C-reactive protein and ankle-brachial index in a Finnish cardiovascular risk population. *Int J Angiol*. 2011; 20:43-48.

IV Syvänen K, Korhonen P, Partanen A, Aarnio P. Endothelial function in a cardiovascular risk population with borderline ankle brachial index. *Vasc Health Risk Manag*. 2011; 7: 97-101

1 INTRODUCTION

Atherosclerotic vascular disease is the leading cause of death in developed countries (AHA 2003). The main three manifestations it has are coronary heart disease (CHD), cerebrovascular disease (CVD) and peripheral arterial disease (PAD). The most widely used method to detect peripheral arterial disease is the measurement of the ankle brachial index (ABI). Different cut-off limits have been used in the literature. TASC II (Inter-Society Consensus for the Management of Peripheral Arterial Disease) was published in 2007 in co-operation between medical and surgical vascular, cardiovascular, vascular radiology and cardiology societies in Europe and North America. It recommends a limit of ≤ 0.90, whereas European guidelines for the management of hypertension and the United States guidelines for treating dyslipidemia, for example, use a cut-off point of < 0.9 (Norgren et al. 2007, Macia et al. 2007 and NCEP 2002). These both figures are used alternatively in studies. It is not only a tool to diagnose peripheral arterial disease, but the ankle brachial index can also be used as a method to assess patients´ cardiovascular risk and functional capacity (McDermott et al. 2000). In addition, subjects with borderline PAD (ABI 0.91-1.00) are also at an increased risk of cardiovascular disease compared to those with ABI 1.11-1.20 (Fowkes et al. 2008)

As there is growing evidence of the significance of inflammation in vascular disease, novel methods have been evolved to study it. For clinical purposes, the most widely studied marker of inflammation has been high-sensitivity C-reactive protein (hsCRP). In population-based studies, the baseline levels of hsCRP have been predictive for future cardiovascular events (Ridker 2001 II). Evidence of the predictive role of hsCRP in PAD is still scarce and quite conflicting. Elevated hsCRP levels in persons with PAD have been linked to short-term cardiovascular and all-cause mortality (Vidula et al. 2008), but standard risk markers (for example ABI) have been better at predicting later outcomes (Criqui et al. 2010). Most of the studies have not found a relation between ABI and hsCRP (McDermott et al. 2003, Santos et al. 2004, Ubronaviciene et al. 2011).

A novel field in cardiovascular research is the assessment of endothelial function. Alterations in it have been considered as the earliest pathological vascular changes that can be detected clinically. Nowadays, flow mediated dilation of brachial artery (FMD) can be considered as the gold standard (Anderson et al. 1989, Laurent et al. 1990, Celermajer et al. 1992 and Sorensen et al. 1995). However, it is technically demanding and unsuitable to be used in an ambulatory setting. This has led to the development of new methods, such as pulse amplitude tonometry after reactive hyperemia (RH-PAT). It detects endothelial dependent vasomotor function in the fingertips. As yet, little is known if this kind of testing can give additional information when assessing subjects at a cardiovascular risk.

In this thesis, the main objectives were to study the risk factor profile and novel methods of risk factor assessment in a cardiovascular risk population. Special attention was paid to hsCRP and RH-PAT and to providing additional information above the traditional risk factors. We also studied the prevalence of endothelial dysfunction in subjects with borderline PAD.

2 REVIEW OF THE LITERATURE

2.1 Peripheral arterial disease

2.1.1 Epidemiology of peripheral arterial disease

Atherosclerotic vascular disease, with its main three manifestations, coronary heart disease (CHD), cerebrovascular disease (CVD) and peripheral arterial disease (PAD), have been the leading causes of death in adults for decades (AHA 2003). The most widely used objective method to detect PAD is the measurement of the ankle brachial index (ABI). Values ≤ 0.90 are considered as PAD (Norgren et al. 2007). The total prevalence of PAD based on objective testing has been evaluated in several epidemiological studies (Criqui et al. 1985, Hiatt et al. 1995 and Selvine et al. 2004). In adult population it has been fluctuating between 3-10% and increasing to 15 to 20% in persons aged over 70 years. Asymptomatic PAD is at least three to four times more common than symptomatic PAD, which occurs as intermittent claudication (IC) or critical leg ischemia (CLI) (Norgren et al. 2007). In epidemiological studies (Diehm et al. 2004, Menke et al. 2006 and Lee et al. 2004), the prevalence of asymptomatic PAD in patients aged over 40 years in the United States is 5%, in patients aged 55-74 years in Scotland 16% and in patients aged over 65 years in Germany 18%. In a study made by Fowkes at al. (1991), a third of the patients with asymptomatic PAD had a complete occlusion of a major artery (common femoral, profunda femoris, superficial femoral or popliteal) to the leg assessed by duplex ultrasound. In a Finnish primary care cohort the prevalence of PAD based on ABI and the documented history of PAD (previous peripheral arterial vascular procedure) ranged from 25.7% to 47.7% (Oksala et al. 2009). These subjects were either symptomatic or risk patients.

2.1.2 Risk factors for peripheral arterial disease

2.1.2.1 Smoking

Basically in every study, smoking is the single most important risk factor. The relation between smoking and IC was first determined at the beginning of the 20th century by Erb (1911). Therefore, efforts to decrease or completely eliminate cigarette smoking have long been the cornerstone of risk factor management in these patients. The effect of smoking on the development of PAD might even be stronger than on that of CHD and a diagnosis of PAD can be made even a decade earlier than in non-smokers (Norgren et al. 2007). For current smoking, the attributable risk of PAD is between 18 and 26% in different studies and current smoking at least doubles the odds of PAD versus nonsmoking (Vogt et al. 1993 and Meijer et al. 2000). In some estimates, this risk is as high as four times greater (Curb et al. 1996 and Selvin et al. 2004). Smoking cessation seems to be related to the decreased incidence of IC. In Edinburgh artery study (Fowkes et al. 1991),

the relative risk of IC was 3.7 in the smokers compared with 3.0 in the ex-smokers (the subjects had discontinued smoking less than five years ago).

2.1.2.2 Diabetes

The role of diabetes (DM) as a risk factor for PAD has been shown in many studies. In addition, insulin resistance even in subjects without DM seems to be a risk factor. Muntner et al. (2005) showed that insulin resistance increases the risk of PAD from 40 to 50%. Symptomatic PAD (IC and CLI) is overall twice as common among diabetics compared to non-diabetic patients (Norgren et al. 2007) and the adjusted risk excess for PAD in diabetics ranged from + 53% in a study by Curb et al. (1996) to almost + 700 % in a study by Criqui et al. (2005). In the study by Selvin et al. (2004 II), for every 1 % increase in hemoglobin A1c, there was a 26% increase in the risk of PAD. The nature of PAD seems to be more aggressive in patients with DM compared to non-diabetics. Compared to the PAD patients without DM, subjects with DM and PAD are five times more likely to undergo an amputation (low or high) than other patients with PAD, and their mortality rate is over three times higher (Jude et al. 2001). Diabetics had tendency towards foot preserving amputations, but they were more likely to undergo repeated operations. In 2003, the American Diabetes Association gave a recommendation to measure ABI every five years in patients with DM to screen for PAD (ADA 2003). In addition, TASC II recommends PAD screening using ABI in all patients aged between 50-69 years with DM (Norgren et al. 2007)

2.1.2.3 Hypertension

Hypertension is considered to be a risk factor for all forms of cardiovascular disease even though the relative risk of developing PAD seems to be less for hypertension than for DM or smoking. Epidemiological studies report an odds ratio for PAD between 1.32 (Meijer et al. 2000) and 2.7 (Cui et al. 2003) in hypertensive patients. Systolic blood pressure seems to be the main contributor since diastolic pressure was not significantly associated with PAD or they had a nonlinear relationship (Meijer et al. 2000, Newman et al. 1993 and Curb et al. 1996). The high prevalence of hypertension, particularly among older patients, makes it a significant contributor to the total risk of PAD in a population. In the Rotterdam study, Meijer et al. (2000) showed that the attributable risk of PAD related to this factor was 17%. It was second only to current smoking in this study population. Likewise in a Framingham study, 30% of the risk of IC was attributable to blood pressure in excess of 160/100 mmHg (Murabito et al. 1997).

2.1.2.4 Dyslipidemia

The total cholesterol level has been the most studied lipid measure in epidemiological surveys, and in the majority of them, it has been significantly associated with PAD in a multivariate analysis (Newman et al. 1993, Curb et al. 1996, Stoffers 1996, Meijer et al. 2000 and Carbayo et al. 2007). These studies have also shown the protective effect of high-density lipoprotein (HDL) cholesterol against PAD. However, in two studies, (Meijer

et al. 1998 and Cui et al. 2003), the total cholesterol level was statistically a significant risk factor in the univariate analysis, but dropped out in the multivariate models in which other lipid measures were considered. In a study by Senti et al. (1992), the patients with PAD had significantly higher levels of serum triglycerides, very low-density lipoprotein (VLDL) cholesterol, VLDL triglycerides, VLDL proteins, intermediate density lipoprotein (IDL) cholesterol, IDL triglycerides and lower levels of HDL cholesterol than the controls. The independent role of high triglyceride levels as a risk factor for PAD is fragmentary. In the Framingham Offspring Study and the Cardiovascular Health Study, triglycerides were significant in a univariate analysis, but they dropped out in multivariable models after adjustment for other lipid measures (Meijer et al. 1998 and Newman et al. 1993). However, there are also studies to suggest their significant and independent association with PAD (Bainton et al. 1994, Katsilambros NL et al. 1996 and Cheng SW et al. 1997) in a multivariate analysis and the others to join triglycerides in a disease progression and more severe PAD (Aboyans et al. 2006, Fowkes et al. 1993 and Smith et al. 1996). To sum up, these results suggest that while the total cholesterol, HDL cholesterol and triglycerides all appear to be associated with PAD in a univariate analysis, in multivariate models triglycerides frequently drop out as an independent risk factor. In addition, many early studies took only account of the total cholesterol leaving the question of the most strongly and independently PAD related lipid-component still open. Finally, increasing evidence is adding lipoprotein(a) to the risk factors of PAD. Aboyans et al. (2006) linked it to the progression of PAD, and it has been observed relate to PAD occurring at a young age (Valentine et al. 1994) and in patients with diabetes (Tseng et al. 2004). To confirm this hypothesis, large population-based studies are still required.

2.1.2.5 Obesity

Despite the fact that obesity is without question harmful to the cardiovascular system, its independent positive association with PAD is controversial. A number of large, population-based studies have failed to find a significant association between obesity and PAD or IC after multivariable adjustment (Meijer et al. 2000, Meijer et al. 1998, Selvin et al. 2004 and Carbayo et al. 2007), but in a study by Bowlin et al. (1994) there was an odds ratio of 1.24 for incident claudication related to a difference of 5.0 kg/m^2 in the body mass index (BMI). The reason for these findings is that obesity is implicated in the etiology of other risk factors for PAD (hypertension, type II DM and dyslipidemia) and adjusting these factors ignores most of the mechanisms by which obesity might cause PAD. In addition, there is some evidence that it is central adiposity, not obesity per se, that is more closely related to a risk of PAD. Vogt et al. (1993) showed that after adjustment for BMI, a higher waist/hip ratio was associated with a significantly higher risk of PAD. Also in diabetic patients, incident PAD was associated with baseline waist/hip ratio, but not BMI (Wattanakit et al. 2005).

2.1.2.6 Ethnicity

Most of the epidemiological studies of PAD have been conducted in non-Hispanic white populations. However, there is pooled data of seven U.S population based cross sectional

studies reporting different rates of PAD among five ethnic groups (Allison et al. 2007). Patients in this study were >40 years old and the prevalence of PAD was 5.5%, 8.8%, 2.8%, 2.6% and 6.1% in non-Hispanic whites, African Americans, Hispanics, Asians and Native Americans, respectively. Also the GENOA (Genetic Epidemiology Network of Arteriopathy) study showed that the higher prevalence of PAD in non-Hispanic blacks was not explained by a difference in the classical risk factors for atherosclerosis (Kullo et al. 2003).

2.1.2.7 Gender

Particularly in younger age groups, the prevalence of PAD is slightly greater in men than in women. When examining patients with IC, the ratio of men to women is between 1:1 and 2:1, increasing to 3:1 in more severe cases such as CLI (Norgren et al. 2007).

2.1.2.8 Hyperhomocysteinemia

Some older studies have suggested that hyperhomocystinemia might actually be the single most powerful risk factor for PAD (Boushey et al. 1995). These were case-control studies suggesting an odds ratio of 6.8 for every 5 mmol/l increase in fasting homocysteine. Obviously, this is not the case since more recent studies have produced much lower and even non-significant estimates of the PAD risk associated with homocysteine. Graham et al. (1997) estimated only a borderline statistically significant odds ratio of 1.7 for subjects with the highest levels of homocysteine in their case-control study, and Meijer et al. (2000) found no significant relationship between fasting homocysteine levels and PAD in the Rotterdam study cohort. The role of homocysteine in PAD is also questioned because there is lack of evidence that supplementation of vitamin B or folate improves the condition despite the fact that they decrease the blood homocysteine concentration (Taylor et al. 1999). These substances are co-enzymes participating in the homocysteine metabolism.

2.1.2.9 Chronic kidney disease

Fisbane et al. (1995) studied vascular disease in hemodialysis patients in whom the prevalence of PAD (ABI < 0.9) was 38% to which should be added 14% of cases with incompressible arteries. In a Finnish study by Leskinen et al. (2002), the prevalence of PAD was 22% in the predialysis patients and 31% in the dialysis patients. Renal transplantation seems to have a favorable effect on vascular health, and the prevalence of PAD was 15% in this patient group (Leskinen et al. 2002). PAD is also an important source of morbidity and mortality in uremic patients (Cheung et al. 2002). Although chronic kidney disease and PAD share many common risk factors (age, hypertension and DM), it has been shown that high creatinine levels are also independently associated with PAD (Newman et al. 1993). Wattanakit et al. (2007) followed 14 280 patients for a mean period of 13 years in the Atherosclerosis Risk in Communities study. They showed that chronic kidney disease is significantly associated with PAD with a hazard ratio of 1.56 in a multivariable model including the classical risk factors.

2.1.2.10 Infection

Infectious agents may also lie behind the process of atherogenesis (Libby et al. 1997 and Danesh et al. 1997). Acute infections may alter hemodynamics and the clotting status in ways that can precipitate ischemic events. Chronic extravascular infections such as gingivitis, prostatitis, bronchitis etc. can play a role in this process through augmenting the extravascular production of inflammatory cytokines. They may then accelerate the evolution of remote atherosclerotic lesions. There is also evidence that periodontal infection and anti-cardiolipin antibodies are involved in the development of Buerger´s disease (Chen et al. 2009). Intravascular infections might also provide a local inflammatory stimulus that could lead to atherogenesis. Researchers have found signs of infection by microbial agents such as *Chlamydia pneumoniae.* These bacteria may release lipopolysaccharide (endotoxin) and heat shock proteins that can stimulate the production of proinflammatory mediators by vascular endothelial cells, smooth muscle cells and infiltrating leukocytes alike. Other infectious agents suspected to take part in atherogenesis are herpes simplex virus, cytomegalovirus and *Helicobacter pylori.* However, there is little prospective evidence that antibodies against these microbes predict vascular risk (Kol et al. 1999 and Libby et al. 2002).

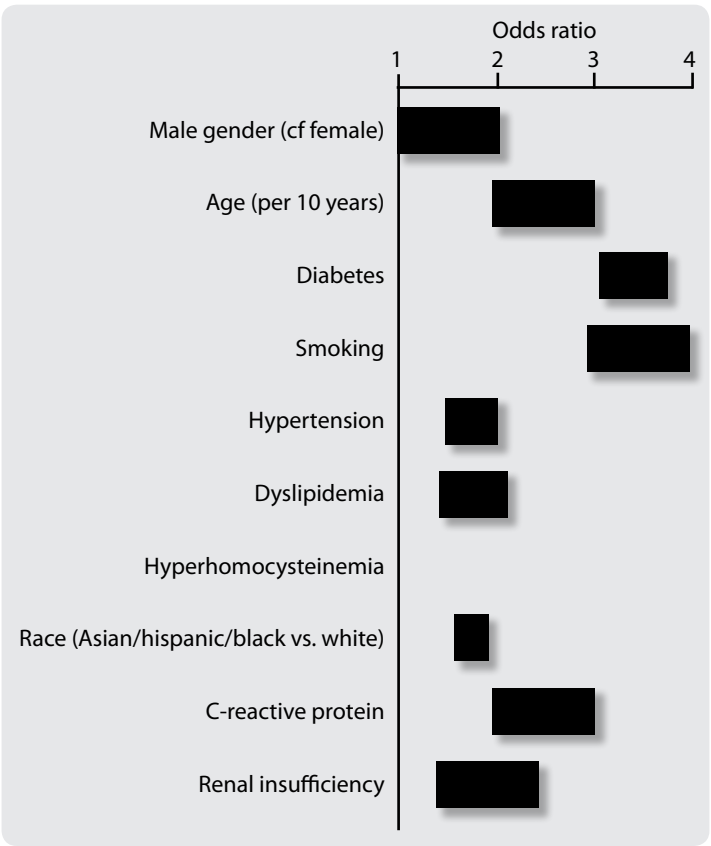

Figure 1. Approximate range of odds ratios of the risk factors for symtomatic peripheral arterial disease. (Norgren et al. 2007)

2.1.3 Progression of peripheral arterial disease and PAD as a predictor of mortality and morbidity

The natural course of PAD can be estimated through studies performed prior to the development of novel revascularization techniques and medications. The classical cohort of patients, diagnosed by symptoms and clinical examination only, were followed in the Mayo Clinic between 1939 and 1948 (Juergens et al. 1960). Two-thirds of the patients suffered from IC, while the remainder presented pain at rest and/or trophic lesions. After five and 10 years, the survival rates were around 75% and 50%. Three fourths of the deaths were related to cardiovascular causes. Almost 15% of the survivors needed amputation, a minority of these patients who initially had an IC. None of the patients who abstained from smoking needed amputation, versus 11.3% of those who continued smoking.

More recent data is available from Hirsch et al. (2006). They gathered information from the PAD population aged 50 years or older. At the presentation, 20-50% of the patients had asymptomatic PAD and 10-35% typical IC. Their five-year outcome of limb morbidity and cardiovascular morbidity and mortality is practically the same. Of them, 70-80% will have stable IC, 10-20% worsening IC and 5-10 % will develop CLI. Every fifth of these patients will have non-fatal cardiovascular event during the five year follow up. The overall mortality in this population is 10-15%, and 75% of these deaths are related to cardiovascular causes. The prognosis is much worse if the patient has CLI: during the one-year follow up 45% are alive with two limbs, 30% require amputation and the mortality rate is 25%. This reflects the increased total cardiovascular risk in this patient group.

The risk of a patient with PAD to develop CLI is multifactorial. These factors appear to be independent and are probably additional. It is estimated that there is between 500 and 1000 new cases of CLI every year in the European or North American population of one million (Norgren et. al 2007).

The prognosis of the limb in patients with symptomatic PAD has not substantially improved over the years. There are factors that have an impact on this data such as access to health care for treatment of PAD in different countries, the changing patterns of risk factors – especially the prevalence of DM – and the improved overall survival. In the study by Goessens et al. (2007), 7.6% of the patients referred to vascular centers underwent amputation. There are also two large epidemiological studies from the 1980s to show that only 1.6 and 1.8% of the patients who developed IC had to undergo amputation (Kannel et al. 1985 and Widmer et al. 1985). In the Edinburgh Artery Study, 8.2% of the patients with IC had undergone vascular surgery or amputation (Leng et al. 1996). A nationwide Finnish study from the early 2000s suggested that increasing infrapopliteal reconstruction surgeries reduce the need for below-knee amputations (Luther et al. 2000). Even in the elderly patients ≥ 80 years of age, infrapopliteal bybass seems to be effective in reducing the requirements of amputation (Eskelinen et al. 2003).

The first study of the cardiovascular prognosis of patients with clinical PAD is that conducted by Juergens et al. (1960) as discussed above, and it was followed by the Framingham cohort (Kannel et al. 1970, Kannel et al. 1971 and Kannel et al. 1985).

These studies showed the excessive risk of cardiovascular morbidity and mortality related to PAD, but it was markedly attenuated when the subjects with baseline CVD and CHD were excluded. There is also Finnish data from early 1980s that failed to find an association between IC and total or cardiovascular mortality in men after adjusting for other cardiovascular risk factors and baseline cardiovascular disease (Reunanen et al. 1982). However, in the Whitehall cohort from England, IC was a significant predictor of cardiovascular disease mortality after adjusting for cardiovascular risk factors. This finding remained after excluding the patients with baseline cardiovascular disease. The survival of PAD patients is universally poor if they develop CLI. Of these patients, 20 to 25% die during the first year of medical management (Bertele et al. 1999).

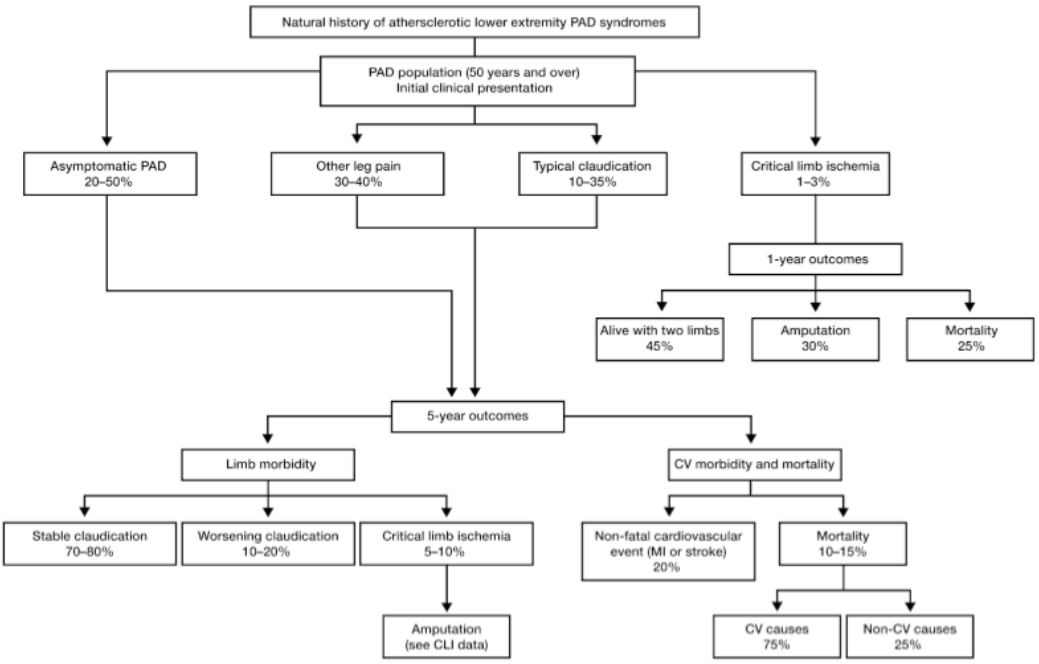

Figure 2. Fate of the claudicant over the next 5 years. PAD – peripheral arterial disease; CLI – critical leg ischemia; CV – cardiovascular; MI – myocardial infarction (Norgren et al. 2007)

The development of ABI and other noninvasive measures of PAD permitted further investigation into the association of PAD with cardiovascular disease. Now there is evidence, that ABI is related to cardiovascular disease mortality and morbidity in a variety of populations. Studies have included patients from vascular laboratories (McKenna et al. 1991 and McDermott et al. 1994), elderly patients with hypertension (Newman et al. 1993 II), elderly women (Vogt et al. 1993 II) and patients from large epidemiological cohorts including data from the Edinburgh Artery Study and the Cardiovascular Health Study (Kornitzer et al. 1995, Leng et al. 1996 II, Newman et al. 1998). In these studies, other known cardiovascular risk factors were controlled as well as the presence of cardiovascular risk at baseline. Many of these studies also connected PAD to the incident CHD. A large meta-analysis including nine studies and over 28 000 subjects was made

by Doobay et al. in 2005. It showed that the low ABI (between 0.80 and 0.90) predicted all-cause mortality (a positive likelihood ratio of 4.0) and cardiovascular mortality (a positive likelihood ratio of 5.6), but with low sensitivity.

2.1.4 Relationship between PAD and other atherosclerotic disease

Peripheral arterial disease, coronary heart disease and cerebrovascular disease, all manifestations of atherosclerotic disease, commonly occur together. They are reported to occur with different frequencies in different studies. In the PARTNERS study by Hirsch et al. (2001), 13% of the subjects screened had ABI $\leq$ 0.9 and no other manifestations of cardiovascular disease defined as history of atherosclerotic coronary, cerebral, or abdominal aortic aneurysmal disease. Of these subjects, 16% had PAD and other cardiovascular disease, and 24% had some other cardiovascular disease manifestation, but a normal ABI. As reported by a TASC II working group in primary care, roughly every second patient diagnosed with PAD will also have CHD and CVD and this number might be even higher in the PAD patients referred to vascular clinics. Autopsy studies have shown that patients dying from myocardial infarction are twice as likely to have a significant stenosis in the iliac and carotid arteries compared to patients dying from other causes (Norgren et al. 2007)

Carotid artery disease occurs in 26 to 50% of patients with IC, but only 5% of patients with PAD have a history of CVD (Norgren et al. 2007). Bhatt et al. (2006) studied patients with symptomatic PAD and found a concomitant prevalence of CVD of 1.2 %. Of these subjects, 1.6% had all the three major manifestations of atherosclerotic disease and 65% of the patients with PAD had clinical evidence of other vascular disease.

In addition, the prevalence of renal artery stenosis of 50% or more is common in patients with PAD (ranging from 23 to 42%) compared to hypertensive general population. Their prevalence of renal artery stenosis is around 3%. This may also be an independent risk factor for mortality in patients with PAD (Norgren et al. 2007).

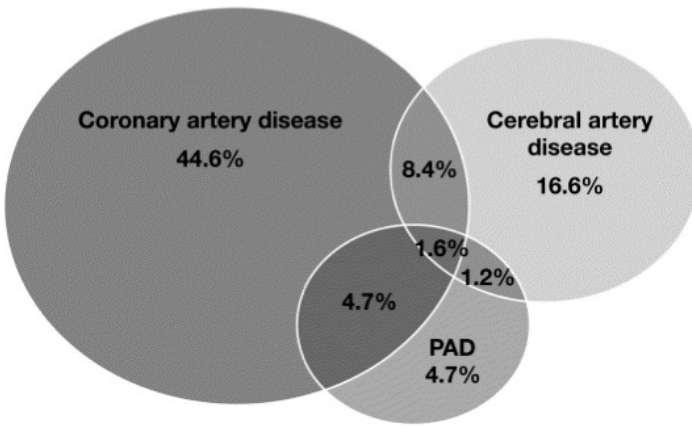

Figure 3. Typical overlap in vascular disease affecting different territories (Adapted from Norgren et al. 2007 and based on data from Bhatt et al. 2006)

2.2 Ankle brachial index

2.2.1 Measurement of ankle brachial index

The ankle brachial index (ABI) is a non-invasive, relatively simple, inexpensive and reproducible method to assess arterial blood flow impairment in the lower limbs. It is possible to measure it whenever there is access to a sphygmomanometer and a Doppler instrument. TASC II recommends a method using a 10-12 cm sphygmomanometer cuff placed just above the ankle and a Doppler instrument used to measure the systolic pressure of the ATP and ADP of each leg. First, the pressure in the cuff is elevated to the suprasystolic level (disappearance of Doppler signal) and then reduced until the signal reappears. This indicates systolic blood pressure in the artery. These pressures are then normalized to the higher brachial pressure of either arm to form the ABI. Measurement should be performed in a supine position and in a warm room. The patient should rest at least five minutes before measurement (Norgren et al. 2007).

When assessing the perfusion of the leg, it is recommended to use the higher of the ankle pressures. In contrast, when assessing the patient's cardiovascular risk, the lower of the two ankle pressures can be used. This may point out vascular disease confined to the distal vascular bed and detect a cardiovascular risk in a greater number of patients. This has a great impact on the patient´s prognosis, since the risk of CHD, CVD or cardiovascular death is almost equal despite the method used to calculate ABI, and when using a higher ABI, a group of patients at a high risk of cardiovascular events is overlooked (Espinola-Klein et al. 2008).

The normal ABI is over 1.00 and a typical cut point to diagnose PAD is ≤ 0.90 at rest. Fowkes et al. (1988) demonstrated that with an ABI threshold of 0.90, the sensitivity of ABI was 95% and specificity was 100% compared with angiography. ABI values over 1.40 suggest mediasclerosis, which is usually due to diabetes or chronic renal disease. In these patients, the tibial vessels become non-compressible leading to a false elevation of the ankle pressure. In the different threshold levels of ABI the specificities in identifying PAD have been estimated to be 86% (ABI 1.3), 94% (ABI 1.4) and 96% (ABI 1.5). The corresponding sensitivities were only satisfactory; 44%, 38% and 36%, respectively (Suominen et al. 2008). On these patients, additional non-invasive diagnostic testing (toe pressures or toe brachial index) should be performed to evaluate the patient for PAD (Norgren et al. 2007). In a Finnish study by Suominen et al. (2010) the mortality was highest in the elevated ABI group (ABI ≥ 1.3) compared to the low and normal ABI group. 35.7% in the high ABI group, 30.1% in the low ABI group and 16.0% in the normal ABI group died during follow-up. PAD was an independent risk factor for mortality among patients with elevated ABI. When an ABI value is < 0.50, the risk of a progression to CLI in the subsequent 6.5 years is likely (Jelnes et al. 1986).

2.2.2 Reproducibility of the ankle brachial index

Multiple investigations have evaluated the inter-observer variability of the ABI measurement. In the study by Endres et al. (2006), three different observer groups were compared. The first included six angiologists, the second six primary care physicians and the third six trained medical office assistants. They performed two ABI measurements each on six individuals from a group of 36 unselected subjects and found the mean difference between the ABI measurements close to zero. However, in the study by Mätzke et al. (2003), in patients with CLI, 16% of ABI values differed by 0.15 or more from the median when the measurements were taken by doctors with little or no instructions. The conclusion was that training is the key to obtain reproducibility and quantitative measurement values.

2.2.3 Prevalence of PAD and borderline PAD

Menke et al. (2006) studied the prevalence of PAD and borderline PAD using ABI measurements in a large cohort of patients aged over 40 years. The prevalence of PAD was 5.0% and the prevalence of borderline PAD was 8.7%. Low ABI values were more common in the older patient groups. In a previously mentioned PARTNERS study (Hirsch et al. 2001), in a primary care setting the prevalence of ABI $\leq$ 0.9 was 29%. This study group consisted of patients over 70 years old or 50 to 69 years old with diabetes or prolonged history of smoking. In the German GetABI study (Diehm et al. 2004) in which primary care physicians measured ABI from patients aged $\geq$ 65 years, the prevalence of ABI $\leq$ 0.90 was 18%. In Scotland with the same kind of setting, the prevalence was 16% (Lee et al. 2004). Most of these patients were asymptomatic since only 3-11% of them had a history of IC.

2.2.4 Ankle brachial index and functional capacity

Several studies have shown that ABI is associated with functional capacity. It is also a useful tool in asymptomatic patients. McDermott et al. (2000) launched The Women´s Health and Aging Study in which they evaluated disabled women aged 65 years or older and used ABI as a measure of lower extremity function. The results showed that decreasing ABI values were related to worse functional scores. The results persisted even after adjustment for age, race, smoking and co-morbidities. Lower ABI scores correlated with slower walking velocities, poorer standing balance scores, slower time to rise, and shorter walking distance per week.

2.2.5 Ankle brachial index as a prognostic tool

An abnormal ABI is predictive of both CHD and CVD. There is an inverse relationship between CVD and ABI (Newman et al. 1993). In this study, the participants with ABI < 0.9 were more than twice as likely as those with a normal ABI to have a history of myocardial infarction, congestive heart failure, stroke, angina, or a transient ischemic

attack. In the study by McKenna et al. (1991), five-year mortality was 50% and 30% in the patients with an ABI of 0.40 and 0.70, respectively. Five-year mortality of 37% was associated with ABI less than 0.50, 29% with ABI between 0.50 and 0.69 and 9% with an ABI between 0.70 and 0.89 (Sikkink et al. 1997). Criqui et al. (1992) made a ten-year follow up for 67 patients with ABI 0.80 or lower. There was a dramatic increase in the rate of mortality in men (61.8%) and women (33.3%) when compared with the men (16.9%) and women (11.6%) without PAD.

Also, the ABI values in the borderline range of normal and pathologic (0.91-1.00) are related to the increased cardiovascular risk (Fowkes et al. 2008). The risk of cardiovascular death is 1.7 times higher in men and 1.8 times higher in women with ABI values 0.91-1.00 compared to the reference level of 1.11-1.20.

TASC II concludes that ABI can confirm the diagnosis of PAD, detect PAD in asymptomatic (sedentary) patients, help differentiating PAD from other causes of leg symptoms, identify patients with reduced limb function, provide key information on the long term prognosis and provide further risk stratification in patients at a cardiovascular risk. It should be measured in all patients with exertional leg symptoms and aged between 50-69 with a cardiovascular risk factor (particularly diabetes or smoking). In addition, it should be measured in all patients aged ≥ 70 years regardless of the risk factor status and with the Framingham risk score from 10% to 20% (Norgren et al. 2007).

2.3 Endothelium and inflammation in cardiovascular disease

2.3.1 Structure of the endothelium

Every vessel wall throughout the body is covered on its inner, luminal surface by a monolayer of specialized cells. These are called vascular endothelial cells and they represent the primary anatomical site that separates the compartment of the flowing blood from the body´s interstitium. It has been estimated that the total surface area of these cells is 350 m^2 and the endothelium may account for as many as 6×10^{13} cells. The total weight of these cells can be as much as that of the liver (Furchgott et al. 1980 and Ross 1999.)

2.3.2 Function of the endothelium

The endothelium is far from being just a passive membrane. It is actively involved in many functions, such as the control and regulation of vascular tone, fluid and solute exchange, haemostasis and coagulation and inflammatory responses. Most of these functions depend critically on actions occurring on the surface of the endothelial cells in the vascular lumen (Pries et al. 2000). This position exposes endothelium to hemodynamic forces exerted by blood flow, blood pressure, and vascular distension. In addition, the endothelium receives chemical signals, both blood-borne and tissue-

derived. These stimuli may elicit endothelial responses locally in the vessel wall or on more distant sites to control the passage of solutes, macromolecules, and blood cells across the vascular wall. In respond to injury, the endothelium can initiate angiogenesis and re-endothelialization of an exposed vascular intima (Böger 2009).

Nitric oxide (NO) is a key player in a variety of regulatory mechanisms of the cardiovascular system. It is regarded as the main mediator of endothelium dependent vasodilation (Böger 2009). It also inhibits smooth muscle cell proliferation and platelet and monocyte adhesion. NO has antioxidant effects resulting in a reduced superoxide radical regeneration and it diminishes the oxidation of LDL cholesterol. These are all mechanisms known to contribute to the pathogenesis of atherosclerosis, and thus, NO can be considered as an endogenous anti-atherogenic molecule (Böger 2003).

Endothelial cells also produce vasoconstrictive agents. One of the most potent of them is 21-residue vasoconstrictor peptide endothelin (Yanagisawa et al. 1998). Endothelin functions as a circulating hormone and as a paracrine factor and it is involved in the regulation of vascular tone and systemic blood pressure (Wei et al. 1994). Its production is induced by substances such as epinephrine, interleukine-1, thrombin, and by conditions such as shear stress and damage to the endothelial cells. Endothelin has been linked to a variety of cardiovascular conditions such as atherosclerosis, hypertension, congestive heart failure, acute coronary ischemic syndromes, vasospasm, and acute and chronic renal failure (Dohi et al. 1991, Lerman et al. 1991, Stewart et al. 1991 and Koyama at al. 1989).

2.3.3 Endothelial dysfunction and the role of inflammation

Endothelial dysfunction has been traditionally described as the earliest manifestation of atherosclerosis. By virtue of NO, the endothelium regulates anti-inflammatory, mitogenic, and contractile activities of the vessel wall as well as the hemostatic process within the vessel lumen (Bonetti et al. 2003). Arterial bifurcations and bending points are vulnerable to disturbances of blood flow and they are starting points of the endothelial dysfunction (Ravensbergen et al. 1998 and Nerem et al. 1992). Endothelial dysfunction is also associated with the biohumoral risk factors such as hypercholesterolemia, DM, hypertension, obesity, smoking, and advanced age (Traub et al. 1999, Kunsch et al. 1999 and Cai et al. 2000). This leads to the decreased levels of NO synthesis, increased vessel wall entry and oxidation of circulating lipoproteins, facilitated monocyte entry, facilitated smooth muscle cell proliferation and extracellular matrix deposition and vasoconstriction. Endothelial dysfunction also triggers a prothrombotic state within the vessel lumen (Ignarro et al. 2004 and Voetsch B et al. 2004).

As previously mentioned, endothelial dysfunction increases the vessel wall entry of lipoproteins. LDL particles infiltrate through the arterial endothelium into the intima where they bind themselves to the proteoglycans. This combination makes LDL particles proinflammatory, chemotoxic, cytotoxic, and proatherogenic (Steinberg et

al. 1989), leading to the activation of endothelial cells to express adhesion molecules on their surface. These molecules regulate the interaction of monocytes and T-cells resulting in the recruitment of monocytes and lymphocytes to the atherosclerotic lesion. Cytokines, such as interleukin-8, may also play a role in this migration (Libby 2002 and Hansson 2005). Monocytes then differentiate into macrophages under the influence of macrophage colony stimulating factor and internalize the oxidized LDL. This step is considered critical for the development of atherosclerosis (Smith et al. 1995). If the cholesterol derived from the uptake of oxidized LDL particles cannot be mobilized from the cell to a sufficient extent, it accumulates as cytosolic droplets. Ultimately, this leads to foam cell formation, which is the prototypical cell in the atherosclerosis (Hansson 2005). These foam cells then form the fatty streak, an early and asymptomatic lesion. Through the multiplication of smooth muscle cells, fatty streaks evolve into complicated atheroma (Libby 2002). As these lesions become more bulky, the arterial lumen narrows leading to the clinical manifestations of cardiovascular disease.

Inflammation also plays a role in the process of plaque disruption. The macrophages and mast cells are capable of degrading the extracellular matrix by phagocytosis or secretion of proteolytic enzymes. This may weaken the fibrous cap of the plaque, predisposing it to rupture (Shah et al. 1995 and 2001). This mechanism is unlikely to occur in the peripheral arteries since acute occlusion of the peripheral vasculature frequently results from thromboemboli of cardiac or aortic origin (Dieter et al. 2002 and Faxon et al. 2004).

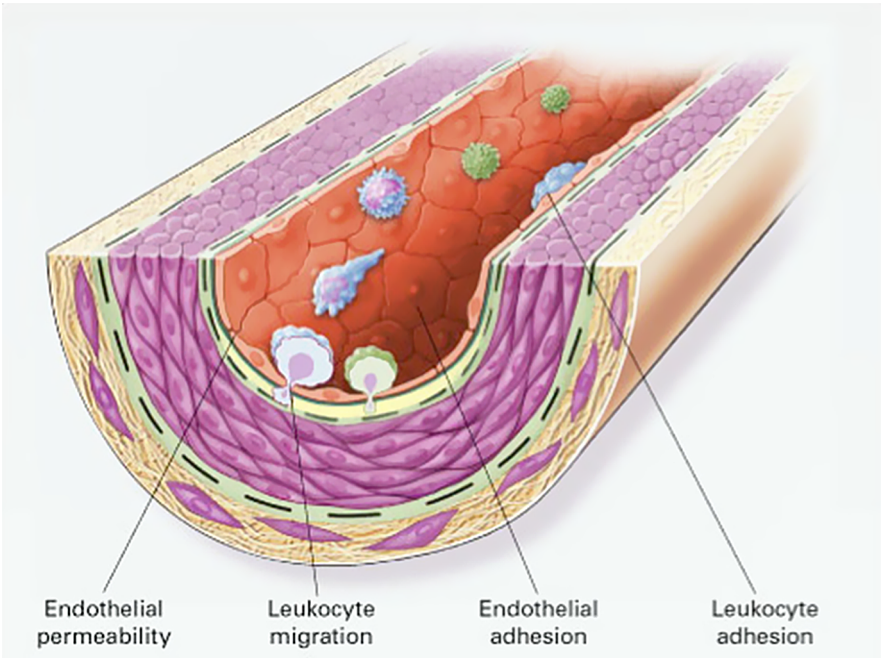

Figure 4. Endothelial dysfunction in atherosclerosis: increased permeability to lipoproteins and other plasma constituents, up-regulation of leukocyte adhesion molecules, up-regulation of endothelial adhesion molecules, and migration of leukocytes into the artery wall, which is mediated by oxidized low-density lipoprotein. Adapted from Ross (1999).

2.3.4 Inflammatory biomarkers in cardiovascular disease

2.3.4.1 Rationale for measuring biomarkers in cardiovascular disease

As discussed above, inflammation is part of the whole spectrum of cardiovascular disease, including the earliest steps in the atherogenesis. Instead of just a disease of lipid accumulation, thrombosis can now be characterized as a disorder of low-grade vascular inflammation. This concept can be used to assess a future cardiovascular risk since elevated levels of several inflammatory mediators among apparently healthy men and women have proven to have predictive value for future vascular events (Libby et al. 2002). There is evidence from prospective epidemiological studies that an increased vascular risk is associated with increased basal levels of cytokines such as interleukine-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Ridker et al. 1997, 2000 and 2000II and Harris et al. 1999); cell adhesion molecules such as soluble intercellular adhesion molecule-1 (ICAM-1), P selectin, and E selectin (Hwang et al. 1997, Ridker et al. 1998 and Ridker et al. 2001); and acute-phase reactants such as c-reactive protein (CRP), fibrinogen and serum amyloid A (Ridker et al. 2000 III, Haverkate et al. 1997, Kuller et al. 1996, Tracy et al. 1997 and Danesh 2000). These inflammatory markers are closely related to the several traditional cardiovascular risk factors such as obesity and BMI, because adipocytes can produce inflammatory cytokines. A common underlying disorder of innate immunity is reported to link obesity, accelerated atherosclerosis, and insulin resistance (Pickup et al. 1998). To support the role of inflammation in diabetogenesis, elevated levels of IL-6 and CRP have been associated with the development of type II DM even among the individuals with no current evidence of insulin resistance (Pradhan et al. 2001).

2.3.4.2 High-sensitivity CRP as a cardiovascular risk marker

For clinical purposes, the most widely used inflammatory biomarker is CRP. It was first discovered by Tillett and Francis in 1930 in the serum of patients with pneumococcal pneumonia (Tillett et al. 1930), and after studies by Löfström (1944), CRP became recognized as a nonspecific acute phase protein. CRP is considered as a major inflammatory cytokine that functions as a nonspecific defense mechanism in response to tissue injury and infection. It is mainly synthesized in the liver, and it belongs to the pentraxin family of innate immune system proteins (Pepys 1981). CRP has a long half-life affording stability of levels with no observable circadian variation (Meier-Ewert et al. 2001). It is easy to measure in a usual outpatient setting, and standardized high sensitivity assays (hsCRP) provide similar results in fresh, stored, and frozen plasma (Rifai et al. 1999). In addition to this, CRP seems to have several direct effects that may affect vascular disease progression. It activates complement, induces the expression of several cell adhesion molecules as well as tissue factor, mediates LDL uptake by endothelial macrophages, induces monocyte recruitment into the arterial wall and enhances the production of monocyte chemoattractant protein-1 (Torzewski et al. 2000, Bhakdi et al. 1999, Pasceri et al. 2000, 2001 and Zwaka et al. 2001).

In population-based studies, the baseline levels of CRP predict future cardiovascular events. Ridker (2001 II) has proposed that CRP testing might thus have a major adjunctive role in the assessment of cardiovascular risk as a part of primary prevention.

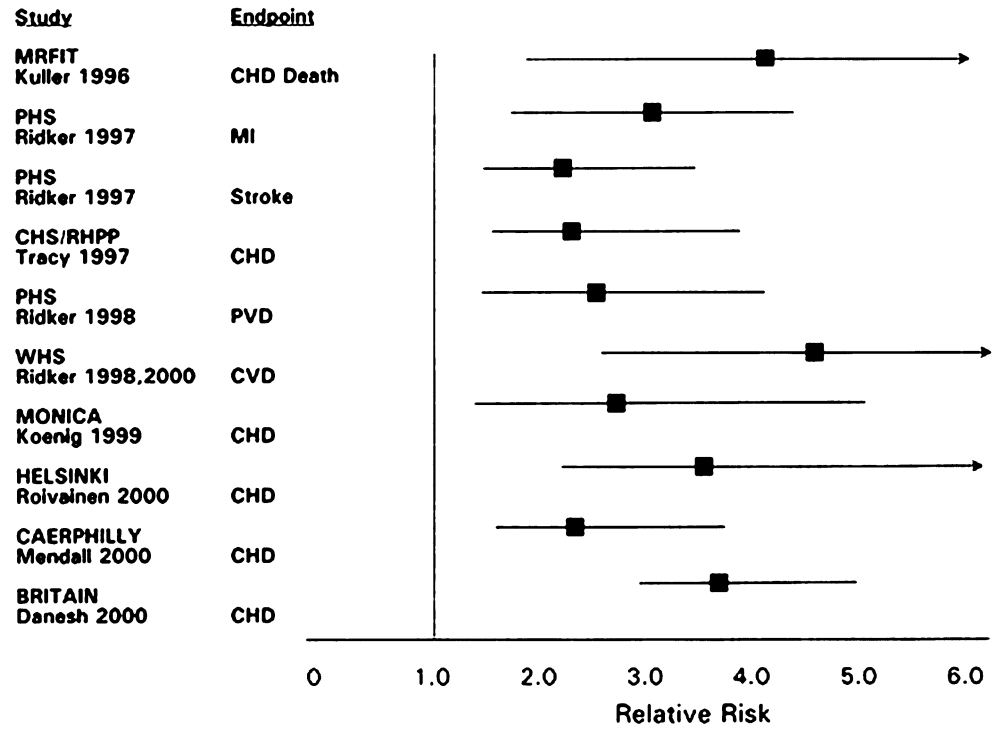

Figure 5. Prospective studies of hsCRP as a marker for future cardiovascular events among individuals without known coronary disease (Ridker 2001 II). MI: myocardial infarction, PVD: peripheral vascular disease

Yet worth mentioning is a study by Reilly et al. (2003) where they found no association between coronary artery calcifications and CRP. This may indicate that the relation between CRP and clinical events might not be related to atherosclerotic burden and these two entities can provide distinct information regarding the cardiovascular risk.

2.3.4.3 High-sensitivity CRP in a peripheral arterial disease

Evidence of the predictive role of CRP in a PAD population is scarce and quite conflicting. Brevetti et al. (2008) studied if CRP predicts an increased risk of major cardiovascular events and adds to the prognostic value of ABI. They found no evidence of that. In a study by Criqui et al. (2010), a cohort of 397 patients with PAD were examined. HsCRP was a strong predictor of short-term mortality at two years, but the standard risk markers (ABI, age, hypertension etc.) were better at predicting longer-term mortality. Elevated hsCRP levels in subjects with PAD also predicted short-term cardiovascular and all-cause mortality in a study by Vidula et al. (2008). Ubronaviciene et al. (2011) concluded in their study that hsCRP is unlikely to be regarded as a useful prognostic clinical marker

alone in patients with PAD because of its limited predictive capability. They studied patients with a symptomatic PAD and found no association between hsCRP and lethal outcome.

2.3.4.4 Relation of high-sensitivity CRP to ankle brachial index

As discussed above, ABI can be used as an indicator of the presence and severity of PAD. McDermott et al. (2003) studied the relation of ABI and hsCRP and found no association in patients without history of CHD or CVD. Also Santos et al. (2004) studied the relation of inflammatory markers to ABI. Their results showed no independent association between hsCRP and ABI. The same finding was reported in a previously mentioned study by Ubronaviciene et al. (2011). This may indicate that CRP is not a perfect tool to assess the total cardiovascular burden. However, Elias-Smale et al. (2007) found an independent and graded relation between CRP and ABI in the longitudinal Rotterdam Elderly Study.

2.3.5 Assessment of endothelial function

Alterations in endothelial function are the earliest pathological vascular changes that can be detected clinically. These processes start well before any structural abnormalities are possible to identify (for example by angiography) leading later to clinical complications (Ross 1999). Topol et al. (1995) showed that angiography may miss significant atherosclerosis that is present in the vessel wall before eventual encroachment on the lumen occurs. In a study by Vane et al. (1990), impaired endothelium-dependent dilation in the coronary circulation was associated with coronary atherosclerosis. It is also related to coronary risk factors and endothelial function can be improved by risk-reduction therapy (Vita et al. 1990 and 2000). The link between coronary endothelial dysfunction and cardiovascular events in the absence of obstructive CHD was demonstrated by two different studies at the beginning of the 21st century (Suwaidi et al. 2000 and Schächinger et al. 2000). Recently, it was suggested that the reduced endothelial function in shift workers might at least partly explain their increased cardiovascular morbidity (Suessenbacher et al. 2011). Endothelial function is also impaired in asymptomatic adolescents with type I diabetes and may thus point out therapeutic and prognostic implications in this young age group (Mahmud et al. 2006)

2.3.5.1 Flow-mediated dilation

The first studies to detect endothelial function in humans involved measurement of the response of the coronary arteries to infused acetylcholine (Ludmer et al. 1986). This kind of methodology is, however, unsuitable to large-scale studies due to its invasiveness. The first non-invasive method to study endothelial function was described by Celermajer et al. (1992). This method involves measuring the diameter of an artery (usually brachial artery) using ultrasound before and after increasing shear stress induced by reactive hyperemia. Detected dilatation reflects arterial endothelial NO release. It has been used

to show disease reversibility (Woo et al. 2004) and it correlates to the findings in the coronary circulation (Anderson et al. 1995). These results were reproduced by Matsuo et al. (2004) nine years later. One limitation of this method is that it is technically demanding, requiring specific training (Celermajer 2008). In addition, FMD can be transiently altered by factors that may not have great importance for a long-term atherosclerotic risk such as an acute infection or a meal and it varies in a circadian pattern. To standardize this technique, guidelines from the International Brachial Artery Reactivity Task Force were published in 2002 (Corretti et al. 2002).

2.3.5.2 Pulse amplitude tonometry after reactive hyperemia

Some new tests have emerged to the field of endothelial function testing in recent years. Particular interest has been shown in testing endothelial vasomotor function after reactive hyperemia in the fingertips. The basis for this testing is that endogenous NO-mediated vasoregulation is particularly prominent in the arterio-venous anastomosis in the human fingertips (Noon et al. 1996) and majority (60%) of the RH-PAT response is mediated by NO release (Nohria et al. 2006). In a comparative study performed by Bonetti et al. (2004), a group of 94 subjects underwent an angiographic assessment of coronary endothelial function and subsequent RH-PAT tests. In this study a reactive hyperemia index (RHI) cut-off value of 1.67 provided a sensitivity of 82% and a specificity of 77% to diagnose coronary endothelial dysfunction.

Digital pulse amplitude is usually measured in a fasting state. A PAT device is placed on the tip of each index finger. A pneumatic plethysmograph that applies uniform pressure to the surface of the distal finger is used. This allows the measurement of pulse volume changes in the finger. A contralateral index finger serves as an internal control. The inflation pressure of the digital device is electronically set to 10 mmHg below diastolic blood pressure or 70 mmHg (whichever is lower). The baseline pulse amplitude is measured from each fingertip for five minutes and then arterial flow is interrupted for another five minutes inflating the cuff 40mmHg above the patients´ systolic blood pressure or to 200mmHg. During this time subject might feel numbness of the hand and this may feel uncomfortable. However, it is harmless. After rapid cuff deflation, the pulse amplitude is recorded electronically up to five minutes in both fingers and analyzed by an automated algorithm provided by the manufacturer of the device. This represents the reactive hyperemia phase.

Kuvin et al. (2003) showed that this method demonstrates patterns of abnormality similar to ultrasound detection of FMD. They concluded that RH-PAT is influenced by factors known to affect endothelial function, and it may be used to study peripheral vascular endothelial function. Its clear advantage is that it can be used in an ambulatory setting (Kuvin et al. 2007). In addition, the results correlate with the CHD risk and the presence or absence of CHD (Bonetti et al. 2004 and Kuvin et al. 2007). In the study by Hamburg et al. (2008), nearly 2000 subjects in the Framingham Third Generation Cohort were assessed. In this cohort, the association with obesity and diabetes was

especially strong and this may mean that metabolic risk factors in particular are reflected in microvessel responses. This study revealed a significant, although relatively weak, correlation between most cardiovascular risk factors and RH-PAT. Unexplained remain the findings that RH-PAT did not correlate with hypertension and that RH-PAT responses were slightly higher with older age. It is also questionable if RH-PAT measurements give any independent prognostic information.

Several studies have also demonstrated the good reproducibility of RH-PAT testing. These results are equal or even above the published reproducibility of brachial artery FMD assessment when operated by a qualified sonographer (Selamet et al. 2009, Tomfohr et al. 2008 and Haller et al 2007).

In addition to cardiovascular disease, RH-PAT has been used in other indications as well. Recent studies have included for example polycystic ovary syndrome (Lowenstein et al. 2007), pre-eclamptic toxemia (Yinon et al. 2006) and inflammatory bowel disease (Rolfman et al. 2007).

3 AIMS

This study was initiated to investigate the risk factor profile and novel methods of risk factor assessment in a Finnish cardiovascular risk population. The present investigation had the following aims:

1. To investigate the relationship between ABI, age, gender and smoking in a cardiovascular risk population

2. To determine the prevalence of PAD among the hypertensive subjects without co-morbidities

3. To study the correlation between hsCRP and ABI, cardiovascular risk factors and PAD.

4. To determine the prevalence of endothelial dysfunction in a cardiovascular risk population with borderline PAD and to test the usefulness of RH-PAT in this setting.

4 MATERIALS AND METHODS

4.1 Study population

The study population originates from a population survey, the Harmonica Project (Harjavalta Risk Monitoring for Cardiovascular Disease). This study was carried out in the rural towns of Harjavalta (7646 inhabitants on 31 December 2007) and Kokemäki (8217 inhabitants on 31 December 2007) in the southwest of Finland from August 2005 to September 2007. An invitation to the project with a cardiovascular risk factor survey, a tape for the measurement of waist circumference, and a type 2 diabetes risk assessment form (FINDRISK, Finnish Diabetes Risk Score) were mailed to inhabitants aged 45 to 70 years (n=6013). ABI measurements were performed for the Harjavalta arm, and they are the study population of this thesis. The number of subjects aged 45-70 in Harjavalta was 2856. The subjects with previously known diabetes (n =199) or cardiovascular disease (n=75) were excluded, because they were already under systematic follow-up at the health center and we wanted to study a cohort in the primary prevention setting.

In the risk factor survey, the subjects were asked to report their waist circumference measured at the level of the navel, latest measured blood pressure, use of antihypertensive medication, history of gestational diabetes or hypertension, and history of CHD, myocardial infarction, or stroke of their parents or siblings to assess family history of cardiovascular disease. If the subjects were willing to participate in the study, they mailed the risk factor survey back to the health center. In the Harjavalta-arm we mailed 2856 invitations and 2085 subjects (73%) responded. Out of them, 1756 persons had at least one of the above-mentioned cardiovascular risk factors. They were invited to the laboratory tests (oral glucose tolerance test and plasma lipids) and a physical examination (measurement of waist circumference, height, weight, BMI and blood pressure) performed by a trained nurse. 1496 (85%) subjects participated to this appointment, 260 (14%) did not participate and 329 (16%) did not have any risk factors. 1059 (71%) of these subjects were diagnosed with hypertension, DM, IGT, MBO, BMI $\geq$30 kg/m^2, or their ten-year risk of cardiovascular death yielded 5% or more based on the SCORE system, and appointment with a physician was scheduled. During the studies we examined 1047 (98%) out of these risk persons and assessed their target organ damage. From these persons also the ABI was measured.

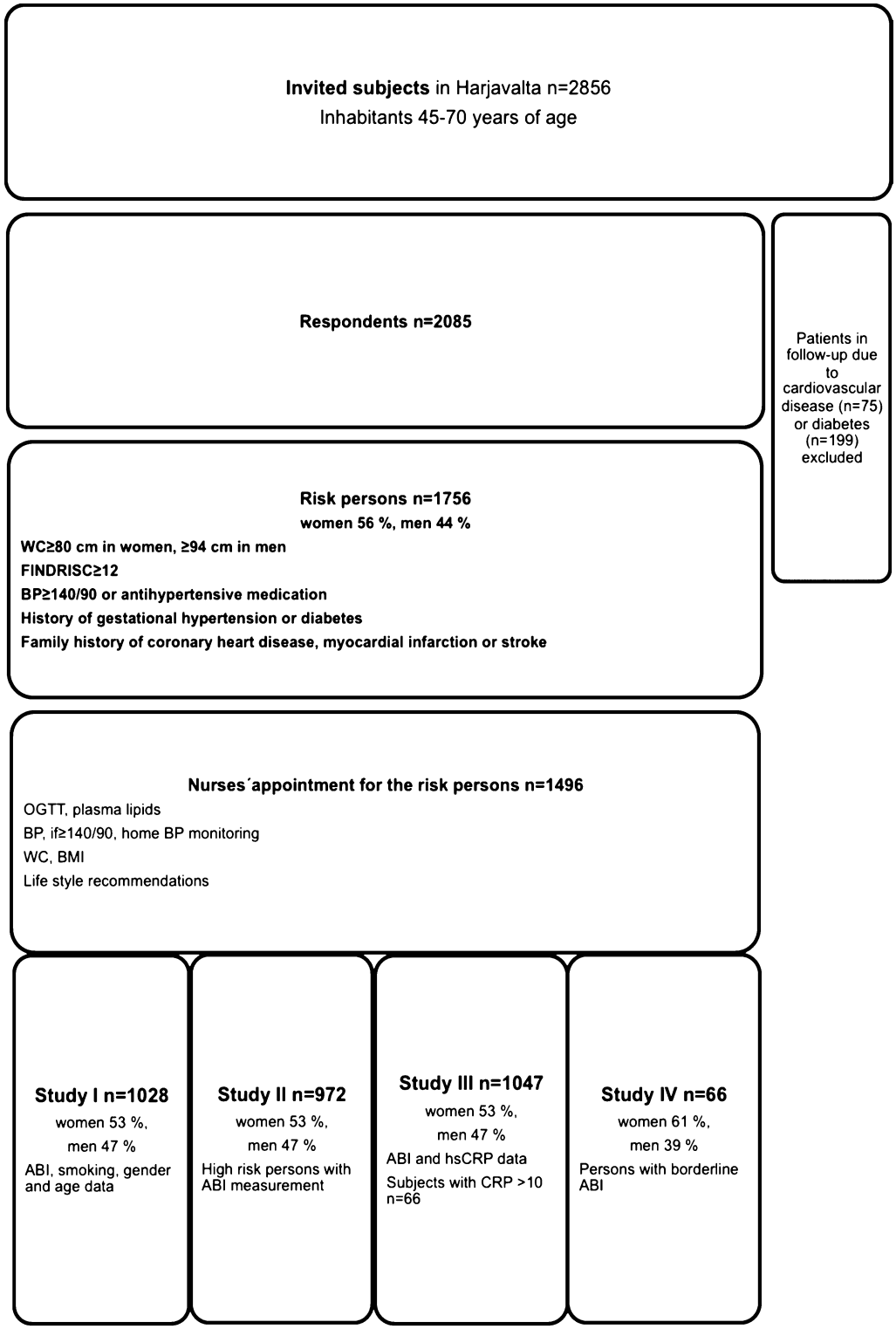

Figure 6. Formation of the study population: WC=waist circumference, FINDRISC=Finnish diabetes risk score, BP=Blood pressure, BMI=Body mass index, ABI=Ankle brachial index

4.2 Methods

4.2.1 Ankle brachial index measurement

ABI was determined from the blood pressure measurements in the arms and ankles with the patient in supine position. SBP in the brachial artery was measured in both upper arms using a blood pressure cuff and a Doppler instrument (UltraTec® PD1v with a vascular probe of 5 MHz; Medema T/A Omega Medical Supplies Ltd., London, United Kingdom) in the antecubital fossa. SBP was measured from both lower limbs, placing the cuff just above the level of malleoli. The ABI was measured from ADP only, and if not found, ATP was used. ABI was the lower ankle SBP divided by the higher brachial SBP. ABI ≤0.90 in either leg was used as a threshold to diagnose PAD. The subjects with an ABI between 0.91 and 1.00 were considered as borderline PAD patients. An ABI between1.01-1.40 was normal and ABI higher than 1.40 was considered as false elevation because of non-compressible vessels.

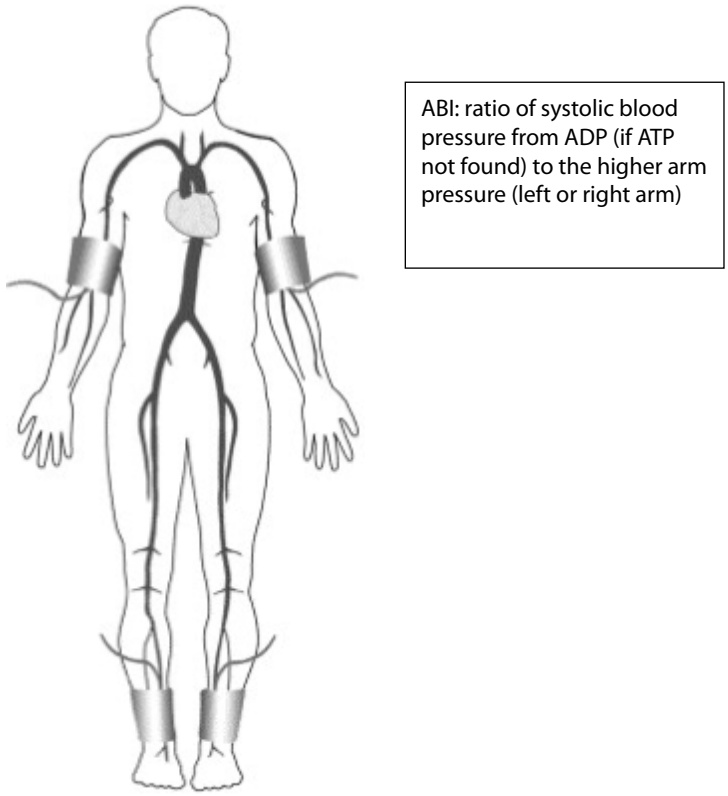

Figure 7. Measurement of the ankle brachial index. ADP=arteria dorsalis pedis; ATP= arteria tibialis posterior.

4.2.2 High-sensitivity CRP

HsCRP was analyzed by a Konelab® 60i analyzer (Thermo Electron, Vantaa, Finland) using a microparticle enhanced turbidometric method. The second analysis was made including only the subjects with hsCRP under 10 mg/l because of the possibility of acute infection in the subjects with hsCRP values over 10 mg/l (Pearson et al. 2003).

4.2.3 Endothelial function

Endothelial function was assessed using an Endo-PAT® device (Itamar Medical Ltd, Caesarea, Israel). During the measurement, the subject sat in a chair with their hands at the level of their heart and fingers hanging freely. Fingertip probes were placed on both index fingers and pulse wave amplitudes were detected and recorded during the study. After a five-minute baseline measurement, arterial flow was occluded using a cuff on the non-dominant arm. The cuff was inflated to 40 mmHg above systolic pressure. After five minutes' occlusion, the cuff was rapidly deflated to allow reactive hyperemia. Pulse wave amplitudes were recorded again for at least five minutes. The software provided by the manufacturer was used to compare the arterial pressure ratio in the two fingers before and after occlusion. It then calculated a reactive hyperemia index which was a ratio of the average pulse wave amplitude measured over 60 seconds, starting one minute after the cuff deflation, to the average pulse wave amplitude measured at the baseline. The other arm served as a control, and the ratio was corrected for changes in the systemic vascular tone. An RHI value of <1.67 was used as a cut-off value to endothelial dysfunction as indicated by the manufacturer and the study by Bonetti et al. (2004).

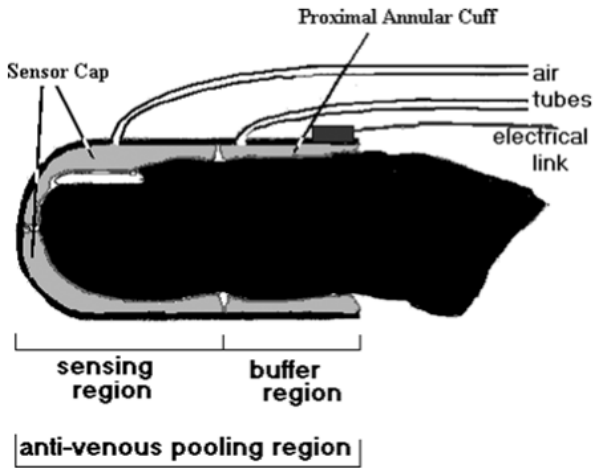

Figure 8. Cross-sectional view through the PAT finger probe; the amplitude of the finger pulse is sensed distally (Celermajer 2008).

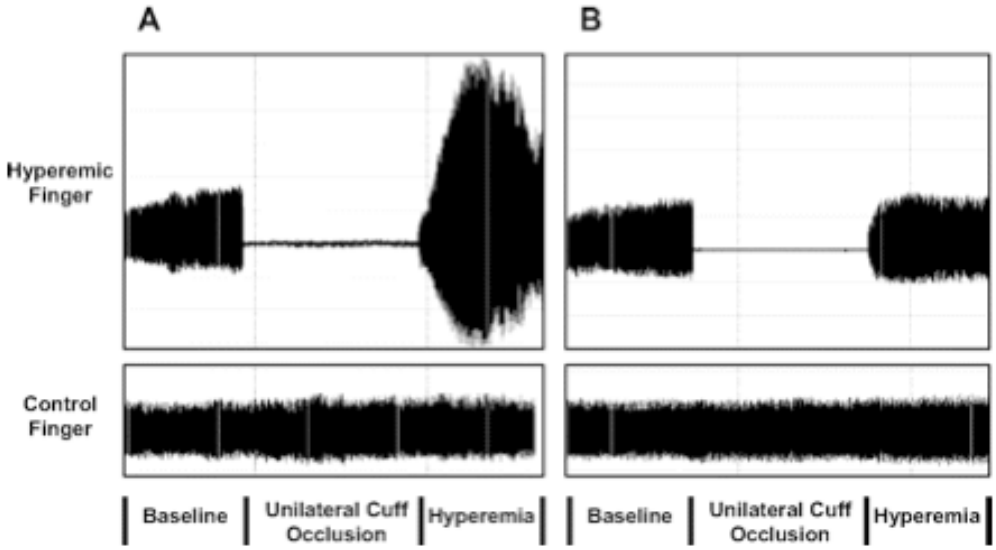

Figure 9. Pulse amplitude tracing in a patient with normal endothelial function (A) and endothelial dysfunction (B). Normal hyperemic response is detected in the subject A (Hamburg et al. 2008).

4.2.4 Blood pressure measurement

Blood pressure measurement was made with a mercury sphygmomanometer with the subjects in a sitting posture. Before measurement, the subjects rested at least five minutes with the cuff placed on the arm. In obese subjects a larger cuff was used. The systolic and diastolic blood pressures were defined by using Korotkoff sounds I and V. Two readings were taken at intervals of at least two minutes. If the mean SBP was ≥ 140 mmHg, or the mean DBP ≥ 90 mmHg, the subjects were taught to use an automatic validated blood pressure monitor (Omron® M4-1, the Netherlands) to perform home blood pressure monitoring. The subjects were instructed to take duplicate blood pressure measurements in a seated position after five minutes of rest in the morning and in the evening for one week. A mean blood pressure was then calculated excluding the measurements from the first day. Hypertension was defined as the use of antihypertensive medication, or as the mean of home blood pressure monitoring ≥ 135 mmHg for SBP or ≥ 85 mmHg for DBP (Parati et al. 2008).

4.2.5 Height, weight, and body mass index

The height and weight were measured with the subjects in a standing position without shoes and outerwear. The height was recorded to the nearest 0.5 cm and the weight to the nearest 0.1 kg. Digital scales (Seca® 861, Germany) were used and the scales were calibrated regularly. BMI was calculated as the weight in kilograms divided by the square of the height in meters.

4.2.6 Waist circumference

In the risk factor survey, the WC home measurement was instructed to perform at the level of the navel. The nurses were instructed to measure the WC of the subjects at the level midway between the lower rib margin and the iliac crest, rounded to the nearest 0.1 cm. The subjects were asked to breathe out gently at the time of the measurement, and the tape was held firmly in a horizontal position. The mean difference between the home-measured WC and that measured by the nurse was -3.76 cm ± 6.59 in the women and -2.41 cm ± 4.49 in the men ($p < 0.001$). In the subsequent screening decisions, measurements performed by health care professionals were used.

4.2.7 Oral glucose tolerance test

An OGTT was performed by measuring fasting plasma glucose and a two-hour plasma glucose after ingestion of 75 g anhydrous glucose dissolved in water. A HemoCue® Glucose 201+ system (Ängelholm, Sweden) was used to measure glucose values from capillary whole blood. The analyzer converts the result from capillary whole blood to plasma glucose values (conversion factor 1.11). The glucose disorders were classified according to the World Health Organization (WHO) 1999 criteria updated in 2006 (WHO 2006). On the basis of two-hour plasma glucose alone, the individuals were classified into categories of newly diagnosed DM, IGT and normal glucose tolerance if their two-hour plasma glucose levels were ≥ 12.2, 8.9-12.1, and < 8.9 mmol/l, respectively. Using the fasting plasma glucose level, the individuals were classified into categories of newly diagnosed DM, IFG and normal fasting glucose, using cut-off levels of ≥ 7.0, 6.1-6.9 and ≤ 6.0 mmol/l, respectively. The diagnosis of DM was confirmed with a control fasting plasma glucose value on another day if the fasting plasma glucose on the OGTT was 7.0 mmol/l or higher, but the two-hour plasma glucose was <12.2 mmol/l.

4.2.8 Metabolic syndrome

MBO was diagnosed according to the criteria of the Adult Treatment Panel III (Grundy et al. 2005) and the International Diabetes Foundation (Alberti et al. 2005).

Adult Treatment Panel III defines MBO according to the following criteria: elevated WC (≥102 cm in men and ≥88 cm in women), elevated triglycerides (≥1.7 mmol/l or drug treatment), reduced HDL-cholesterol (<1.03 mmol/l in men and <1.3 mmol/l in women), elevated blood pressure (≥130 mmHg systolic or ≥85 diastolic or drug treatment) and elevated fasting glucose (≥5.6 mmol/l or drug treatment). Any 3 of the 5 criteria constitute diagnosis.

International Diabetes Foundation´s criteria differ slightly. WC has to be ≥94 cm in men and ≥80 cm in women. In addition, there has to be two out of the four following measures: fasting glucose ≥5.6 mmol/l or type II DM, elevated blood pressure (≥130/80 or drug treatment), elevated triglycerides (≥1.7 mmol/l or drug treatment) and reduced HDL-cholesterol (<1.03 mmol/l in men and <1.3 in women or drug treatment).

4.2.9 Other laboratory measurements

Total cholesterol, HDL cholesterol and triglycerides were measured enzymatically (Olympus® AU640, Japan). LDL cholesterol was calculated using Friedewald´s formula (Friedewald et al. 1972).

4.2.10 Statistical analyses

The data is presented as the means with standard deviations or as counts with percentages. *P*-value of < 0.05 is considered as statistically significant.

Study I: SPSS for Windows 15.0 (SPSS Inc, USA) was used. A statistical significance between the groups was calculated using the cross-tabulation and χ^2-test; the means were compared using the Student´s *t* test or ANOVA.

Study II: The 95 % confidence intervals (CIs) are given for the most important outcomes. The statistical comparisons between the hypertensive and non-hypertensive individuals in characteristics were made by using the *t*-test or χ^2-test. The equality of distributions of ABI was tested by the Kolmogorov-Smirnov test. The age and sex-adjusted linearity across ABI levels was determined by using a linear model with an appropriate contrast. The relationship between PAD and important risk factors was analyzed with univariate and multivariate forward stepwise logistic regression models.

Study III: The analyses were performed using the SAS System for Windows, version 9.1 (SAS Institute Inc, Cary, NC, USA). A one-way analysis, the Pearson correlation test, the *t*-test and a stepwise multivariable analysis were used. A natural logarithm transformation was made for the hsCRP data.

Study IV: The statistical significance of the differences between frequency distributions was tested using the Pearson´s χ^2-test. The difference between the mean values was tested using the pairwise *t*-test or the Wilcoxon rank sum test for two samples. The normality of residuals was tested with the Shapiro-Wilks test, and if the residuals were not normally distributed, the difference between RHI values was tested using the Wilcoxon test. The statistical analyses were carried out using SAS/STAT® software, Version 9.2 of the SAS System for Windows (SAS Institute Inc, Cary, NC, USA).

4.2.11 Ethics

The study protocol and consent forms of the Harmonica Project were reviewed and approved by the ethics committee of Satakunta Hospital District. All the participants provided written informed consent to the project and subsequent medical research.

5 RESULTS

5.1 Effects of age, gender and smoking on ABI and the prevalence of PAD (I)

Mean ABI and prevalence of PAD

In this study, ABI was measured from 1028 subjects. Of them, 547 (53%) were women and 481 (47%) men. The mean age was 59 years (45-70). The mean ABI was 1.10 (range 0.56-1.64). Thirty patients had ABI ≤ 0.90, so the prevalence of PAD was 3%. Of the PAD patients, 19 (63%) were men, and these cases concentrated on the older age group. Only 2 (7%) of the patients with PAD were younger than 60 years.

Effects of smoking, age and gender on ABI

Among the study subjects, 195 (19%) were current smokers. Their ABI was lower than that of the non-smokers (1.06 vs. 1.11 ($p<0.001$)). There was no statistically significant difference between the mean ABI in different genders. The mean ABI in the men was 1.10 and in the women 1.11 ($p=0.185$). There was no statistically significant difference in ABI-values between the different age groups, although there was a trend towards a lower ABI in the older persons (Tables 1 and 2).

Table 1. Effects of sex and current smoking on ankle brachial index (Study I)

	n	Mean ABI	P
Sex			
Male	481	1.10	0.185 (NS)
Female	546	1.11	
Current smoker			
Yes	197	1.06	<0.001
No	830	1.11	

NS Nonsignificant

Table 2. Ankle brachial index in the different age groups (Study I)

Age group, years	Mean ABI	P
45-49	1.11	
50-54	1.10	
55-59	1.11	0.248 (NS)
60-64	1.10	
65-70	1.09	

NS Nonsignificant

5.2 Ankle brachial index in the hypertensive subjects (II)

In this study, we had ABI measured from 972 subjects [457 (47%) men], out of whom 532 (55%) had hypertension. Of the 532 hypertensive subjects, 414 (78%) were under antihypertensive treatment. The hypertensive subjects were slightly older, they had more often MBO, higher glucose levels in OGTT, higher fasting glucose and their BMI was higher. In contrast, they had lower total and LDL cholesterol levels. There were more current smokers among the non-hypertensive subjects (Table 3).

Table 3. Characteristics of the study subjects (Study II)

	Hypertension		P value
	Not present n = 440	**Present** n = 532	
Number of females (%)	224 (51)	293 (55)	0.20
Age, years, mean (SD)	57 (7)	60 (7)	<0.001
Current smokers (%)	93 (21)	77 (14)	0.0065
Body mass index, kg/m^2, mean (SD)	28.8 (4.6)	29.9 (5.4)	<0.001
ABI, mean (SD)	1.11 (0.11)	1.06 (0.12)	<0.001
SBP, mmHg, mean (SD)	143 (17)	154 (17)	<0.001
DBP, mmHg, mean (SD)	86 (8)	91 (8)	<0.001
Pulse pressure, mmHg, mean (SD)	57 (13)	63 (14)	<0.001
Total cholesterol, mmol/l, mean (SD)	5.36 (0.97)	5.26 (0.91)	0.099
HDL-C, mmol/l, mean (SD)	1.46 (0.40)	1.50 (0.41)	0.18
LDL-C, mmol/l, mean (SD)	3.28 (0.87)	3.12 (0.82)	0.0038
Triglycerides, mmol/l, mean (SD)	1.39 (0.70)	1.41 (0.67)	0.63
Fasting glucose, mmol/l, mean (SD)	5.61 (1.16)	5.77 (1.12)	0.036
2-hour glucose, mmol/l, mean (SD)	7.40 (2.54)	7.94 (2.56)	0.0012
Metabolic syndrome present (%)			
IDF	206 (47)	350 (66)	<0.001
ATP III	152 (35)	296 (56)	<0.001

IDF: The International Diabetes Federation, ATP III: Third Adult Treatment Panel

Prevalence of PAD and borderline PAD

The prevalence of PAD was 2.3% (95% CI 1.1 to 4.1) among the non-hypertensive subjects and 7.3% (95% CI 5.3 to 9.9) among the hypertensive subjects. Borderline PAD was also more common among the hypertensive subjects [23.7% (95% CI 20.1 to 27.5)] than in the non-hypertensive subjects [15.0% (95% CI 11.8 to 18.7)]. The mean ABI (SD) was lower in the hypertensive subjects compared to the non-hypertensive subjects [1.06 (0.12) vs. 1.11 (0.11), p<0.001] (Figure 10).

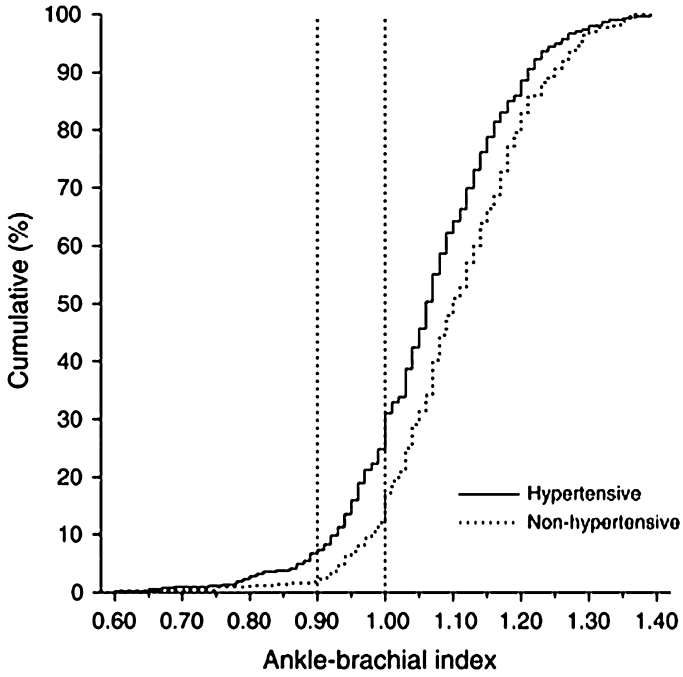

Figure 10. The cumulative distribution of ankle brachial index in the hypertensive and nonhypertensive subjects.

Different risk factors and PAD

Hypertension, age, current smoking and higher triglycerides were significantly associated with PAD in the univariate analysis. After adjustment of other risk factors in the multivariate forward stepwise logistic regression model, hypertension remained an independent factor associated with PAD (adjusted Odds ratio (OR 3.20; 95% CI 1.56 to 6.58).

Systolic blood pressure, diastolic blood pressure and pulse pressure in the different ABI subgroups

In comparison between three ABI subgroups, normal (ABI 1.01-1.40), borderline (ABI 0.91-1.00) and PAD (ABI $\leq$0.90), there was a linear increase in the means of SBP and pulse pressure in the hypertensive subjects ($p<0.001$, adjusted for age and gender). In the normotensive subjects there was no such linear association. There was no statistically significant difference between the mean DBPs in the different groups among the hypertensive ($p=0.16$ adjusted for age and gender) or normotensive ($p=0.13$ adjusted for age and gender) subjects (Figure 11).

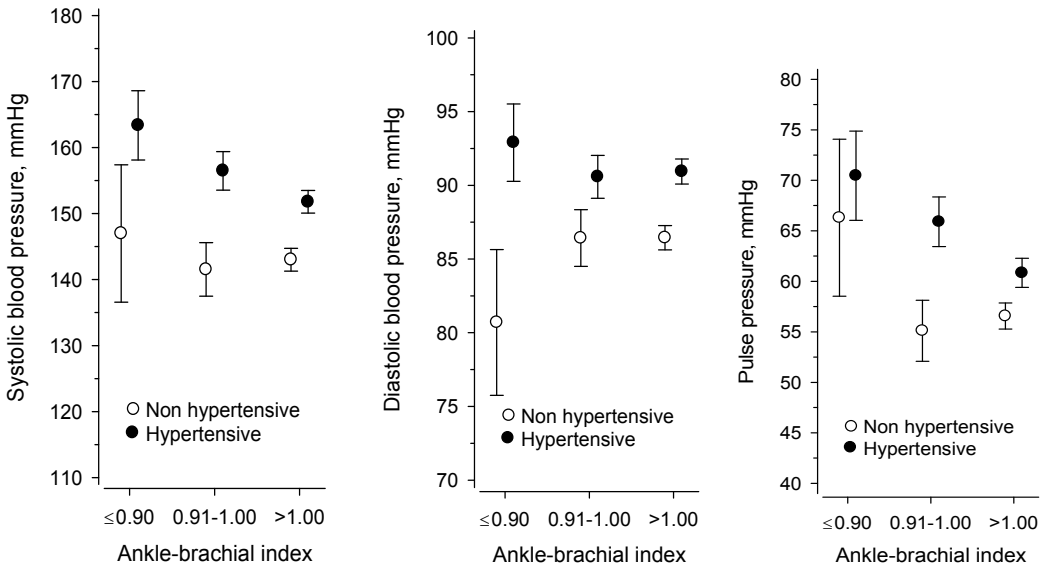

Figure 11. Mean systolic blood pressure, diastolic blood pressure, and pulse pressure according to the ankle brachial index group in the hypertensive and non-hypertensive subjects. The whiskers show 95% confidence intervals (Study II).

Risk factors for PAD in the hypertensive subjects

Among the hypertensive subjects, increasing pulse pressure [OR 1.04 for every 1 mmHg increase (95% CI 1.01 to 1.06)] and SBP [OR 1.03 95% CI 1.01 to 1.05)] were associated with PAD in the age and gender adjusted model. Also, current smoking was associated with PAD defined as ABI ≤ 0.90 [OR 2.72 95% CI 1.23 to 6.04)]. The disorders in glucose metabolism or in lipid profile as well as BMI were not associated with PAD (Table 4).

Table 4. Relationships between peripheral arterial disease (ABI≤0.90 versus ≥0.91) and important risk factors in the subjects with hypertension (adjusted with age and gender) (Study II)

Characteristic	OR (95% CI)	P value
Gender, male	0.68 (0.34 to 1.33)	0.26
Age, years	1.03 (0.98 to 1.09)	0.20
Current smoking	2.72 (1.23 to 6.04)	0.014
Pulse pressure, mmHg	1.04 (1.01 to 1.06)	0.002
SBP, mmHg	1.03 (1.01 to 1.05)	0.001
DBP, mmHg	1.04 (1.00 to 1.08)	0.041
Body mass index, kg/m^2	1.02 (0.96 to 1.08)	0.54
HDL-C ≤1.0, mmol/l	1.59 (0.57 to 4.39)	0.37
LDL-C ≥3.0, mmol/l	1.01 (0.52 to 1.95)	0.98
Triglycerides ≥1.7, mmol/l	1.69 (0.86 to 3.34)	0.13
Glucose homeostasis		
Normal	1.00 (reference)	0.40
IFG	0.82 (0.32 to 2.13)	
IGT	1.19 (0.51 to 2.77)	
T2D	1.86 (0.64 to 5.40)	
MBO		
IDF	1.00 (0.50 to 2.00)	0.99
ATP III	1.30 (0.65 to 2.49)	0.49

5.3 High-sensitivity CRP and ankle brachial index (III)

We had data on ABI and hsCRP from 1047 subjects in a cardiovascular risk population. The mean age was 58.6 years. The prevalence of PAD as indicated by ABI ≤ 0.90 was 3.1%.

High-sensitivity CRP and ankle brachial index

The mean hsCRP (SD) was 1.9 mg/l (3.0 mg/l). The current smokers had slightly higher hsCRP values [mean 2.2 mg/l (2.9 mg/l)] than nonsmokers [mean 1.8 mg/l (3.5 mg/l)] (p=0.050) and there was only minimal difference in the hsCRP values between genders. The mean hsCRP in the women was 2.0 mg/l (3.1 mg/l) and in the men 1.8 mg/l (2.9 mg/l) (p=0.058).

The correlation analysis showed a weak correlation between ABI and hsCRP (r=-0.077; p=0.014), leukocyte count (r=-0.107; p=0.001, and SCORE (r=-0.116; p=0.001). There was a correlation between hsCRP and BMI (r=0.208, p<0.001). HsCRP also correlated to waist circumference (r=0.325, p<0.001).

When we excluded the subjects with hsCRP values >10 mg/l (n=66), the women had higher hsCRP than the men. The mean hsCRP in the women was 1.8 mg/l (2.8 mg/l) and 1.5 mg/l (2.5 mg/l) in the men (p=0.013). No statistically significant difference was

found between hsCRP values in the smokers and nonsmokers. ABI did not correlate with hsCRP. Instead, there was a correlation between hsCRP and waist circumference ($p<0.001$).

Table 5. Correlation between ABI and hsCRP and between waist circumference, leukocyte count and SCORE (Study III).

	ABI	hsCRP	Waist circumference	Leukocyte count	SCORE
ABI		r= -.077 P=.014	r= .032 P=.312	r= -.107 P=.001	r= -.116 P=.001
hsCRP	r= -.077 P=.014		r= .325 P<.001	r= .215 P<.0001	r= .060 P=.077

Pearson correlation analysis.
r: Pearson correlation coefficient
SCORE: Systemic Coronary Risk Evaluation

5.4 Endothelial function in the subjects with borderline ABI (IV)

In this preliminary study we examined 66 subjects with borderline ABI (61% females). The mean age was 60.1 (50-75) years. Hypertension was diagnosed in 35 patients, 27 (41%) had IFG, 21 (33%) IGT and 6 (9%) had DM. Ten were current smokers and 21 former smokers (Table 6). Of the subjects, 36 (55%) had more than one cardiovascular risk factor.

Table 6. Characteristics of the study subjects (Study IV)

Mean age (range) years	60.1	(50-75)
Males (%)	26	(39)
Females (%)	40	(61)
Current smoker (%)	10	(15)
Former smoker (%)	21	(32)
Hypertension (%)	35	(55)
IFG (%)	27	(41)
IGT (%)	22	(33)
T2DM (%)	6	(9)
Mean ABI (SD)	0.95	(0.023)
Mean RHI (SD)	2.11	(0.552)
Endothelial dysfunction (%)	15	(23)

The mean (SD) ABI was 0.95 (0.023) and the mean (SD) RHI 2.11 (0.552). Using a cut-off value of RHI <1.67, endothelial dysfunction was detected in 15 (23%) subjects. The mean RHI was lower in the subjects with disorders in glucose metabolism, but this difference was not statistically significant. The same was true for smoking since the

current smokers had a lower mean RHI. The only statistically significant difference was found between the mean RHI in the subjects with IFG compared to those without IFG (Table 7).

Table 7. Comparison between the subjects with different risk factors and RHI values (Study IV)

Risk factor	**Number of subjects with risk factor**	**Mean RHI (SD) (risk factor)**	**Mean RHI (SD) (no risk factor)**	***P*-value**
IGT and/or IFG and/or T2DM	55	1.91 (0.332)	2.25 (0.587)	0.16
T2DM	6	1.79 (0.156)	2.14 (0.568)	0.14
IGT	22	2.13 (0.390)	2.08 (0.632)	0.30
IFG	27	1.91 (0.397)	2.24 (0.608)	0.02[a]
Hypertension	35	2.04 (0.441)	2.18 (0.656)	0.70
Smoking	10	2.04 (0.643)	2.12 (0.531)	0.62

[a]Statistically significant.

6 DISCUSSION

6.1 Study population

The subpopulations in this study were drawn from a cross-sectional population-based survey, the Harmonica Project. Originally this project had two different arms, Harjavalta and Kokemäki. We studied the Harjavalta-arm, because the ABI measurements were done only in this population.

The subjects with known diabetes or cardiovascular disease were excluded so that we could examine subjects without serious comorbidities. Out of the invited 2856 subjects, 2085 (73%) responded. This can be considered quite high indicating motivation and interest in one's health in the general population.

Out of these respondents, 1756 (84%) had at least one cardiovascular risk factor and to them, a further appointment with a nurse was offered. 1496 (85%) subjects out of these 1756 participated and were further examined. If they were diagnosed with hypertension, diabetes, impaired glucose tolerance, metabolic syndrome, BMI $\geq$30 kg/m^2, or the ten-year risk of cardiovascular death yielded 5% or more based on the SCORE, and they were willing to continue in the study, the physician examined them further. From 1047 subjects the possible target organ damage was assessed, and from these persons also the ABI was measured. They formed the representative cohort of persons at the risk of cardiovascular disease and were examined in our sub-studies.

Unfortunately, we could not obtain all the data from every study person and there was some lost during follow-up. Out of the invited 2856 subjects, 27% (771) did not respond. They were predominantly males, but practically of the same age as the respondents. In general this can be, however, considered quite diminutive. In addition, in the study IV we only took a small sample of persons with borderline ABI to assess their endothelial function. Therefore, this data can only be considered as directional and the need for large-scale studies are needed to confirm these results.

6.2 Methods

We used a simplified method to calculate ABI by measuring ankle systolic pressures from ADP only. The lower of the two measurements was used. ABI was assessed using ATP if ADP pulsation could not be found. Espinola-Klein et al. (2008) have used the same methodology as we did to better assess subjects at an elevated cardiovascular risk. It is possible that in some patients the ABI measured from ATP might have been lower. In these subjects this method used in our study somewhat underestimates the prevalence of PAD and borderline PAD. However, this simplified method might be more

practical and less time-consuming in primary care practices to identify persons at a high cardiovascular risk. In addition, most of our ABI measurements were done by a single doctor. Some additional measurements were done by a trained nurse. Thus, we think that our ABI data can be considered valid.

To detect endothelial function, a novel RH-PAT method was used. The Endo-PAT® device (Itamar Medical Ltd, Caesarea, Israel) is FDA approved (2003) and it has a CE (Conformité Européenne) marking. This method has been tested against the gold standard of endothelial assessment, the flow mediated dilation. As discussed above, Kuvin et al. (2003) showed that this method demonstrates patterns of abnormality similar to ultrasound detection of FMD. There are also studies to demonstrate the good reproducibility of RH-PAT testing. These results are equal or even above the published reproducibility of brachial artery FMD assessment when operated by a qualified sonographer (Selamet et al. 2009, Tomfohr et al. 2008 and Haller et al 2007). However, this method as every method assessing endothelium-dependent dilatation, only gives insights into one of many aspects of endothelial physiology.

6.3 Prevalence of PAD and effects of age, gender, and smoking on ABI (I)

The prevalence of asymptomatic PAD, defined as ABI ≤ 0.90, in this study was 3%. In previous studies the prevalence increases from the age of 50 onward and is in the range of 3-18%, increasing further to 25-30% in persons aged > 70 (Diehm et al. 2004 and Meijer et al. 1998). In Carbayos´ et al. study (2007), the prevalence of PAD was higher (10-11%), but our findings are in line with the Framingham offspring study where the prevalence was 3-4% (Murabito et al. 2002). Our study was based on individuals – drawn from a population survey – who were at a risk of cardiovascular disease. They did not have previously diagnosed cardiovascular disease, chronic renal disease or diabetes. The estimated prevalence of PAD could have been expected to be higher in this population. However, it is possible that the previously mentioned exclusion criteria may have affected to this prevalence we detected. In addition, risk factor survey did not include questions about current or previous smoking as well as possible previously measured cholesterol levels. Also, the mean age in our study was 59 years and we did not study subjects over 70 years. In Carbayo´s et al. study, it was shown that the prevalence of PAD almost doubled after the age of 70.

We found an expected difference between ABI values in the smokers and non-smokers. We only took into account current smoking, so it is possible that there were even heavy former smokers in the group of non-smokers. Previous research has shown that pack-years smoked and smoking history are related to the risk of developing PAD (Carbayo et al. 2007 and Murabito et al. 2002). In our study, ABI values were lower in the smokers than non-smokers.

In our study, only two patients with an ABI ≤ 0.90 were younger than 60 years. The mean ABI values in the oldest age group were lower than those in the youngest group, but this finding was not statistically significant. Carbayo et al (2007) showed that the prevalence of PAD almost doubled after the age of 70. Our study group consisted of subjects between the age of 45 and 70.

The prevalence of ABI >1.40 was low, most likely because there was a low number of diabetics and patients with chronic kidney disease. Only three subjects had ABI >1.40 which has been considered as a limit of false positive elevation of ABI for example in the study by McDermott et al. (2001).

6.4 Ankle brachial index in the hypertensive subjects (II)

The prevalence of asymptomatic PAD was higher (7.3%) in the hypertensive than in the non-hypertensive subjects (2.3%). Mostaza et al. (2006) investigated the relationship between ABI and chronic kidney disease in hypertensive patients with no known cardiovascular disease in the MERITO study. In their study, however, 62% of the subjects had DM, which most likely explains the higher 27% prevalence of PAD. Among an unselected cohort of Chinese subjects the age and gender matched prevalence of PAD (defined as ABI ≤ 0.9) was 8.7% in the hypertensive, and 5.0% in the normotensive subjects (Yang et al. 2007). Of them, 20% had a history of cardiovascular disease and 8% DM. This quite a low prevalence can be mainly explained by ethnicity. In the Multi-Ethnic Study of Atherosclerosis, it was shown that the prevalence of PAD was the highest in African Americans, and the lowest among Chinese in men and Hispanics in women (McDermott et al. 2005). All the subjects in our study were Caucasians. The Systolic Hypertension in the Elderly Program reported a 27% prevalence of PAD (defined as ABI ≤ 0.90) in the subjects aged 60 years or older with isolated systolic hypertension (Newman et al. 1991). This study also included subjects with known diabetes and cardiovascular disease. In our study, the prevalence of PAD was 8.5% among the hypertensive subjects aged over 60 years or older and without co-morbidities (n= 279). Only 6% (30/532) of the hypertensive subjects in our study population had isolated systolic hypertension defined as SBP ≥ 160 mmHg and DBP < 90 mmHg.

The prevalence of borderline PAD in our study (defined as ABI 0.91-1.00) was 24% in the hypertensive subjects and 15% in the other high-risk subjects. ABI values 0.91-1.00 are traditionally considered as “normal”, but recent investigation has revealed the increased risk also in this sub-group of patients. In a meta-analysis by Fowkes et al. (2008), it was shown that in men with ABI 0.91-1.00, compared to a reference ABI of 1.10-1.20, the hazard ratios for total mortality, cardiovascular mortality and major cardiovascular events were 1.61, 1.68 and 1.43, respectively. The corresponding figures in women were 1.52, 1.84 and 1.53. The same kind of elevated risk was confirmed in a study by Diehm et al. (2006). The magnitude of the increased risk in persons with borderline PAD was much lower than in those with ABI ≤ 0.90, but substantially higher

than in those with ABI > 1.40. None of the subjects in our study had ABI > 1.40 referring to mediasclerosis as discussed above.

The TASC II recommendation is that ABI should be measured in all patients aged 70 years or older regardless of the risk factor status. In the case of patients aged between 50 and 69, the TASC II recommends that ABI should be measured when there are one or more cardiovascular risk factors (particularly diabetes or smoking) (Norgren et al. 2007). In our study, the hypertensive subjects aged 50 to 69 years accounted for 95% (506/532) of the hypertensive study population, and among them the prevalence of PAD and borderline PAD was 8% and 24%, respectively. In addition, among the subjects aged less than 50 years none of them had PAD, but 22% had borderline PAD. Since there is evidence of increased cardiovascular events in these patients the decision of measuring ABI in the hypertensive subjects should not be based on age or other cardiovascular risk factors.

The previously mentioned TASC II guidelines recommend the use of ABI ≤ 0.90 to diagnose PAD. However, for example European guidelines for the management of hypertension and the United States guidelines for treating dyslipidemia use the cut-off point of ABI <0.9 to initiate medical therapy for cardiovascular risk factors (Macia et al. 2007 and NCEP 2002). If we compare these two cut-off points, using the former leads to the greater number of subjects with PAD. This discrepancy points out the importance of having uniform diagnostic criteria for PAD and borderline PAD not only for scientific purposes, but also to facilitate clinical decision-making.

We found no association between impaired glucose homeostasis or the metabolic syndrome and PAD. Pande et al. (2008) showed in their study that insulin resistance, indicated by the homeostasis model of insulin resistance, is associated with PAD. However, when the subjects with known DM were excluded, the associations between insulin resistance and PAD were no longer statistically significant. In our study, the subjects with previously known DM were excluded. It seems that newly diagnosed pre-diabetes or DM is not strongly associated with PAD, whereas long-lasting diabetes remains a well-established risk factor for this condition.

We found a significant association between blood pressure levels and PAD. When a linear model was used to determine the age and sex adjusted linearity across ABI levels > 1.00, 0.91-1.00 and ≤ 0.90, the relationship between ABI and pulse pressure or SBP, but not DBP, was linear across ABI subgroups in the hypertensive subjects. The majority (67%) of the PAD cases were found among patients with widened pulse pressure; the prevalence of PAD was 12% (26/216) among the hypertensive patients with a pulse pressure of ≥ 65 mmHg.

In the hypertensive patients, arterial compliance – estimated indirectly by increasing pulse pressure – was reduced already with borderline PAD. Instead, in the non-hypertensive patients, an increase in pulse pressure was only seen in the patients with established PAD. While we cannot determine any causal relationship from our cross-sectional study,

we find it possible that increasing arterial stiffness plays a major pathophysiological role in the development of both increased pulse pressure and atherosclerotic lesions in the peripheral arterial tree.

6.5 High-sensitivity CRP in a cardiovascular risk population (III)

HsCRP has been previously linked with PAD. In the study by Pande et al. (2008), a 2.2-fold increase in the prevalence of PAD was found in the subjects with a high CRP level (> 3 mg/l) compared with those with a low CRP level (< 1 mg/l). However, this relationship only persisted in the subjects without insulin resistance. As atherosclerosis can be considered a sum of multiple risk factors, it might be that in individuals with a few risk factors, inflammation greatly contributes to the development of vascular disease, whereas in the presence of a strong risk factor like insulin resistance, this contribution is diminished. Eldrup et al. (2006) concluded in their study that ABI <0.9 identified the individuals with severe atherosclerosis in the general population, while CRP did not do that.

Our study indicates that there is only a weak correlation between ABI and hsCRP; the subjects with a lower ABI had higher hsCRP values. This relation disappeared in the multivariate analysis and when the subjects with hsCRP >10 mg/l were excluded. One reason for this may be that we studied a cardiovascular risk population without a previous diagnosis of cardiovascular disease or diabetes. In addition, as discussed above in chapter 2.3.4, CRP might not be a perfect tool to assess the total cardiovascular burden. Our finding is also in line with the study by Bo et al. (2009) where among the asymptomatic, moderate- to high cardiovascular risk subjects, hsCRP had no independent association with subclinical PAD.

We found a correlation between hsCRP and BMI, as well as, hsCRP and waist circumference. This finding is similar to that in the study by Hak et al. (1999), where hsCRP was related to BMI and to waist and hip circumferences separately. However, after adjustment of BMI, hip circumference was no longer related to CRP, whereas waist circumference remained related. Thus, it seems that abdominal fat deposition may be the most important factor contributing to inflammation detected using hsCRP. In a study by Ford (1999), the same kind of finding was made about the correlation between hsCRP and BMI. He concluded that his results confirm cross-sectional findings from previous studies that show elevated C-reactive protein concentrations among individuals who are obese or have DM.

As we know that obesity is closely associated with MBO, it is not surprising that it was diagnosed according to previously mentioned International Diabetes Foundation´s criteria in 57% of the subjects and according to Adult Treatment Panel III criteria in 46% of the subjects. In our study population 7% had newly diagnosed DM, 37% IFG and 17% IGT, all of these are conditions associated with elevated inflammatory markers.

Previous studies have shown that PAD is associated with waist-hip ratio, but not with BMI (Planas 2001). BMI is possibly not the best measure of intra-abdominal adiposity, which drives the progression of cardiovascular risk factors through secretion of adipokines and exacerbation of insulin resistance (Despres 2006). In a study by Carbayo et al. (2007) lower BMI values were, however, detected in the patients with ABI < 0.9. This may also indicate that adipose tissue driven inflammation is not a major factor in the development of PAD. Our study found no statistically significant correlation between BMI and ABI.

In our study, the smokers had higher hsCRP values than the nonsmokers. A recent study showed that hsCRP was higher in the smoking men compared with the nonsmoking men (Ahonen et al. 2008). They could not find the same association in the women. This may indicate that smoking associates differently with subclinical inflammation in different genders. Elevated inflammatory markers among the smokers were detected also in the study by Yanbaeva et al. (2007).

In conclusion, it can be said that in our study hsCRP was not associated with ABI. So it seems that inflammatory markers are not strongly dependent on the severity of PAD. The prevalence of PAD defined by ABI ≤ 0.90 was 3.1%, so there were only 32 subjects with that condition, which means that definite conclusions about the relationship between hsCRP and PAD cannot be drawn based on this study only. HsCRP correlated to the measures of obesity (WC and BMI), indicating its role as a marker of adipose tissue driven inflammation.

6.6 Endothelial function in a cardiovascular risk population with borderline PAD (IV)

The endothelium plays an important role in the regulation of vascular tone and structure as first recognized by the experiments of Robert Fuchgott´s group (Furchgott et al. 1980). The endothelium is also involved in vascular inflammation and thrombosis, the key events of the atherosclerotic disease process and its clinical implications, such as myocardial infarction and stroke. With the presence of cardiovascular risk factors such as hypercholesterolemia, hypertension, and smoking, the endothelium has reduced nitric oxide availability, impairing its function and endothelium-dependent vasodilation (Giannotti et al. 2007). The endothelial dysfunction is associated with a risk of major cardiovascular events (Lerman et al. 2005). In the study carried out by Hamburg et al. (2008), male sex, BMI, the ratio of total to HDL cholesterol, DM, smoking, and lipid-lowering treatment were inversely related to the PAT ratio. An improvement in endothelial function and a reduction in the risk of vascular events can be achieved by intensively treating risk factors such as hypertension (Modena et al. 2002, Iwatsubo H et al. 1997, Giugliano et al. 1998).

For decades, ABI has been used as a tool of vascular practice to quantify the severity of occlusive disease among patients with leg symptoms, and to decide whether vascular

interventions are needed. More recently the role of ABI has been expanded, since it can be utilized as a marker of cardiovascular risk. A normal ABI value is between 1.00-1.40 and PAD is defined as an ABI value ≤ 0.90. An ABI of > 1.40 might indicate medial sclerosis. The presence of an abnormal ABI value increases the risk of myocardial infarction and death, among other adverse outcomes (Fowkes et al. 2008, Lee et al. 2004 and Criqui et al. 1992). In the Walking and Leg Circulation Study (WALCS), the subjects with low normal ABI (1.00-1.09) and borderline PAD (ABI 0.90-0.99) also had a significant risk of mobility loss during follow up (McDermott et al. 2009). The likely mechanism was progression towards PAD indicating preventive measures to improve the outcome of these subjects.

We studied a cardiovascular risk population that had not been previously diagnosed with DM or cardiovascular disease. We took a small sample of subjects with borderline ABI to evaluate the prevalence of endothelial dysfunction in this group and to test the methodology. To our knowledge, this is the first study in this kind of setting. The homogenous nature of this group may be the main reason why we did not find differences in RHI values between different types of risk factors (i.e., all the subjects had at least one cardiovascular risk factor). We used the cut-off value RHI < 1.67, determined in previous studies, to diagnose endothelial dysfunction (Yeo et al. 2007, Yinon et al. 2006 and Ohno et al. 2010). Almost every fourth subject with borderline ABI had endothelial dysfunction. This may point to a subgroup of patients who need intensive risk factor management in order to prevent cardiovascular events and loss of mobility.

PAT testing is free of operator dependent errors, because results are analyzed with a computerized, automated algorithm. The observations can be considered as being free of subjective bias when compared to flow mediated dilation measurement with ultrasound from brachial arteries. Reliability is the strength of the method we used. However, PAT can only measure one aspect of endothelial function. Further, as discussed above in this thesis, the reproducibility of the ABI measurements can be questioned, if they are performed by inexperienced personnel. In the study carried out by Mätzke et al. (2003), 16% of the ABI values differed from the median by 0.15 or more when the measurements were taken by doctors with little or no instructions. To obtain reproducibility, the measurements have to be performed by trained personnel.

Although the PAT results are free of intra and inter-observer variation, this method may be influenced by changes in the physiological state of the study subject. To diminish the influence of external factors, we made all the measurements in a controlled environment, including a quiet, darkened room at a steady room temperature. The other arm served as an internal control. Because dietary intake may influence the results, all the measurements were taken with the subject in a fasting state (Giannattasio et al. 2005 and Gaenzer et al. 2001). Obviously, the small number of study subjects can be considered as a limitation to our study, but the study group is a representative sample of people at a cardiovascular risk in the general population.

Now that there is growing evidence to link low RHI scores to the risk of cardiovascular disease, it is encouraging that we can use this non-invasive method to provide an additional level of risk stratification to initiate early and aggressive treatments to prevent future cardiovascular events. As pointed out by Celermajer (2008), the exact role of PAT in this regard is not clear, but there is evidence that it gives relevant, predictive and prognostic data about endothelial function. However, the prognostic usefulness of this technique in clinical practice is only beginning to emerge. The technique per se is suitable for clinical use because it is reasonably priced, non-invasive and it requires minimal training. In the future, it will be necessary to perform large-scale clinical studies to provide us with further information about the diagnostic usefulness of PAT in everyday clinical practice. There might still be unknown factors to be discovered that influence PAT measurements and their results. Some clinical studies are currently under way and we can expect results in the near future.

7 CONCLUSIONS

On the basis of the present investigations, the following conclusions can be drawn:

1. Smoking was a crucial risk factor for PAD. In addition, age was not statistically significantly related to the ABI, but the majority of the subjects with PAD were in the oldest age group. Gender had no effect on ABI.

2. Subclinical PAD is common in hypertensive patients even without co-morbidities. The measurement of ABI is an efficient method to identify patients with an increased cardiovascular risk and worth performing on hypertensive patients, particularly those with pulse pressure above 65 mmHg.

3. HsCRP did not correlate with ABI or PAD. Instead, it correlated to measures of obesity indicating its role as a marker of adipose tissue driven inflammation.

4. In a cardiovascular risk population with borderline ABI nearly every fourth subject had endothelial dysfunction, indicating an elevated risk of cardiovascular events. This might point out a subgroup of individuals in need of more aggressive treatment for their risk factors.

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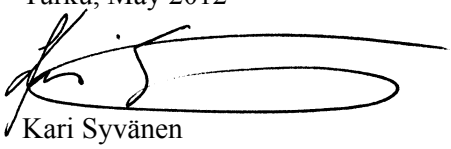

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