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ENDOTHELIAL FUNCTION AND ARTERIAL STIFFNESS IN PATIENTS WITH ATHEROSCLEROSIS AND IN HEALTHY SUBJECTS

A clinical and biochemical study

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To my parents

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred to in the text by their Roman numerals:

I Kals J, Kampus P, Kals M, Pulges A, Teesalu R, Zilmer M. Effects of stimulation of nitric oxide synthesis on large artery stiffness in patients with peripheral arterial disease. Atherosclerosis 2006;185:368–374.

II Kals J, Kampus P, Kals M, Teesalu R, Zilmer K, Pulges A, Zilmer M. Arterial elasticity is associated with endothelial vasodilatory function and asymmetric dimethylarginine level in healthy subjects. The Scandinavian Journal of Clinical and Laboratory Investigation 2007 (in press).

III Kals J, Kampus P, Kals M, Zilmer K, Kullisaar T, Teesalu R, Pulges A, Zilmer M. Impact of oxidative stress on arterial elasticity in patients with atherosclerosis. American Journal of Hypertension 2006;19:902–908.

IV Kals J, Kampus P, Kals M, Pulges A, Teesalu R, Zilmer K, Kullisaar T, Salum T, Eha J, Zilmer M. Inflammation and oxidative stress are differently associated with endothelial function and arterial stiffness in healthy subjects and in patients with atherosclerosis (submitted).

ABBREVIATIONS

ABPI	ankle brachial pressure index
ADMA	asymmetric dimethylarginine
AIx	augmentation index
AIx@75	augmentation index corrected for a heart rate of 75 beats *per* minute
BMI	body mass index
BP	blood pressure
C1	large artery elasticity index
C2	small artery elasticity index
CAD	coronary artery disease
CRP	C-reactive protein
CV	cardiovascular
EDV	endothelium-dependent vasodilatation
EF	endothelial function
EFI	endothelial function index
EIDV	endothelium-independent vasodilatation
ELISA	enzyme-linked immunosorbentassay
ESRD	end-stage renal disease
F_2-IsoPs	8-iso-prostaglandin F_{2a}
HDL	high-density lipoprotein
HR	heart rate
ICAM-1	intercellular adhesion molecule-1
LDL	low-density lipoprotein
LDL-BDC	baseline diene conjugates of low-density lipoprotein
MAP	mean arterial pressure
MI	myocardial infarction
MPO	myeloperoxidase
NO	nitric oxide
NOS	nitric oxide synthase
NTG	nitroglycerin
oxLDL	oxidized low-density lipoprotein
OxS	oxidative stress
PAD	peripheral arterial disease
PP	pulse pressure
PWA	pulse wave analysis
PWV	pulse wave velocity
ROS	reactive oxygen species
Salb	salbutamol
Tr	travel time of the reflected wave
VCAM-1	vascular adhesion molecule-1

1. INTRODUCTION

Modern understanding of the circulation of blood and the cardiovascular (CV) system can be traced back to the publication in 1628 of Dr. William Harvey's famous treatise "On the Motion of the Heart and Blood in Animals" (Modern History Sourcebook: William Harvey (1578–1657)). Four years before the foundation of Tartu University English physician Harvey showed that the blood passed through the lungs, propelled through the arteries by the pulsations caused by contractions of the left ventricle of the heart, and returned to the heart through the veins. Prior to 1980 it was generally thought that the major mechanism whereby vessels *in vivo* constricted and dilated was *via* an action of neurohumoral substances on the vascular smooth muscle. This view was dramatically altered when Robert Furchgott and his co-workers demonstrated that endothelial cells produce an endothelium-derived relaxing factor in response to stimulation by acetylcholine in vessels with an intact endothelium (Furchgott and Zawadzki 1980). In 1987, Salvador Moncada and Louis Ignarro independently published chemical evidence that endothelium-derived relaxing factor is nitric oxide (NO) (Ignarro *et al.* 1987; Palmer *et al.* 1987).

Today, we no longer consider the blood vessel as an inert tube but rather as a living structure composed of cells, as endothelium and smooth muscle. Furthermore, it is widely recognised that endothelium serves as an independent organ, with a weight of almost 1 kilogram, which releases a number of mediators to regulate vascular homeostasis (Sumpio *et al.* 2002). The most potent endothelial vasodilator molecule NO plays a key role in circulatory control. Besides fulfilling several biofunctions, the endothelium regulates also vascular tone, structure and stiffness (Schmitt *et al.* 2005). Being a single monolayer of cells, the endothelium possesses little tensile strength but can profoundly alter the mechanical characteristics of the blood vessels through the elaboration of vasoactive substances (e.g. NO) (Wilkinson *et al.* 2004). Decreased bioavailability of NO is responsible for altered regulation of the vasomotor response of endothelial cells, which results in a diminished vasculo-protective action of NO and increased arterial stiffness (Arcaro *et al.* 2004). Both endothelial dysfunction (Gocke *et al.* 2003) and arterial stiffening (Mattace-Raso *et al.* 2006) are early mechanisms of vascular alterations, and important contributors of disease progression, are increasingly considered independent CV risk factors and specific targets for therapy (Plantinga *et al.* 2007; Tonetti *et al.* 2007).

The wall of the artery is the primary site of disease process and has therefore become an attractive target for demonstrating functional or structural alterations that may precede morbid events. Early identification of pathological changes in the vasculature is of great importance in stratification of CV risk as endothelial dysfunction and increased arterial stiffness are modifiable (Mäki-Petaja *et al.* 2006; Plantinga *et al.* 2007). Furthermore, reduction of CV risk factors, conventional treatment of diseases and/or specific therapy may improve vascular

function and thereby reduce vascular risk. Although vascular function can be measured using several devices, pulse wave analysis (PWA) is a non-invasive, simple and reproducible method for assessing arterial stiffness and endothelial function (EF) in large-scale studies (Wilkinson *et al.* 2002a; Hayward *et al.* 2002).

Several studies have considered that systemic inflammation and high-grade oxidative stress (OxS) may be pathophysiologically involved in vascular damage (Ross 1999; Loffredo *et al.* 2007; Steinberg *et al.* 1989). It has been shown that inflammation and OxS are also independent predictors of CV disease progression and mortality (Ridker *et al.* 2001; Mueller *et al.* 2004). However, their impact on progression of endothelial dysfunction and arterial stiffening has not been fully elucidated. Furthermore, a better understanding of the associations between biochemical and functional vascular parameters will facilitate identification of specific therapeutic targets for individuals at risk and will help monitor the success of treatment.

Lower extremity peripheral arterial disease (PAD) is a manifestation of systemic atherosclerosis and is associated with an increased CV risk (Ouriel 2001). Because endothelial dysfunction and arterial stiffness are responsible for initiation and progression of lower extremity PAD and may contribute to the explanation of their increasingly poor prognosis (Safar 2007a; Gocke *et al.* 2003), assessment of vascular function in patients with PAD is a significant clinical challenge. This thesis will focus on assessment of EF and arterial stiffness in patients with lower extremity PAD and in healthy subjects using PWA; on determination of possible associations between EF and arterial stiffness as well as on demonstration of the current evidence linking OxS and inflammation to vascular dysfunction.

2. REVIEW OF THE LITERATURE

2.1. Vascular function and cardiovascular diseases

2.1.1. Vascular endothelium

Since the pioneering discovery that the intact endothelium was essential for vasodilatation (Furchgott and Zawadzki 1980), and the subsequent discovery of endothelium-derived NO (Ignarro *et al.* 1987; Palmer *et al.* 1987), the fundamental importance of the endothelium for vascular homeostasis has been increasingly acknowledged. Today there is recognition that the endothelium, a ubiquitous monolayer of cells lining the entire vascular tree, consists of approximately $1–6\times10^{13}$ cells and forms an "organ" with a weight of almost 1 kilogram (Sumpio *et al.* 2002). The vascular endothelium plays a pivotal role in health, and has important metabolic, autocrine, paracrine, endocrine, and innate immunological functions in CV and other chronic diseases (Mensah *et al.* 2007; Deanfield *et al.* 2007; Hayoz and Mazzolai 2007; Asselbergs *et al.* 2005). Endothelial cells control a variety of critical processes including regulation of vascular permeability and tone, vascular smooth muscle proliferation and migration, vasculogenesis, inflammation, immunity, fibrinolysis, and the delicate balance between pro-thrombosis and anti-coagulation (Sumpio *et al.* 2002).

The vascular endothelium releases a large number of vascular substances that participate in the regulation of vascular homeostasis (Constans and Conri 2006). The endothelium synthesizes vascular relaxing substances, such as NO, prostacyclin, endothelium-derived hyperpolarizing factor, adrenomedullin and C-type natriuretic peptide. Endothelium-derived vasoconstrictors include endothelins, angiotensin II, prostaglandin H_2, superoxide anion radical, leukotriens and thomboxane A_2. Inflammatory modulators include cytokines, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, receptor CD 40, interleukins, etc. Modulation of hemostasis includes release of thrombomodulin, heparin sulfate, antithrombin III, tissue-type plasminogen activator, plasminogen activator inhibitor-1 and -2, von Willebrand factor, thromboplastin and fibrinogen. Additionally, endothelial cells secrete several other molecules such as matrix products, growth factors, lipid metabolism substances, etc. These products are released in response to a range of neurohumoral and chemical stimuli, such as thrombin, bradykinin, serotonin, histamine or adenosine diphosphate, as well as changes in haemodynamic forces such as alterations in blood pressure (BP) or flow (Hayoz and Mazzolai 2007; Deanfield *et al.* 2007; Sumpio *et al.* 2002).

The myriad of functions of the endothelial cells makes the endothelium indispensable for whole body vascular homeostasis. In this regard, endothelial dysfunction is a major factor in development of a wide range of diseases

including atherosclerosis, allograft vasculopathy, hypertension, sepsis, congestive heart failure, primary pulmonary hypertension and inflammatory syndromes (Mensah 2007; Cockcroft 2005; Brunner *et al.* 2005; Endemann and Schiffrin 2004).

2.1.2. Endothelial dysfunction and atherogenesis

Endothelial cells are of significance in initiating, development and progression of atherosclerosis (Deanfield *et al.* 2007; Trepels *et al.* 2006). Impaired EF is caused by several CV risk factors, e.g. hypercholecterolemia, cigarette smoking, diabetes, hypertension, obesity, hyperhomocysteinaemia, chronic inflammation, OxS, etc. In fact, despite numerous other endothelial functions, endothelial dysfunction has now become synonymous with reduced biological activity of NO, the most potent anti-atherogenic molecule. Nitric oxide not only regulates vascular tone by directly acting on smooth muscle cells, but it counterbalances the action of other vasoconstrictors, inhibits platelet adhesion and aggregation and smooth muscle cell proliferation (Hayoz and Mazzolai 2007). Moreover, NO also protects lipoproteins from oxidation and prevents leucocyte adherence to the endothelium. Thus, although not limited to NO metabolism, endothelial homeostasis greatly depends on NO-balanced release owing to the pleiotropic actions of NO on control of most other endothelial factors.

Shear stress, the tangential drag force, appears to be a predominant mechanical stimulus inducing changes in the structure and function of endothelial cells. The areas of the vessels exposed to high laminar flow tend to be disease-free, whereas atherosclerotic vessels tend to develop at bends or bifurcations where flow is disturbed. Chronic high levels of laminar shear stress in the physiologic range down-regulates the genes that contribute to atherosclerosis, as well as augments endothelial NO synthase (NOS) and up-regulates the other atheroprotective genes (Hayoz and Mazzolai 2007). In contrast, perturbations in local haemodynamics, i.e. turbulent flow occurring at sites of arterial branching, attenuates NO release and potentiates expression of adhesion molecules (Gimbrone *et al.* 1997).

Most CV risk factors activate molecular machinery in the endothelium that results in expression of chemokines, cytokines, and adhesion molecules, designed to interact with leukocytes and platelets and target inflammation to specific tissues to clear microorganisms (Aikawa *et al.* 2002; Johnson *et al.* 1998; Hansson 2005). Adhesion molecules play a crucial role in the interaction of the endothelial surface with circulating leucocytes and mediate the recruitment of leucocytes in the vessel wall (Esper *et al.* 2006). This process is crucial in the clearance of low-density lipoprotein (LDL), and especially oxidized LDL (oxLDL), in the arterial wall. Low density lipoprotein is cleaned by ligation to LDL receptors, and oxLDL may also bind by scavenger receptors on monocytes

in the subendothelial compartment. Monocytes ingest oxLDL and turn into macrophages; macrophages generate reactive oxygen species (ROS), which convert oxLDL into highly oxidized LDL. The uptake of highly oxidized LDL by macrophages leads to formation of foam cells. Foam cells combine with leukocytes to become fatty streaks, and as the process continues, foam cells secrete growth factors that induce smooth muscle cell migration into the intima. Smooth muscle cell proliferation, coupled with the continuous influx and propagation of monocytes and macrophages, converts fatty streaks to more advanced lesions and ultimately to fibrous plaque that will protrude into the arterial lumen (Libby and Ridker 2006; Mehta 2006; Ross 1999).

2.1.3. Endothelial function and clinical outcome

A study by Ludmer and co-workers, using the acetylcholine test, provided the first evidence of paradoxical constriction in the coronary arteries of patients with mild coronary artery disease (CAD), as well as in those with advanced CAD, indicating that endothelial dysfunction is present in the early stage of atherosclerosis (Ludmer *et al.* 1986). Coronary endothelial dysfunction may be also associated with myocardial ischaemia, despite absence of angiographic evidence of atherosclerosis (Quyyumi *et al.* 1995). In addition to its role in early atherosclerosis (Celermajer *et al.* 1992, Pepine *et al.* 1998; Chauhan *et al.* 1996), there is growing recognition that endothelial dysfunction also contributes to the later stages of the disease when patients develop clinical symptoms (Okumura *et al.* 1992; van Boven *et al.* 1996). Impaired EF may play a fundamental role in the pathogenesis of acute coronary syndromes, such as unstable angina and acute myocardial infarction (MI) (Libby 2001). Thus, EF rather than presence and severity of fixed anatomic disease is likely to be relevant to the pathogenesis of vascular events.

Studies of the prognostic value of EF in coronary circulation during diagnostic catheterisation (patients with or without CAD) demonstrated that impaired EF predicted occurrence of CV events (Targonski *et al.* 2003; Halcox *et al.* 2002; Suwaidi *et al.* 2000; Schachinger *et al.* 2000), as well as development of in-stent restenosis (Thanyasiri *et al.* in press). However, endothelial dysfunction is not confined to the coronary arteries but rather represents a systemic disorder that also affects peripheral vascular beds, including both conduit arteries and small resistance vessels in the extremities (Anderson *et al.* 1995). Studies involving patients with CAD suggested that peripheral EF has also a significant prognostic value of CV events (Fichtlscherer *et al.* 2004) as well as in-stent restenosis (Patti *et al.* 2005). Furthermore, studies in patients with angiographically normal coronary arteries provide further evidence that endothelial dysfunction precedes and portends development of atherosclerosis (Nitenberg *et al.* 2004; Schindler *et al.* 2003). In addition, several reports have

considered the prognostic value of EF in patients with PAD (Gocke *et al.* 2002; Gocke *et al.* 2003; Pasqualini *et al.* 2003), heart failure (Heitzer *et al.* 2005) and hypertension (Perticone *et al.* 2005).

The ability to improve EF is a common feature of many other interventions proved to reduce CV risk. A number of interventions have been shown to be effective in improving EF or reversing endothelial dysfunction in the coronary and peripheral circulation. These include lipid-lowering (Ott *et al.* in press; Fichtlscherer *et al.* 2006), anti-inflammatory (Booth *et al.* 2004) and anti-hypertensive therapy (Modena *et al.* 2002), hyperhomocysteinemia and hyperglucaemia reduction (Yasuda *et al.* 2006), administration of L-arginine (Böger *et al.* 1998a) and vitamins C and E (Plantinga *et al.* 2007), hormone replacement (Brown *et al.* 2002), diet (Brown *et al.* 2001), exercise (Fuchsjager-Mayrl *et al.* 2002), smoking cessation (Celermajer *et al.* 1993), etc. Moreover, evidence now suggests that EF may be modulated not only by factors causing vascular injury but also by repair mechanisms, potentially mediated *via* circulating endothelial progenitor cells (Hill *et al.* 2003). These findings add support to the concept that restoration of EF can prevent/restabilize the atherosclerotic disease process.

A number of potential mechanisms have been proposed to explain the relationship between endothelial dysfunction and CV risk. It is possible that endothelial dysfunction simply reflects the presence and extent of atherosclerosis and the severity of traditional risk factor "burden," thus implying that combined or repeated injury to the vascular endothelium results in greater dysfunction. Nevertheless, there is considerable heterogeneity in the magnitude of dysfunction, observed in individuals with similar risk factor profiles indicating probably different impacts of aging and other confounders on vascular function (Quyyumi *et al.* 1995; Anderson *et al.* 1995). However, EF varies widely among patients with atherosclerosis, and many patients with advanced disease display EF comparable to that observed in healthy subjects (Yataco *et al.* 1999). Papers I and IV of the current thesis assessed the degree of EF in patients with lower extremity PAD in comparison with healthy subjects.

2.1.4. Arterial stiffness

Great emphasis has been placed on the role of arterial stiffness in the development of CV disease. Stiffening affects predominantly the aorta and the proximal elastic arteries, and to a lesser degree the peripheral muscular arteries. Stiff arteries lead to left ventricular hypertrophy, aneurysm formation and rupture and are also a major contributor to atherosclerotic and small vessel disease and thus to stroke, MI, renal failure and PAD (Laurent *et al.* 2006; Safar 2007a).

The terms arterial stiffness, elasticity and compliance are used interchangeably, although their physical definitions are somewhat different (Cohn *et al.* 2004; O'Rourke *et al.* 2002). All these terms relate to volume or dimensional change in the artery in response to a transmural pressure change. However, as a generic term, arterial stiffness is preferable (O'Rourke *et al.* 2002).

In addition, there is confusion in the clinical literature on terminology, especially between the terms arteriosclerosis and atherosclerosis (Safar 2007b; O'Rourke 2003). The arterial wall has two principal layers. The innermost (or intima) functions to separate flowing blood from the vascular structures, to prevent adherence of formed elements from the blood on the wall, and to control muscular tone in the media. The second layer (or media) comprises the structural components (elastin fibres, collagen fibres and smooth muscle) which are responsible for the biomechanical properties of the arterial wall (Nichols and O'Rourke 1998). Atherosclerosis (intima disease) is characterized by lipid accumulation, inflammation, fibrosis and development of focal plaques. However, it may secondarily involve the media and weaken the arterial wall, but its principal effects exert through flow limitation. In contrast, the two forms of arteriosclerosis – hypertensive and senile – are diffuse and affect mainly the media increasing stiffness in the whole arterial tree (Mackey *et al.* 2007; Safar 2007b; O'Rourke 2003).

Increasing arterial stiffness is the hallmark of the ageing process and has emergences as an important determinant of increased systolic BP and pulse pressure (PP). Peripheral PP, i.e. the difference between systolic and diastolic BP obtained from the brachial artery, has been used as a simple surrogate measure of arterial stiffness. This is based on the assumption that peripheral PP is an accurate surrogate for central BP and hence represents the workload experienced by the heart. While diastolic and mean BP remain relatively constant throughout the arterial tree, then systolic BP and PP do not; therefore peripheral BP may not accurately reflect actual aortic PP (Schiffrin 2004).

Pulse pressure varies throughout the arterial system due to the difference in vessel stiffness and the phenomenon of wave reflection. A pressure wave which propagates along a visolelastic tube is progressively amplified, from central to distal conduit arteries. In peripheral arteries, wave reflections can amplify more the pressure wave because reflection sites are closer to peripheral sites than to central arteries, and pulse wave velocity (PWV) is higher in a peripheral stiffer

artery. Thus, amplitude of pressure wave is higher in peripheral arteries than in central arteries. This is likely to be important clinically, because the left ventricle, the kidneys, and the brain are influenced by central, not peripheral haemodynamics (O'Rourke and Safar 2005). Furthermore, central PP is a stronger predictor, compared with peripheral PP, of all-cause mortality (Safar *et al.* 2002) and CV events (Roman *et al.* in press). Central PP is also a better predictor of left ventricular mass (Saba *et al.* 1993), carotid intima-media thickness (Boutouyrie *et al.* 1999) and atherosclerotic plaque score (Roman *et al.* in press).

Several studies have revealed positive significant associations between arterial stiffness and atherosclerosis. There exists significant correlation of aortic PWV with carotid intima-media thickness and severity of plaques in the carotid artery, in the aorta (van Popele *et al.* 2001) and in the coronary arteries (McLeod *et al.* 2004). However, there is no clear evidence of association between atherosclerosis and arterial stiffness, or of the validity of the indices of arterial stiffness in identification of persons with preclinical atherosclerosis. Avolio and co-workers showed that aortic PWV was virtually identical in populations with low and high prevalence of atherosclerosis (Avolio *et al.* 1983). Studies have not found association between aortic PWV and coronary and extracoronary atherosclerosis (Megnien *et al.* 1998) or between arterial stiffness and severity of CAD, either (Wykretowicz *et al.* 2005).

2.1.5. Arterial stiffness is regulated by endothelium

Traditionally, vessel stiffness was regarded simply as a function of the structural elements of the vessel wall (mainly collagen and elastin) and transmural (mean arterial) pressure. However, arterial stiffness is a dynamic parameter that depends, at least in part, on smooth muscle tone which is actively regulated by endothelium-derived NO. Inhibition of basal NO production in the endothelium with L-monomethyl-N^G-arginine increases iliac PWV in sheep (Wilkinson *et al.* 2002b), and, in humans, increases wave reflections and aortic stiffness (Wilkinson *et al.* 2002c) and brachial artery stiffness (Kinlay *et al.* 2001). Recent data suggest also that conditions associated with endothelial dysfunction (Chowienczyk *et al.* 1992) are correlated with increased arterial stiffness (Toikka *et al.* 1999), and therapeutic interventions which improve EF also reduce arterial stiffness (McEniery *et al.* 2004). It has been shown that brachial artery EF independently predicts arterial stiffness in humans (Smith *et al.* 2002). However, only a few data have been published about the interrelationships between arterial elasticity and endothelial vasomotor properties (Parvathaneni *et al.* 2002; Tao *et al.* 2004; Wilson *et al.* 2004), but relevant studies have not used PWA for endothelial assessment. Furthermore, direct evidence of a relationship between EF and measures of arterial stiffness is limited to studies in patients

with CV diseases and risk factors (Ramsey *et al.* 1995; Cheung *et al.* 2002; Ravikumar *et al.* 2002; Nigam *et al.* 2003). Moreover, data about NO and regulation of large artery stiffness in humans have been somewhat contradictory (Stewart *et al.* 2003). However, there were no data about associations between arterial stiffness and EF, both measured by PWA, in healthy normotensive individuals, who are free of the potentially confounding influence of CV diseases.

In Paper I of this thesis we investigated the effects of stimulation of NO synthesis on wave reflections and on aortic stiffness in patients with lower extremity PAD and in healthy subjects. We focused on testing the hypothesis that release of NO increases aortic stiffness independently of changes in mean arterial pressure (MAP), which is *per se* a strong contributor to arterial stiffness. In Paper II we determined possible associations between EF and arterial stiffness in healthy subjects.

2.1.6. Asymmetric dimethylarginine

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of all three isoforms of NOS (Vallance *et al.* 1992). Asymmetric dimethylarginine is produced by methylation of arginine residues in intracellular proteins *via* arginine *N*-methyltransferases (Moncada and Higgs 1993). Circulating ADMA is metabolized by the specific enzyme dimethylarginine dimethylaminohydrolase into L-citrulline and dimethylamine (MacAllister *et al.* 1996a).

When administered to healthy volunteers, ADMA produces elevation of BP, vasoconstriction, increased renovascular resistance, reduced forearm blood flow, reduced heart rate (HR) and reduced cardiac output (Achan *et al.* 2003; Kilestein *et al.* 2004; Calver *et al.* 1993). It has been shown that ADMA plasma levels are elevated in patients with hypertension (Goonasekera *et al.* 1997), CAD (Valkonen *et al.* 2001), PAD (Böger *et al.* 1997), hypercholesterolemia (Böger *et al.* 1998b), hyperhomocysteinaemia (Böger *et al.* 2001), diabetes (Abbasi *et al.* 2001), end-stage renal disease (ESRD) (MacAllister *et al.* 1996b), etc. Moreover, ADMA is a strong and independent predictor of CV events (Zoccali *et al.* 2001; Krempl *et al.* 2005; Hedner *et al.* 2002).

Accumulation of ADMA contributes to endothelial dysfunction (Perticone *et al.* 2005) and may lead potentially to arterial stiffening. Chronic inhibition of NOS, or clinical conditions associated with endothelial dysfunction may increase arterial stiffness which in the long run may favour development of atherosclerosis. Increased ADMA concentrations represent a strong and independent risk marker for progression of CAD (Schnabel *et al.* 2005) and renal damage (Ravani *et al.* 2005), in which arterial stiffness is also patophysiologically involved (Weber *et al.* 2005; Blacher *et al.* 1999). These data suggest the

existence of a potential link between arterial stiffness and ADMA. However, there are no data about arterial stiffness, measured by PWA, and plasma ADMA level. Paper II of this thesis elucidated the possible relationship between arterial stiffness and ADMA plasma level in healthy subjects.

2.1.7. Clinical implications of arterial stiffness

A variety of clinical conditions are associated with increased arterial stiffness including hypercholesterolaemia, smoking, metabolic syndrome, hypertension, CAD, stroke, ESRD, etc (Laurent *et al.* 2006). Arterial stiffness is now widely considered as the most important determinant of increased systolic BP and PP and hence the intrinsic cause of a host of MI and stroke. An increase in central PP and a decrease in diastolic BP may directly cause subendocardial ischemia and lead to left ventricular hypertrophy (Roman *et al.* 2000), which is *per se* an independent CV risk factor (Boutouyrie *et al.* 1995). However, in addition to increase in central BP, an increase in arterial stiffness can increase the risk of MI and stroke through several mechanisms. Stiffer arteries influence arterial remodelling, increase arterial wall thickness and promote development of plaques. Several studies have demonstrated that aortic PWV has an independent predictive value for all-cause and CV mortality (Laurent *et al.* 2001), coronary events (Boutouyrie *et al.* 2002) and strokes (Laurent *et al.* 2003) in patients with uncomplicated essential hypertension and in general population (Mattace-Raso *et al.* 2006; Shokawa *et al.* 2005; Willum-Hansen *et al.* 2006). Arterial wave reflections independently stratify all-cause and CV mortality in patients with ESRD (London *et al.* 2001), CV events in patients with CAD (Weber *et al.* 2004) and hypertension (Williams *et al.* 2006). Reduced small artery elasticity is significantly associated with CV events independently of age (Grey *et al.* 2003). These data provide further support for the concept that the biological process in the artery wall is a better guide to future CV morbid events than standard risk factors.

Arterial stiffness would be a novel therapeutic target for prevention of CV diseases. Antihypertensive agents (β-blockers, diuretics, angiotensin converting enzyme inhibitors, etc.) may influence arterial stiffness in a number of ways; indirectly *via* reduction in MAP, or directly *via* an effect on the various components of the arterial wall (Ahimastos *et al.* 2005). Theoretically, an ideal antihypertensive agent should reduce BP and stiffness. In subjects with ESRD, treated with antihypertensive drugs, survival is the highest in those in whom therapy reduces PWV and MAP rather than MAP alone (Guerin *et al.* 2001). More importantly, increasing data indicate that despite similar effects on peripheral BP and a greater effect on aortic stiffness, atenolol had less impact on central systolic BP than amplodipin (Williams *et al.* 2006) or eprosartan (Dhakam *et al.* 2006) because it failed to reduce wave reflections. This provides

one potential explanation for the failure of atenolol to improve outcome in older patients with essential hypertension. Pharmacological treatment which is able to reduce arterial stiffness involves also nitrates, aldosterone antagonists, hypolipidaemic agents, antidiabetic agents, sildenafil and advanced glycation endproduct breakers. An expert consensus document on arterial stiffness declares that arterial stiffness provides direct evidence of target organ damage, and measurements of arterial stiffness and central pressure should be considered recommended tests for evaluation of CV risk (Laurent *et al.* 2006).

Data about arterial stiffness using novel non-invasive PWA methodology in patients with PAD is largely limited. Few studies have demonstrated increased arterial stiffness in patients with lower extremity PAD (van Popele *et al.* 2001; Duprez *et al.* 2001). However, other authors have reported controversial results about links between arterial stiffness and atherosclerosis (Megnien *et al.* 1998; Wykretowicz *et al.* 2005). Papers I, III and IV of this thesis investigated the impact of lower extremity atherosclerosis on several indices of arterial stiffness in comparison with healthy controls.

2.2. Assessment of vascular function

"Surely it must be to our advantage to appreciate fully all the pulse tells us, and to draw from the pulse all that it is capable of imparting" (Mahomed 1872).

2.2.1. Testing of endothelial vasodilatory function

The improved understanding of the vascular biology of the endothelium has permitted the development of clinical tests that evaluate the functional properties of the endothelium. Ideally, such tests should be safe, non-invasive, reproducible, cheap, and standardized between laboratories. The results should also reflect the dynamic biology of the endothelium throughout the natural history of atherosclerotic disease, define subclinical disease processes, as well as provide prognostic information for risk stratification.

Endothelial dysfunction is a generalized process that affects both the conduit and resistance arteries (coronary and peripheral) and can be identified in vascular beds remote from circulation where events occur (Gocke *et al.* 2002; Kuvin *et al.* 2001). In response to physiologic stimuli, such as shear stress, the endothelium releases NO. Nitric oxide synthesized by NOS from the L-arginine in the presence of cofactors such as tetrahydrobiopterin (Palmer *et al.* 1988). There are at least three isoforms of NOS, endothelial NOS, neuronal NOS and inducible NOS. The gaseous NO diffuses to the vascular smooth muscle cells and activates guanylate cyclase, which leads to cyclic guanosine monophosphate-mediated vasodilation (Arnold *et al.* 1977). Nitric oxide is not only a mediator of endothelium-dependent vasodilatation (EDV) but is also critically involved

in the regulation of other protective properties of the healthy endothelium. Thus, measurement EDV may serve as surrogate for the bioavailability of NO (Lane *et al.* 2006; Deanfield *et al.* 2005). Established methods for assessing EF commonly focus on measuring stimulated NO release in response to pharmacologic or physical stimuli such as acetylcholine, metacholine, bradykinine, serotonin, substance P, and shear stress in different vascular beds. Basal release of NO can be assessed by using the specific L-arginine analogue NOS inhibitor N^G-nitro-L-arginine methyl ester (Rees *et al.* 1989). It is usual also to assess the response to a direct NO donor drug, like sodium nitroprusside or nitroglycerin (NTG) (endothelium-independent agonist), in order to exclude any concomitant alteration in vascular smooth muscle sensitivity to NO.

A well-established technique for the evaluation of coronary conduit EF is quantitative coronary angiography with pharmacological provocative tests (Ludmer *et al.* 1986). This method has been refined with the use of the Doppler flow wires to measure myocardial microvascular EF. Non-invasive tests for assessment of coronary EF include Doppler echocardiography, positron emission tomography, and phase-contrast magnetic resonance imaging (Verma and Anderson 2002; Behrendt and Ganz 2002). Brachial artery ultrasound is a widely used non-invasive measure of peripheral EF in conduit vessels (Celermajer *et al.* 1992) and strain gauge plethysmography is similarly used for measurement of peripheral EF in resistance vessels (Wilkinson and Webb 2001). Laser digital Doppler flowmetry and wire- or perfusion-pressure micromyographs can be used also to assess EF in the microvasculature (Deanfield *et al.* 2005). In addition, several biological markers have been used as indicators of endothelial dysfunction (e.g. von Willebrand factor, thrombomodullin, adhesion molecules, endothelial cells, etc) (Constans and Conri 2006).

Recently, an alternative non-invasive PWA technique has been developed with the β_2-adrenoreceptor agonist salbutamol (Salb) and NTG administration, using measurement of the response with radial artery applanation tonometry (Wilkinson *et al.* 2002a; Hayward *et al.* 2002) or digital photopletysmography (Chowienczyk *et al.* 1999). Salbutamol is a β_2-adrenergic receptor agonist that reduces arterial stiffness in a NO-dependent manner (Dawes *et al.* 1997). Therefore, reduction of stiffness that occurs with Salb (i.e. EDV) can be used as a measure of global EF which includes the conduit and resistance vessels. Improvement in arterial stiffness after NTG is a marker of endothelium-independent vasodilatation (EIDV) in case PWA methodology used. The endothelial function index (EFI), defined as the EDV/EIDV ratio, is supposed to reflect more specifically the contribution of the endothelium to the vasodilatory process (Lind *et al.* 2002).

Pulse wave analysis technique for assessment of EF is reproducible and correlates with the response to acetylcholine in the forearm vascular bed, assessed using venous occlusion plethysmography (Wilkinson *et al.* 2002a). Only preliminary results of EF in humans have been obtained by PWA method.

An impaired pulse wave response with Salb has been demonstrated in patients with diabetes (Chowienczyk *et al.* 1999), hypercholesterolemia (Wilkinson *et al.* 2002a), ESRD (Covic *et al.* 2003) and CAD (Hayward *et al.* 2002). However, there are no data of the applicability of the PWA method for assessment of EF in patients with lower extremity PAD. Furthermore, patients with PAD have generalized atherosclerotic process and are therefore appropriate candidates for basic mechanistic studies using PWA. In the current thesis all EF tests were carried out using PWA methodology.

2.2.2. Measurement of arterial stiffness

Assessment of the elastic behaviour of the arteries may provide an insight into the early functional and structural abnormalities of atherosclerosis as well as serve as a surrogate end point for prediction and treatment of CV diseases. Three main methodologies of measuring arterial stiffness include: 1) assessment of PWV, 2) use of ultrasound or magnet resonance imaging to relate the change in the diameter of an artery to distending pressure, and 3) PWA, for the regional, local and systemic determination of stiffness, respectively (Pannier *et al.* 2002; Oliver and Webb 2003; Laurent *et al.* 2006).

Analysis of the specific components of the arterial pressure waveform are used in a number of non-invasive methodologies designed to measure arterial stiffness (Laurent *et al.* 2006; O'Rourke *et al.* 2001). Methods for analysing the pulse wave involve evaluation of both the systole and the diastole. The technique of systolic PWA depends on the accurate recording of the radial pressure wave, its calibration against brachial pressure and further generation of the ascending aortic pressure waveform through use of a validated transfer function in a computerized process (Chen *et al.* 1997; Pauca *et al.* 2001).

The systolic part of the central arterial pressure waveform is characterized by two pressure peaks: the first peak is caused by left ventricular ejection, while the second peak is the result of wave reflection. From the central aortic waveform central BP values and indices of arterial stiffness, the augmentation index (AIx) and the travel time of the reflected wave (Tr) (Figure 1) can be calculated. The AIx was defined as the difference between the second and the first systolic peaks of the central arterial waveform, expressed as the percentage of central PP. The AIx, a predominant determinant of wave reflections, depends also on several factors, including sex, ventricular ejection, height, HR, MAP and aortic PWV. The Tr represents the composite travel time of the pulse wave to the periphery and its return to the ascending aorta, thus providing estimated aortic PWV or aortic stiffness (Wilkinson *et al.* 2002b; Wilkinson *et al.* 2001).

Pulse wave analysis, based on the modified Windkessel model of the vasculature, concentrates exclusively on the diastolic part of the arterial pressure waveform, and can be used to derive information on the stiffness/elasticity of

both the proximal and the distal arteries (Zimlichman *et al.* 2005; Cohn *et al.* 2004). Two components of the diastolic waveform are distinguished in diastolic PWA. The exponential decay curve represents large (capacitative) artery elasticity (C1), whereas the other component, oscillatory or reflective elasticity, consists of peripheral wave reflections and provides a measure of small artery elasticity (C2). (Parameters C1 and C2 are defined by the manufacturer as the elasticity indices. Therefore as in the text the general term arterial stiffness is used, both C1 and C2 decreased with increasing stiffness). In this thesis both systolic (Papers I, II and IV) and diastolic PWA (Papers II and III) are used.

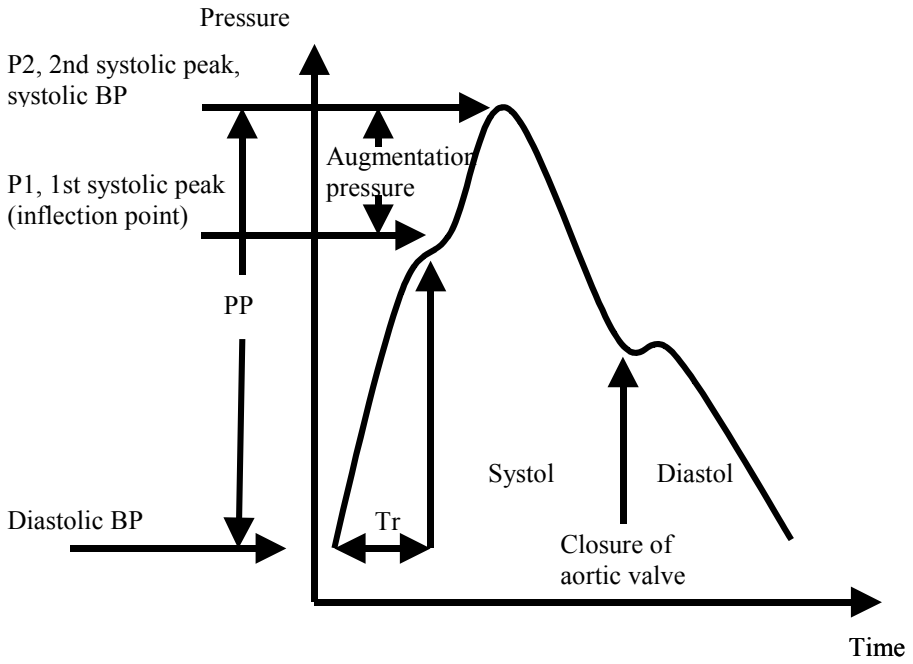

Figure 1. The systolic part of the central arterial pressure waveform is characterized by two pressure peaks. The first peak is caused by left ventricular ejection, while the second peak is the result of wave reflection. Augmentation pressure is the difference between P2 and P1. The AIx is augmentation pressure expressed as the percentage of central PP. The Tr is calculated as the time between the foot of the wave and the inflection point (wave reflection). Modified from (Kals *et al.* 2003).

2.3. Mechanisms of vascular dysfunction

2.3.1. Oxidative stress

Free radicals, largely derived from molecular oxygen, have been implicated in a variety of human conditions and diseases. Reactive oxygen species are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells. Reactive oxygen species are involved in regulation of signal transduction and gene expression, in inflammatory and vasodilatory responses, in activation of receptors and nuclear transcription factors, in oxidative damage to cell components, in the antimicrobial and cytotoxic action of immune system cells, neutrophiles and macrophages, in apoptosis as well as in aging (Packer and Cadenas 2005).

To compensate for the effects of ROS, cells have evolved both enzymatic and nonenzymatic mechanisms to protect against the toxic effects of oxidants (Halliwell 1997). Excessive production of ROS, outstripping endogenous anti-oxidant defence mechanisms, has been implicated in processes in which they oxidize biological macromolecules, such as deoxyribonucleic acid, protein, carbohydrates, and lipids. The term "oxidative stress" generally indicates that the antioxidant status of cells and tissues is altered by exposure to pro-oxidants (e.g. free radicals, ROS), which can lead to cell and tissue injury (Sies 1991). An increasing body of evidence suggests that OxS is involved in the pathogenesis of many CV diseases, including atherosclerosis, hypertension, diabetes, heart failure (Cai and Harrison 2000), but also specifically in endothelial dysfunction (Loffredo *et al.* 2007) and arterial stiffness (Toikka *et al.* 1999). Determination of OxS is mainly based on measurements of oxidatively modified compounds.

2.3.1.1. 8-iso-prostaglandin F_{2a}

In the Biomarkers of Oxidative Stress Study, a recent multi-investigator study, it was found that the most accurate method to assess *in vivo* the status of OxS is the quantification of plasma or urinary 8-iso-prostaglandin F_{2a} (F_2-IsoPs) (Milne *et al.* 2007; Kadiiska *et al.* 2005; Morrow 2005). The 8-iso-prostaglandin F_{2a} are a series of prostaglandin F_{2a}-like compounds produced by the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase (Belton *et al.* 2000). The 8-iso-prostaglandin F_{2a} are stable, robust molecules detectable in all human tissues and biological fluids.

The 8-iso-prostaglandin F_{2a} been shown to be increased in association in with a number of atherosclerotic risk factors, including cigarette smoking (Morrow *et al.* 1995), hypercholesterolemia (Davi *et al.* 2004), diabetes mellitus (Davi *et al.* 1999), and obesity (Keaney *et al.* 2003), etc. There has been

demonstrated accumulation of F_2-IsoPs in human atherosclerotic plaques (Pratico *et al.* 1997). Furthermore, their levels are related to plaque instability (Cipollone *et al.* 2000). The 8-iso-prostaglandin F_{2a} is an indicator of presence and extent of CAD (Wang *et al.* 2006) as well as of atherosclerotic risk (Mueller *et al.* 2004). A preliminary study demonstrated increased F_2-IsoPs levels and impaired EF and increased arterial stiffness in healthy subjects after methionine administration (Arcaro *et al.* 2004).

2.3.1.2. Oxidized low-density lipoprotein

A number of studies suggest that oxLDL is a more potent pro-atherosclerotic stimulus than native unmodified LDL (Berliner and Watson 2005). The endothelium exposed to oxLDL develops early signs of injury (Li *et al.* 1998), decreases the gene expression of endothelial NOS and enhances generation of ROS (Mehta *et al.* 2001). The oxLDL itself activates inflammatory cells and facilitates release of growth factors from monocytes/macrophages (Absood *et al.* 2002). *In vivo* studies in human tissue have demonstrated the accumulation of oxLDL in the vessel wall at all stages of atherosclerosis (Mehta 2006).

Measurement of oxLDL (Holvoet *et al.* 2001) or measurement of the baseline diene conjugates of LDL (LDL-BDC) (Ahutopa *et al.* 1999) are reliable methods to monitor oxidative modifications of LDL. Studies have shown that circulating oxLDL is a sensitive marker of CAD (Holvoet *et al.* 2001; Shimada *et al.* 2004) correlated with plaque progression (Wallenfeldt *et al.* 2004) and intima-media thickness (Liu *et al.* 2004). However, only preliminary data are available about correlations of oxLDL with arterial stiffness (Toikka *et al.* 1999).

2.3.1.3. Myeloperoxidase

Recent studies have emphasized the importance of myeloperoxidase (MPO) for CV diseases (Baldus *et al.* 2003; Zhang *et al.* 2001). Activation of leukocytes prompts the secretion of MPO and generation of oxidants with an important physiological role in host defence (Klebanoff *et al.* 1984; Eiserich *et al.* 2002). Myeloperoxidase level is higher in patients with CAD (Baldus *et al.* 2003) and this compound predicts future CV events (Brennan *et al.* 2003) and endothelial dysfunction in humans (Vita *et al.* 2004).

However, no earlier study has demonstrated associations between the functional parameters of arterial stiffness such as C1, C2, AIx and Tr and OxS-related biomarkers, F_2-IsoPs, oxLDL, LDL-BDC and MPO. Papers III and IV of this thesis investigated possible association between arterial stiffness and OxS in patients with lower extremity PAD and in healthy subjects.

2.3.2. Inflammation

Recent advances have established the pivotal role of inflammation in mediating all stages of atherosclerosis (Ross 1999). At the site of an endothelial injury, in response to mechanical, immunologic, and chemical injuries, the invading inflammatory cells producing numerous pro-inflammatory factors promote and amplify both local and systemic inflammation that lead to atherosclerosis (Libby and Ridker 2006). Both the vascular endothelium (Marchesi *et al.* 2007; Hingorani *et al.* 2000; Cleland *et al.* 2000; Booth *et al.* 2004) and arterial stiffness (Yasmin *et al.* 2004; Kampus *et al.* 2004) are affected by inflammatory processes. Inflammatory biomarkers possess substantial clinical utility for improving detection of CV risk, and for seeking novel anti-inflammatory therapies with the potential to treat and prevent vascular complications.

2.3.2.1. Intercellular adhesion molecule-1

Adherence of leucocytes to the endothelium and their transmigration into the arterial wall are dependent on a cascade of events mediated by a family of cellular adhesion molecules in response to several inflammatory stimuli. Membrane bound VCAM-1, ICAM-1, endothelial leucocyte adhesion molecule and E-selectin are expressed on endothelial cells, smooth muscle cells and tissue macrophages (O'Brien *et al.* 1996)

Elevated levels of ICAM-1 were reported in patients with acute coronary syndromes (Shyu *et al.* 1996), diabetes (Jude *et al*. 2002), PAD (Silvestro *et al.* 2003), etc. Moreover, studies have shown that ICAM-1 concentrations are predictive of development of CAD (Malik *et al.* 2001) and both carotid atherosclerosis and future CV events (Ridker *et al.* 1998a; Luc *et al.* 2003; Blankenberg *et al.* 2001; Hwang *et al.* 1997). It has been demonstrated that human ICAM-1 correlates with femoral and carotid artery plaque severity in patients with PAD (Hulthe *et al.* 2002), with carotid intima-media thickness (Rohde *et al.* 1998) and with EF (Holmlund *et al.* 2002).

2.3.2.2. C-reactive protein

C-reactive protein (CRP) is a pentameric protein synthesised in the liver. Its main action is to activate the complement and to counteract infections. The main stimuli for secretion of CRP are interleukin-1 and -6, and indirectly also tumour necrosis factor alpha (Libby and Ridker 2006).

Circulating levels of CRP have been found to be related to a number of well known CV risk factors, such as obesity, smoking, serum fibrinogen, LDL cholesterol, BP, serum triglycerides, fasting blood glucose, apolipoprotein B and inversely to high-density lipoprotein (HDL) cholesterol levels both in children and in adults (Libby and Ridker 2006; Cook *et al.* 2000; Mendall *et al.* 1996). In healthy women and men CRP predicts future CV risk independently

of traditional CV risk factors (Ridker *et al.* 1997; Ridker *et al.* 1998b). Moreover, CRP is a stronger predictor of future CV events than LDL cholesterol (Ridker *et al.* 2002). Studies have shown that CRP is also closely associated with EF (Cleland *et al.* 2000; Brevetti *et al.* 2003a), arterial stiffness (Kampus *et al.* 2007) and ankle-brachial pressure index (ABPI) (Ridker *et al.* 2001; Brevetti *et al.* 2003a).

Recently, much attention has been given to the possibility that inflammation is a primary process of atherosclerosis and oxidative events may be a consequence, rather than a cause, of the atherosclerosis (Stocker and Keaney 2004). If inflammation precedes high-grade OxS in atherogenesis, the markers of inflammation and OxS may be linked differently to EF and arterial stiffening both in atherosclerosis and in a healthy condition. Paper IV of this thesis investigated possible different relationships between EF, arterial stiffness and several indices of inflammation (CRP, ICAM-1) and OxS (F_2-IsoPs, LDL-BDC, oxLDL, MPO) in patients with lower extremity PAD and in healthy subjects.

2.4. Patients with lower extremity peripheral arterial disease

The term "peripheral arterial disease" broadly encompasses a range of noncoronary arterial syndromes that are caused by the altered structure and function of the arteries that supply the brain, visceral organs, and the limbs (Hirsch *et al.* 2006). Lower extremity PAD includes disorders that affect the leg arteries and does not include disease of the aorta, carotid, upper extremity, or visceral arteries. The major cause of lower extremity PAD is atherosclerosis, and thus the epidemiology and clinical consequences of PAD are closely associated with classic atherosclerotic risk factors (e.g. cigarette smoking, diabetes, dyslipidemia, hypertension, family history, and postmenopausal state) and with more recently defined risk factors (e.g. hyperhomocysteinemia and elevated CRP level) (Hirsch *et al.* 2006; Norgen *et al.* 2007). Severity of leg ischaemia in the patients with lower extremity PAD can be classified according to either the Fontaine or the Rutherford categories (Hirsch *et al.* 2006). We have used Fontaine classification, which categorizes leg symptoms as follows: stage I – asymptomatic, stage IIa – mild intermittent claudication, stage IIb – moderate-severe intermittent claudication, stage III – leg pain at rest, and stage IV – tissue loss due to ischemic ulcer or gangrene (Hirsch *et al.* 2006).

Total prevalence of lower extremity PAD, based on objective testing, has been evaluated in several epidemiologic studies and is in the range of 3% to 10%, increasing to 15% to 20% in persons aged over 70 years (Criqui *et al.* 1985; Hiatt *et al.* 1995; Ouriel 2001; Selvin and Erlinger 2004). The prevalence of asymptomatic PAD in the leg can only be estimated by using non-invasive

measurements in a general population. A commonly used non-invasive test for lower extremity PAD is the measurement of systolic BP in the ankles and arms with a Doppler ultrasonic instrument, from which the ABPI is derived (Carter 1968). A resting ABPI of ≤ 0.90 is caused by haemodynamically significant arterial stenosis and is most often used as a haemodynamic definition of PAD. In symptomatic individuals, an ABPI of ≤ 0.90 is approximately 95% sensitive in detecting angiogram-positive PAD and almost 100% specific in identifying healthy individuals (Norgen *et al.* 2007). A low ABPI is highly predictive not only of the presence of PAD but also of subsequent CV mortality (Resnick *et al.* 2004). The Edinburgh Artery Study has shown that the ABPI is a good predictor of non-fatal and fatal CV events as well as total mortality, in an unselected general population (Fowkes *et al.* 1991).

Several studies have demonstrated that patients with PAD have a 3- to 5-fold increased risk of CV mortality compared with age-matched controls (Criqui *et al.* 1992; Vogt *et al.* 1993). The prognosis for patients with lower extremity PAD is characterized by an increased risk for CV ischemic events due to concomitant CAD and cerebrovascular disease (Ness *et al.* 1999; Leng *et al.* 1996). These CV ischemic events are more frequent than ischemic limb events in any lower extremity PAD cohort (Weitz *et al.* 1996).

Endothelium-dependent vasodilation mediated by NO is impaired in patients with lower extremity PAD (Yataco *et al.* 1999; Harris *et al*. 1995). These patients have also stiffer arteries (van Popele *et al.* 2001) and elevated markers of OxS (Mueller *et al.* 2004) and inflammation (Silvestro *et al.* 2003). However, besides severity of PAD (defined by ABPI), the increased CV risk in these patients appears to be related also to EF, arterial stiffness, OxS and inflammation. Altered production of NO in patients with PAD does not mediate only blood flow in the large conduit arteries, but impaired EF on the microvascular level contributes to leucocyte adherence to the endothelium and further impedes nutritive blood flow. Patients with PAD with preserved EF are at low risk for peri-operative and long-term events and might be managed differently than patients with poor EF (Gocke *et al.* 2002; Gocke *et al.* 2003; Pasqualini *et al.* 2003). Furthermore, alterations of systemic haemodynamics which characterize patients with lower extremity PAD, such as increase in systolic BP and decrease in diastolic BP due to increased arterial stiffness may be possible links between PAD and mortality from CAD and stroke (Safar 2007a).

It has been shown that the F_2-IsoPs level is an independent predictor of lower extremity PAD (Mueller *et al.* 2004). Additionally, elevated levels of CRP (Ridker *et al.* 1998c; Ridker *et al.* 2001) and ICAM-1 (Pradhan *et al.* 2002; Tzoulaki *et al.* 2005; Silvestro *et al.* 2003; Brevetti *et al.* 2003a) are independent risk factors for PAD development and severity, and predict fatal and non-fatal MI (Rossi *et al.* 2002) as well as postoperative vascular graft-related events in these patients (Owens *et al.* 2007).

Vascular function may be a target of therapy to improve functional capacity in patients with lower extremity PAD. It has been demonstrated that interventions for reversing endothelial dysfunction could limit CV risk and enhancement of NO production may help maintain bypass patency and limb salvage in patients with lower extremity PAD (Komori *et al.* 1997). Restoring EF by L-arginine administration (Böger *et al.* 1998), by autologue bone-marrow mononuclear cell implantation (Higashi *et al.* 2004), by exercise rehabilitation (Brendle *et al.* 2001), by antihypertensive treatment (Okuro *et al.* 2006) and by administration of vitamin C (Silvestro *et al.* 2002) could improve also the clinical symptoms in patients with lower extremity PAD.

Recently it has been pointed out that traditional risk factors explain less than 20 % of variance in vascular dysfunction (Chan *et al.* 2001), intimating the emergence of novel surrogate risk markers for evaluation of subclinical CV disease (Griedling and Fitzgerald 2003; Tardif *et al.* 2006). Therefore, measurement of the biomarkers related to OxS and inflammation, and non-invasive assessment of the subclinical changes of the arteries will facilitate identification of therapeutic targets for individuals at risk and will help monitor the success of treatment. In the current thesis we measured several functional and biochemical parameters of the arteries, attempting to provide a mechanistic insight into vascular dysfunction, inflammation and OxS in health and in disease.

3. AIMS OF THE STUDY

Overall aim

To assess endothelial function and arterial stiffness by pulse wave analysis in patients with lower extremity peripheral arterial disease and in healthy subjects; to determine associations between the functional and biochemical properties of the endothelium and arterial stiffness, and to provide mechanistic insights into the relative contribution of inflammation and oxidative stress to vascular alteration in patients with lower extremity peripheral arterial disease and in healthy subjects.

Specific aims

1. To measure aortic stiffness and wave reflections in patients with lower extremity peripheral arterial disease as well as in healthy subjects, and to evaluate changes in aortic stiffness and wave reflections following nitroglycerin and β_2-agonist salbutamol administration in these subjects.

2. To test if systemic stimulation of nitric oxide synthesis decreases aortic stiffness independently of changes in mean arterial pressure in whole study group consisting of patients with lower extremity peripheral arterial disease and healthy subjects.

3. To evaluate weather large and small artery elasticity indices are correlated with plasma level of asymmetric dimethylarginine and endothelial function in healthy subjects.

4. To measure large and small artery elasticity indices as well as oxidative stress markers, plasma level of baseline diene conjugates of low-density lipoprotein and urinary level of 8-iso-prostaglandin F_{2a} in patients with lower extremity peripheral arterial disease as well as in healthy subjects, and to determine associations between arterial stiffness and oxidative stress.

5. To assess endothelial function, aortic stiffness, wave reflections, and plasma levels of high sensitivity C-reactive protein, intercellular adhesion molecule-1, myeloperoxidase, oxidized low-density lipoprotein and urinary level of 8-iso-prostaglandin F_{2a} in patients with lower extremity peripheral arterial disease as well as in healthy subjects, and to test if endothelial function and arterial stiffness are determined by levels of inflammation and oxidative stress in a similar manner in atherosclerosis as well as in healthy condition.

4. SUBJECTS AND METHODS

4.1. Subjects

4.1.1. Patients with lower extremity peripheral arterial disease

The study population consisted of 39 patients with lower extremity PAD. In Paper I were included 24, in Paper III 38 and in Paper IV 39 patients. All patients who responded to the advertisement and in whom the inclusion criteria were met were recruited from the Clinic of Cardiovascular and Thoracic Surgery, University Hospital of Tartu, Estonia. All patients had stages II–IV as defined by Fontaine. The patients were all male with angiographically proven lower extremity PAD, i.e. with stenosis or occlusion of the arteries of the lower extremities. Ankle-brachial pressure index was less than 0.90 in the patients with lower extremity PAD. The patients' exclusion criteria were the following (based on clinical examination, electrocardiogram and blood tests): any concomitant acute or chronic inflammatory disease, MI, coronary revascularization or cerebrovascular events during the last 6 months, earlier revascularization procedures at the lower limb, upper limb occlusive arterial disease, hypertension (BP $\geq$ 140/90 mmHg), cardiac arrhythmias, or valve pathologies, diabetes mellitus (fasting serum glucose level > 6 mmol/L), malignancies and renal failure. No dietary restrictions were imposed.

4.1.2. Apparently healthy subjects

The total number of the control subjects was 63. The control group for comparison with patients with PAD in Paper I comprised 24, in Paper III 28 and in Paper IV 34 age-matched males. The study group in Paper II included 63 apparently healthy persons (17 women and 46 men). All controls who responded to the advertisement and in whom the inclusion criteria were met were recruited from general population. The exclusion criteria for the control group were the following (based on clinical examination, electrocardiogram and blood tests): any acute or chronic inflammatory disease, CAD, cardiac arrhythmias, or valve pathologies, hypertension (BP $\geq$ 140/90 mmHg), cerebral or peripheral atherosclerotic disease, diabetes mellitus (fasting serum glucose level > 6 mmol/L), malignancies, renal failure and regular use of any medications. No dietary restrictions were imposed.

4.2. Study design and protocol

All substudies (Papers I–IV) were designed as cross-sectional studies. The subjects were assessed and blood/urine samples were collected in all studies between 8:00 and 10:00 am, after an overnight fast and abstinence from any medications, tobacco, alcohol and tea or coffee. After 15 minutes of rest in a quiet, temperature-controlled room, ABPI (Papers I, III and IV) and BP were measured and PWA (systolic and/or diastolic) was performed.

In Papers I, II and IV systolic PWA was performed to measure aortic stiffness (Tr), wave reflections (AIx@75, AIx) and endothelial function (EDV, EIDV, EFI). In Papers II and III diastolic PWA was used for assessing small and large artery elasticity indices (C1 and C2) (Table 1). All haemodynamic and PWA recordings were made in duplicate for each time point. Thereafter, venous blood samples were drawn from the antecubital fossa, and urine samples were collected. Finally, height and weight were recorded, and body mass index (BMI) was calculated.

This study protocol was approved by the Ethics Committee, University of Tartu. Informed written consent was obtained from each participant.

Table 1. Subjects and methods.

Paper	Subjects				Vascular parameters							
	Patients		Healthy subjects		AIx@75	AIx	Tr	EDV	EIDV	EFI	C1	C2
	Male	Age	Male/Female	Age								
I	24	57.5±8.7	24	57.2±6.4	+	+	+	+	+			
II			46 17	46.9±14.2				+	+	+	+	+
III	38	58.6±7.8	28	55.6±6.7							+	+
IV	39	57.6±7.4	34	54.9±7.0	+	+	+	+	+	+		

4.3. Biochemical analyses

All regular biochemical parameters (plasma glucose, total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride and creatinine levels as well as urinary creatinine concentration) were determined by standard laboratory methods using certified assays in a local clinical laboratory. Lipid levels were measured by the Hitachi 912 analyser (Roche Diagnostics®, Basel, Switzerland). Specific parameters of OxS (F_2-IsoPs, LDL-BDC, oxLDL, MPO) and inflammation (ICAM-1,CRP), as well as ADMA were measured from plasma or urine, which was collected and stored at –70ºC until analysed. All determination procedures were performed in accordance with the manufacturer's recommendation. Glomerular filtration rate was estimated using the Modification of Diet in Renal Disease formula 1 (Brosius *et al.* 2006).

4.3.1. Measurement of plasma level of asymmetric dimethylarginine

Plasma level of ADMA (Paper II) was determined from plasma by a competitive enzyme-linked immunosorbentassay (ELISA) using a commercially available kit (Catalogue No EA201/96 (DLD Gesellschaft für Diagnostika und Medizinische GmbH®; Adlerhorst 15 D-22459 Hamburg, Germany) in 49 individuals. The competitive ADMA-ELISA uses the microtitre plate format. The ADMA is bound to the solid phase of the microtitre plate. In samples ADMA is acylated and competes with solid-phase-bound ADMA for a fixed number of rabbit anti-ADMA anti-serum binding sites. When the system is at equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase ADMA is detected by anti-rabbit/peroxidase. The substrate TMB/peroxidase reaction is monitored at 450 nm by a photometer Sunrise (Tecan Austria GmbH®, Salzburg, Austria).

4.3.2. Measurement of oxidative stress-related biomarkers

4.3.2.1. Urinary content of 8-iso-prostaglandin F_{2a}

The urinary content of F_2-IsoPs (Papers III and IV) was analysed by competitive ELISA (BIOXYTECH® 8-Isoprostane Assay, catalogue number 21019, Oxis-Research®, Portland, USA). Briefly, F_2-IsoPs in the samples competed for binding (to the antibody coated on the plate) with F_2-IsoPs conjugated to horse-radish (*Amoracia rusticana*) peroxidase. Peroxidase activity resulted in colour development when the substrate was added, and was measured by the photometer Multiscan MCC/340 (LabSystems®, Helsinki, Finland) at 450 nm. The intensity of the colour was proportional to the amount of bound F_2-IsoPs-horseradish peroxidase and inversely proportional to the amount of F_2-IsoPs-horseradish peroxidase in the samples or standards. The urinary concentrations of F_2-IsoPs were corrected by urinary creatinine concentrations to account for the differences in renal function.

4.3.2.2. Plasma level of baseline diene conjugates of low-density lipoprotein

Plasma level of LDL-BDC (Paper III) was measured by determining the level of LDL diene conjugation using a method that has been recently validated and reported in detail (Ahutopa *et al.* 1998). In brief, serum LDL was isolated by precipitation with buffered heparin-citrate. The amount of peroxidized lipids in the samples was determined by the degree of conjugated diene double bonds. Lipids were extracted from the samples by a mixture of chloroform and methanol (2:1), dried under nitrogen, redissolved in cyclohexane, and analysed spectro-photometrically at 234 nm by a photometer Spectronic Genesys

(Rochester®, New York, USA). For LDL-BDC, the coefficient of variance for within-assay and between-assay precision was 4.4% and 4.5%, respectively.

4.3.2.3. Plasma level of oxidized low-density lipoprotein

Plasma level of oxLDL was measured using an ELISA kit (Mercodia AB®, Uppsala, Sweden; Cat No 10-1143-01) (Paper IV). Mercodia oxLDL-ELISA is a solid phase two-site enzyme immunoassay, based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. During incubation and a simple washing step that removes non-reactive plasma components, a peroxidase conjugated anti-apolipoprotein B antibody recognizes oxLDL bound to the solid phase. After a second incubation and a simple washing step that removes unbound enzyme-labelled antibody, the bound conjugate is detected by reaction with 3,3´,5,5´-tetramethylbenzidine. The reaction is stopped by adding acid to obtain a colorimetric endpoint that is read spectrophotometrically at 450 nm by a photometer Sunrise (Tecan Austria GmbH®, Salzburg, Austria).

4.3.2.4. Plasma level of myeloperoxidase

Plasma level of MPO was determined by ELISA kit (BIOXYTECH® MPO-EIA, Cat No 21013, OxisResearch®, Portland, USA) (Paper IV). The MPO-EIA assay system is a "sandwich" ELISA. The antigen captured by a solid phase monoclonal antibody is detected with a biotin-labelled goat polyclonal anti-MPO. An avidin alkaline phosphatase conjugate then binds to the biotinylated antibody. The alkaline phosphatase substrate p-nitrophenyl phosphate is added and the yellow product (p-nitrophenol) is monitored at 405 nm by a photometer Sunrise (Tecan Austria GmbH®, Salzburg, Austria).

4.3.3. Measurement of inflammatory biomarkers

4.3.3.1. Plasma level of intercellular adhesion molecule-1

Plasma level of soluble ICAM-1 (Paper IV) was measured by an ELISA using a commercially available kit (Human soluble ICAM-1 Immunoassay, catalogue number BBE 1B, R&D Systems Inc.®, Minneapolis, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody, specific for ICAM-1, is pre-coated onto a microplate. Standards, samples, controls and the conjugate are pipetted into wells and any ICAM-1 present is sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for ICAM-1. Following a wash to remove unbound substances and/or the antibody-enzyme reagent, a substrate solution is added and colour

develops in proprtion to the amount of ICAM-1 bound. The colour development is stopped and the intensity of the colour is measured at 450 nm, with the correction wavelength set at 620 nm, by a photometer Sunrise (Tecan Austria GmbH®, Salzburg, Austria). The intra- and inter-assay precision coefficients of variation for ICAM-1 were 4.8% and 7.4%, respectively.

4.3.3.2. Plasma level of C-reactive protein

Plasma concentration of CRP was determined by using a validated latex particle-enhanced high-sensitivity immunoturbidimetric assay (CRP (Latex) HS, Roche Diagnostics Gmbh®, Mannheim, Germany), and analysed by the Hitachi 912 analyser (Roche Diagnostics®, Basel, Switzerland) (Paper IV).

4.4. Measurement of vascular function

4.4.1. Blood pressure measurement

Peripheral BP was measured in the dominant arm using a validated oscillometric technique (OMRON M4-I; Omron Healthcare Europe BV®, Hoofddorp, The Netherlands), with the subject seated for 10–15 minutes (Papers I–IV). Mean arterial pressure was calculated from the integration of the radial pressure waveform using the Sphygmocor (SCOR Px, 7.0; AtCor Medical®, Sydney, Australia) (Papers I and IV) or Hypertension Diagnostics (HDI/Pulse Wave CR-2000, Hypertension Diagnostics Inc®, Eagan, USA) software (Papers II and III). Peripheral PP was calculated as the difference between peripheral systolic BP and peripheral diastolic BP. Pulse pressure amplification was defined as the peripheral PP/central PP ratio (Paper IV). The mean of the two readings was used in all analyses.

4.4.2. Assessment of arterial stiffness and endothelial function using systolic pulse wave analysis

Arterial stiffness and EF were assessed by PWA using an Sphygmocor apparatus (SCOR Px, 7.0; AtCor Medical®, Sydney, Australia) as described previously (Wilkinson *et al.* 2002a). The peripheral pressure waveforms were recorded from the radial artery of the dominant arm at the wrist employing a high fidelity micromanometer (SPT-301B; Millar Instruments®, Texas, USA). Using a transfer function, the corresponding ascending aortic waveforms were then generated, from which central haemodynamics, AIx and Tr were calculated. The AIx was corrected for a HR of 75 beats *per* minute (AIx@75).

After the baseline measurements of haemodynamics, a 500-µg tablet of NTG (Nycomed®, Roskilde, Denmark) was placed under the tongue for 3 minutes and pulse waves were recorded 3, 5, 10, 15 and 20 minutes after NTG administration. Next, after restoration of haemodynamics, 400µg of the β_2-agonist Salb (GlaxoWellcome Production®, Evreux, France) was given by inhalation and recordings were made 5, 10, 15 and 20 minutes after administration. A maximum improvement in AIx@75 following Salb administration (due to stimulation of NO synthesis) was defined as EDV, while an improvement in AIx@75 after NTG was interpreted as a marker of EIDV. We used EFI, defined as the EDV/EIDV ratio, to represent endothelial function. All PWA recordings were performed consecutively by a single operator.

4.4.3. Assessment of arterial stiffness by diastolic pulse wave analysis

The arterial waveform was measured in the dominant arm by a Cardiovascular Profiling Instrument (HDI/Pulse Wave CR-2000, Hypertension Diagnostics Inc®, Eagan, USA). Briefly, the tonometer was applied to the patient's radial artery at the wrist overlying the radial bony prominence. The subject's arm was supported by a wrist stabilizer for optimal positioning and minimal movement during the measurements. The elasticity indices of the arteries (C1 and C2) were quantified during the diastolic portion of the cardiac cycle (mean of 30-sec recording). Heart rate and stroke volume were also calculated from the radial pressure waveform using the HDI/Pulse Wave CR-2000 software.

4.4.4. Measurement of ankle brachial pressure index

The ABPI was measured using Mini Dopplex D900 (Huntleigh Healtcare Ltd.®, Cardiff, UK). A 10–12 cm sphygmomanometer cuff was placed just above the ankle and a Doppler instrument used to measure the systolic BP of the posterior tibial and dorsalis pedis arteries of each leg. These pressures were then normalized to the higher brachial pressure of either arm to form ABPI. The value of ABPI was calculated as an average of two resting measurements.

4.5. Statistical analysis

All data were tested for normality (Papers I–IV). The response to NTG and Salb was defined as a maximum change in each parameter after drug administration (Papers I, II and IV). In all analyses the values of AIx@75 were used. Raw AIx was inserted in the multiple linear regression models (Paper I and IV). An aver-

age of two closest measurements of all vascular parameters was included in all statistical analyses (Papers I–IV).

The continuous data are expressed as the means ± SD if distributed normally, or otherwise by medians with 25% and 75% percentiles. The dichotomous variables are given in count and percentage. The skewed data were log-transformed to obtain a normal distribution and then analysed. Comparisons between the patients and the controls were assessed using unpaired Student's *t*-test (for the means) and Mann-Whitney *U* test. The effects of NTG and Salb on haemodynamics were investigated by paired *t*-test.

The correlations between the variables were examined using multiple linear regression analysis (free software R, version 2.2.1 for Windows) (Papers I–IV). For multiple regression model building, forward and backward variable selection procedures were applied. The variables entered into the model were chosen from simple correlation analyses, and the variables known or likely to be associated with corresponding parameters were taken from published observations. Significance was defined as $P < 0.05$.

5. RESULTS

5.1. Nitric oxide-mediated changes in arterial stiffness in patients with lower extremity atherosclerosis and in healthy subjects (Paper I)

Characteristics of the patients and the controls

The clinical characteristics of the 24 patients and 24 matched controls are summarized in Table 2. There was no significant difference between the groups in age, height, BMI, peripheral and central BP, MAP, HR, glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride and creatinine level. However, there occurred a difference in AIx, AIx@75 and Tr (also in Tr corrected for body height) between the groups ($P = 0.001$, $P < 0.001$, $P < 0.001$, respectively).

Haemodynamic changes after administration of nitroglycerin and salbutamol

We did not find no significant difference between the groups in the response to Salb for any parameter except for AIx, AIx@75 and Tr (Table 3). The Salb-produced absolute changes in AIx, AIx@75 and Tr from baseline were significant only in healthy subjects ($P < 0.001$, $P = 0.006$, respectively), however, Salb did not affect significantly HR or MAP in either group. After the administration of NTG, MAP decreased both in the patient group and in the control group ($P = 0.004$, $P = 0.001$, respectively), while NTG caused an increase in HR only in the patient group ($P = 0.01$). Nevertheless, there was no significant difference in the changes in MAP and HR between the groups after the administration of either drug. The Salb-induced changes in Tr, AIx and AIx@75 were significantly reduced in the patients with lower extremity PAD compared with the control subjects ($P < 0.001$, $P < 0.001$, respectively), while the NTG-produced changes were not significantly different ($P = 0.25$, $P = 0.35$, respectively).

Relationship between arterial stiffness and mean arterial pressure

Linear regression analysis was performed to compare the effects of the drugs on AIx@75 and Tr with reference to MAP, a key determinant of large artery stiffness. The regression line for AIx@75 and MAP shows linear correlation between the changes in AIx@75 and MAP after the administration of NTG and Salb (Figure 2). In contrast, there was no correlation between the changes in Tr and MAP produced by either drug (Figure 3). There occurred a significant

linear correlation between the drug-produced changes in AIx@75 and Tr as shown in Figure 4.

Table 2. Baseline characteristics.

Variable	PAD patients (n = 24)	Controls (n = 24)	P value
Age (y)	57.5±8.7	57.2±6.4	0.88
Weight (kg)	72.6±13.0	77.2±8.7	0.16
Height (cm)	174.6±6.1	175.4±6.1	0.65
BMI (kg/m^2)	24.0±3.8	25.1±2.7	0.26
ABPI	0.5±0.2	1.2±0.2	< 0.001
Peripheral systolic BP (mmHg)	125.7±11.4	122.1±11.7	0.30
Peripheral diastolic BP (mmHg)	75.8±7.1	74.3±6.1	0.45
Peripheral PP (mmHg)	52.0±8.7	47.9±9.4	0.12
MAP (mmHg)	94.3±8.8	91.5±7.9	0.26
Central systolic BP (mmHg)	120.1±12.6	113.8±11.7	0.08
Central diastolic BP (mmHg)	76.7±7.3	75.0±6.3	0.39
Central PP (mmHg)	43.5±8.2	38.8±9.2	0.07
HR (beats/min)	64.5±7.8	60.0±7.8	0.051
AIx (%)	34.4±7.9	27.3±6.7	0.001
AIx@75 (%)	29.3±8.2	20.0±6.7	< 0.001
Tr (ms)	135.5±14.1	147.5±8.3	< 0.001
Height/Tr (cm/ms)	1.30±0.12	1.19±0.08	0.001
Total cholesterol (mmol/L)	5.6±1.0	5.3±1.3	0.43
HDL cholesterol (mmol/L)	1.3±0.3	1.4±0.4	0.13
LDL cholesterol (mmol/L)	3.9±1.0	3.7±0.7	0.52
Triglycerides (mmol/L)	1.6±0.6	1.3±0.5	0.07
Glucose (mmol/L)	5.1±0.5	5.3±0.4	0.16
Creatinine (mmol/L)	81.9±17.9	86.5±12.0	0.31
Medication, n (%)			
Pentoxyfylline	24 (100)	0 (0)	–
Aspirin	20 (83.3)	0 (0)	< 0.001
Calcium channel blockers	6 (25)	0 (0)	0.008
Current smoking, n (%)	23 (95.8)	3 (12.5)	< 0.001

All values are expressed as mean ± SD unless otherwise indicated.

Multivariate association of the changes in wave reflections and aortic stiffness following β_2-stimulation with the other variables

To investigate further the factors influencing the response of AIx to Salb, a multiple linear regression model was developed with the change in AIx after Salb as the dependent variable. Age, weight, smoking status (defined as 0 – non-smoker, 1 – smoker), baseline MAP and AIx, LDL and HDL cholesterol,

triglycerides, glucose, and change in HR and MAP were entered in the model. The final model (Table 4) accounted for about 50% of the variability in the response of AIx to Salb. The change in AIx was negatively correlated with smoking, age and change in MAP.

Table 3. The maximum changes from baseline in each parameter after NTG and Salb administration.

Variable	PAD patients (n = 24)		Controls (n = 24)	
	Salb	NTG	Salb	NTG
AIx (%)	–3.5±4.6	–22.5±6.6[c]	–9.3±4.2[c,d]	–22.2±6.1[c]
AIx@75 (%)	–3.4±3.8	–19.0±5.6[c]	–9.3±3.5[c,d]	–20.5±5.1[c]
Tr (ms)	1.1±4.5	18.6±9.7[c]	10.6±11.7[b,d]	15.6±8.2[c]
MAP (mmHg)	–1.7±8.1	–7.5±7.1[b]	–2.9±7.7	–8.2±6.4[b]
HR (beats/min)	–0.3±5.8	5.8±5.1[a]	0.9±5.9	3.1±5.9

All data are presented as means ± SD, and significance from baseline are indicated by [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$. Significant differences in the response after administration of both drugs between the groups are indicated in column by [d] $P < 0.001$.

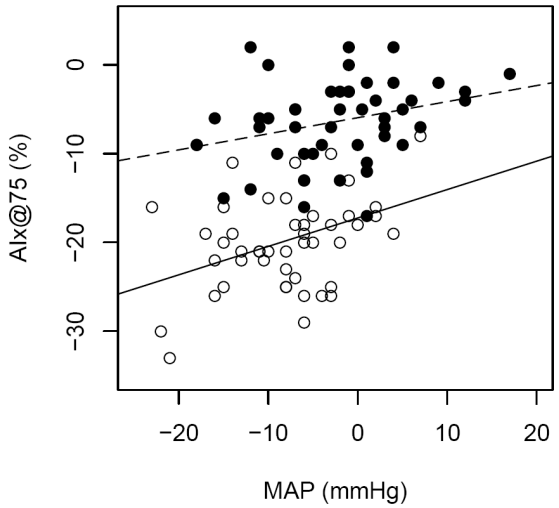

Figure 2. The relationship between maximum change in AIx@75 and in MAP after NTG (○) and Salb (●) administration in all study subjects (n = 48). Linear regression lines are shown for NTG (continuous line; R = 0.40, P = 0.005) and Salb (dashed line; R = 0.30, P = 0.04).

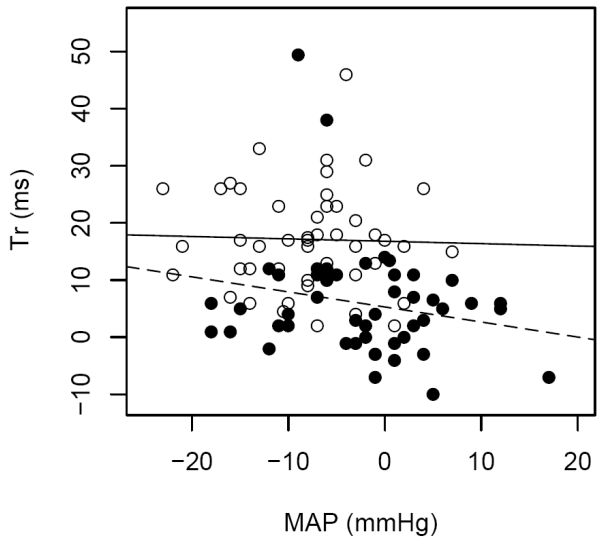

Figure 3. The relationship between maximum change in Tr and in MAP after NTG (○) and Salb (●) administration in all study subjects (n = 48). Linear regression lines are shown for NTG (continuous line; R = –0.03, P = 0.84) and Salb (dashed line; R = –0.21, P = 0.16)

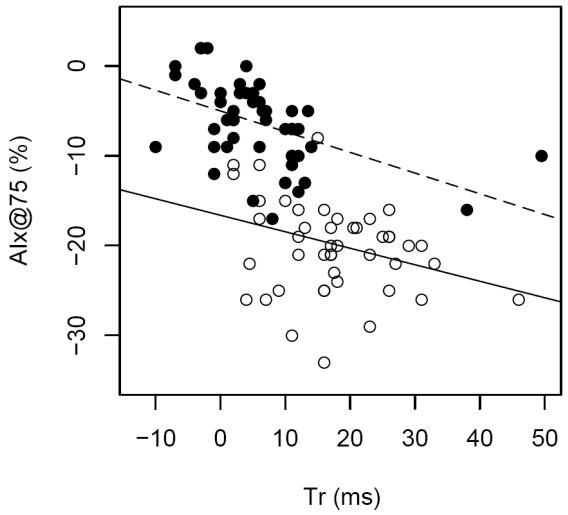

Figure 4. The relationship between maximum change in AIx@75 and in Tr after NTG (○) and Salb (●) administration in all study subjects (n = 48). Linear regression lines are shown for NTG (continuous line; R = –0.31, P = 0.03) and Salb (dashed line; R = –0.50, P < 0.001).

The data for either group were used to develop another multiple linear regression model with the changes in Tr after Salb as the dependent variable. Age, weight, smoking status, baseline MAP and Tr, LDL and HDL cholesterol, triglycerides, glucose, and change in HR and MAP were entered into the model. The final model only accounted for about 25% ($R^2 = 0.27$, $P < 0.02$) of the variability in Tr, indicating that the change in Tr after Salb administration was significantly inversely correlated with plasma triglyceride only ($P = 0.03$).

Table 4. Results of the multiple regression analysis with change in AIx after Salb as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Smoking	–5.28	1.12	< 0.001
Change in MAP (mmHg)	–0.18	0.07	0.02
Age (y)	–0.17	0.08	0.03
Change in HR (beats/min)	0.19	0.10	0.08

$R^2 = 0.51$, $P < 0.00001$ for all study subjects (n = 48).

5.2. Relationship of arterial stiffness with endothelial function and asymmetric dimethylarginine level in healthy subjects (Paper II)

The main characteristics of the study population are listed in Table 5. The subjects' age was in the range 19 to 70 years. Linear regression analysis was used to establish whether EF correlated with C1 and C2. There was a significant positive association of EFI with C1 and C2 (Figure 5). Significant inverse correlations were observed also between ADMA and C1 as well as C2 (Figure 6). The results of univariate correlation analysis encouraged elucidation of relationships between arterial elasticity and EFI or ADMA also in multivariate models, adjusted for age and BMI. After adjustment for potential confounders in multivariate models, the final model demonstrated that C1 was significantly inversely related to age and positively to BMI and EFI (Table 6a) but not to ADMA level (Table 6b). The data were also used in multivariate models to find out independent determinants of C2. The final model (Table 7) showed that C2 was correlated inversely to age and ADMA and positively to EFI and BMI.

Table 5. Main characteristics of the study population.

Variable	
Female/male, n	17/46
Age (y)	46.9±14.2
BMI (kg/m^2)	24.1±2.8
Total cholesterol (mmol/L)	5.4±1.2
HDL cholesterol (mmol/L)	1.4±0.4
LDL cholesterol (mmol/L)	3.6±0.8
Triglycerides (mmol/L)	1.2±0.5
Glucose (mmol/L)	5.2±0.5
Glomerular filtration rate (mL/min *per* 1.73 m^2)	90.3±12.4
ADMA (µmol/L)	0.55±0.1
Systolic BP (mmHg)	121.1±10.6
Diastolic BP (mmHg)	71.4±7.8
MAP (mmHg)	88.9±9.2
C1(mL/mmHg×10)	17.9±5.5
C2 (mL/mmHg×100)	7.9±3.3
EDV (%)	9.6±4.6
EIDV (%)	18.0±6.9
EFI	0.6±0.3
Current smokers, n (%)	13 (20.6)

Values are means ± standard deviation, unless otherwise indicated.

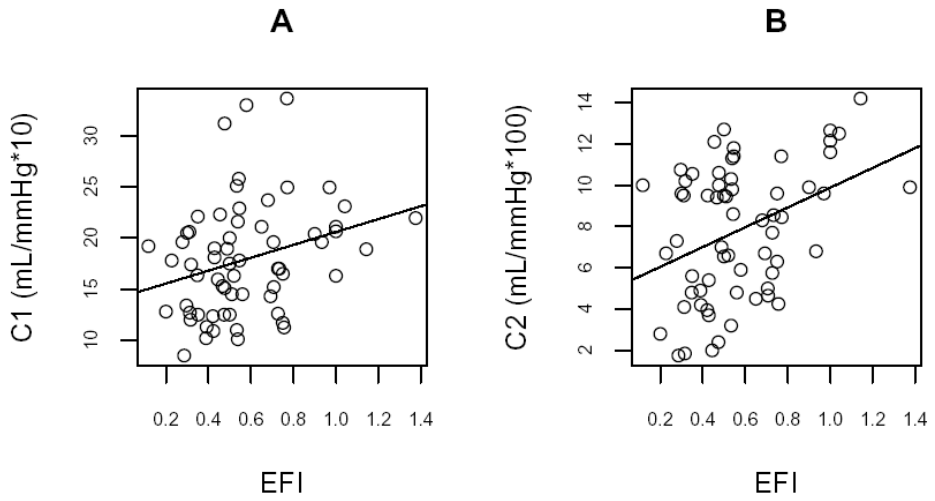

Figure 5. Scatterplot of C1 and C2 and EFI in 63 healthy subjects. C1 is positively correlated to EFI (R = 0.29, P = 0.02) (A), and C2 is positively associated with EFI (R = 0.38, P = 0.002) (B).

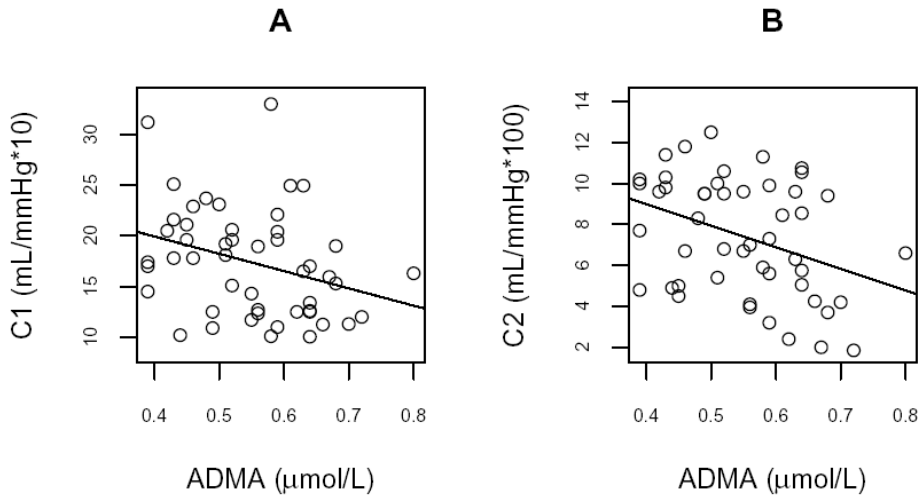

Figure 6. Scatterplot of C1 and C2 and ADMA level in 49 healthy subjects. C1 is inversely correlated to ADMA (R = –0.32, P = 0.03) (A), and C2 is inversely associated with ADMA (R = –0.37, P = 0.009) (B).

Table 6a. Multiple regression model with C1 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.009	0.002	< 0.001
BMI (kg/m^2)	0.05	0.01	< 0.001
EFI	0.37	0.14	0.01

$R^2 = 0.37$, $P < 0.001$.

Table 6b. Multiple regression model with C1 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.007	0.003	0.03
BMI (kg/m^2)	0.04	0.01	0.004
ADMA (μmol/L)	–0.44	0.42	0.3

$R^2 = 0.27$, $P < 0.003$.

Table 7a. Multiple regression model with C2 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.11	0.03	< 0.001
BMI (kg/m^2)	0.44	0.12	< 0.001
EFI	3.85	1.46	0.01

$R^2 = 0.41$, $P < 0.001$.

Table 7b. Multiple regression model with C2 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.08	0.03	0.02
BMI (kg/m^2)	0.28	0.14	0.05
ADMA (μmol/L)	–6.9	4.08	0.04

$R^2 = 0.26$, $P < 0.004$.

5.3. Association between arterial stiffness and oxidative stress in patients with atherosclerosis and in healthy subjects (Paper III)

Subject characteristics

The clinical characteristics of the 38 patients and the 28 controls are summarized in Table 8. There was no significant difference between the groups in age, height, systolic and diastolic BP, MAP, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, glucose and urinary creatinine level. However, there occurred a significant difference in ABPI, C1, C2, LDL-BDC, F_2-IsoPs (also in creatinine indexed F_2-IsoPs) between the groups. After adjustment for LDL-BDC and F_2-IsoPs, the differences between the groups in small artery elasticity remained significant ($P < 0.001$), but the differences in large artery elasticity did not achieve significance ($P = 0.16$).

Relationship between arterial stiffness and oxidative stress

Linear regression analysis was used to establish whether arterial stiffness correlated with the OxS-related biomarkers. There was a significant association between C1 and F_2-IsoPs, as well as between C2 and F_2-IsoPs only in the patient group, but not in the controls (Figure 7 and Figure 8).

Table 8. Baseline characteristics.

Variable	PAD patients (n = 38)	Controls (n = 28)	P value
Age (y)	58.6±7.8	55.6±6.7	0.11
Height (m)	1.73±0.06	1.75±0.04	0.15
BMI (kg/m^2)	23.5±3.5	25.3±2.8	0.02
Systolic BP (mmHg)	125.3±8.7	121.0±11.6	0.09
Diastolic BP (mmHg)	76.9±7.6	74.0±7.4	0.14
MAP (mmHg)	92.9±6.4	90.7±9.3	0.27
HR (beats/min)	64.9±8.6	58.5±7.1	0.002
ABPI	0.41±0.12	1.34±0.29	< 0.001
Total cholesterol (mmol/L)	5.59±0.87	5.21±0.82	0.07
HDL cholesterol (mmol/L)	1.28±0.33	1.36±0.33	0.34
LDL cholesterol (mmol/L)	3.93±1.13	3.59±0.76	0.17
Triglycerides (mmol/L)	1.33±0.31	1.25±0.50	0.46
Glucose (mmol/L)	5.13±0.59	5.25±0.48	0.36
LDL-BDC (μmol/L)	23.70±8.13	18.34±5.74	0.004
Urinary creatinine (mmol/L)	12.72±4.43	14.59±5.83	0.20
F_2-IsoPs (ng/mL)	5.34±1.95	2.34±0.79	< 0.001
F_2-IsoPs (ng/mg creatinine)	9.47±5.71	4.29±2.58	< 0.001
Stroke volume (mL/beat)	72.5±12.7	89.6±12.6	< 0.001
C1 (mL/mmHg×10)	14.5±4.3	18.6±4.9	< 0.001
C2 (mL/mmHg×100)	2.7(1.9–3.4)	7.5(5–9.7)	< 0.001
Medication, n (%)			
Pentoxyfylline	38 (100)	0 (0)	–
Aspirin	30 (78.9)	0 (0)	< 0.001
Calcium channel blockers	10 (26.3)	0 (0)	< 0.001
Current smoking, n (%)	31 (81.6)	6 (21.4)	< 0.001

All values are expressed as means (±SD), medians (with 25% and 75% percentiles).

The results of correlation analysis and clinical data encouraged elucidation of the relationships between arterial stiffness and F_2-IsoPs also in multivariate models, adjusted for age and BMI, separately for both groups. The final model with these covariates (Table 9a) revealed that C1 was not significantly related to F_2-IsoPs level in the controls but was inversely associated with age and positively with BMI in the healthy subjects. In the patient group C1 clearly tended to inversely correlate with F_2-IsoPs after such adjustment (Table 9b).

The data for either group were used separately in multivariate models to determine also correlations between C2 and F_2-IsoPs. Table 10 shows that C2 is determined inversely by age and positively by BMI in the healthy controls. There was no relationship between C2 and isoprostanes in the controls. However, C2 was significantly inversely associated with F_2-IsoPs and age in the patient group.

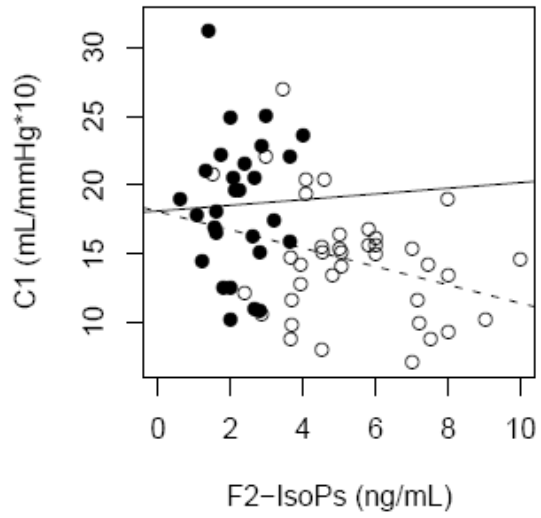

Figure 7. Scatterplot of C1 and F_2-IsoPs in 38 patients with PAD (○) and in 28 controls (●). The parameters were significantly correlated in the whole group (R = –0.41, P < 0.001). In separate analyses correlation was found only in the patients (dashed line; R = –0.30, P = 0.04), but not in the controls (continuous line; R = 0.04, P = 0.86).

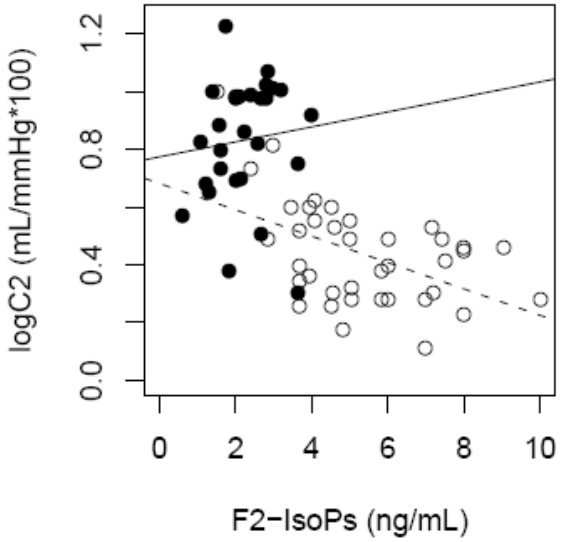

Figure 8. Scatterplot of logC2 and F_2-IsoPs. The parameters were significantly correlated in the whole group (n = 66) (R = –0.65, P < 0.001). In separate analyses correlation was found only in the patients (○) (dashed line; R = –0.49, P = 0.002), but not in the controls (●) (continuous line; R = 0.10, P = 0.61).

Table 9a. Multiple regression model for the controls with C1 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.361	0.118	0.005
BMI (kg/m^2)	0.682	0.269	0.018
F_2-IsoPs (ng/mL)	–0.424	0.956	0.661

$R^2 = 0.43$, $P < 0.004$.

Table 9b. Multiple regression model for the patients with C1 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.137	0.088	0.127
BMI (kg/m^2)	0.186	0.197	0.351
F_2-IsoPs (ng/mL)	–0.662	0.341	0.06

$R^2 = 0.20$, $P < 0.057$.

Table 10a. Multiple regression model for the controls with logC2 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.014	0.005	0.01
BMI (kg/m^2)	0.038	0.011	0.003
F_2-IsoPs (ng/mL)	0.004	0.04	0.908

$R^2 = 0.47$, $P < 0.001$.

Table 10b. Multiple regression model for the patients with logC2 as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Age (y)	–0.009	0.003	0.007
BMI (kg/m^2)	0.009	0.007	0.18
F_2-IsoPs (ng/mL)	–0.046	0.012	< 0.001

$R^2 = 0.45$, $P < 0.0001$.

5.4. Relationships between oxidative stress, inflammation, endothelial function and arterial stiffness in patients with atherosclerosis and in healthy subjects (Paper IV)

Subject characteristics

The clinical characteristics of study groups are summarized in Table 11. There was no significant difference between the patients and the controls in age, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, glucose or EIDV. Nor occurred there differences in height (1.73±0.06 m *vs* 1.75±0.06 m, P = 0.07), peripheral systolic and diastolic BP (128.3±11.6 mmHg *vs* 122.4±11.4 mmHg, P = 0.08; 76.9±7.5 mmHg *vs* 73.9±7.0 mmHg, P = 0.31, respectively), central diastolic BP (77.8±7.7 mmHg *vs* 74.6±7.1 mmHg, P = 0.26) or MAP (95.8±10.2 mmHg *vs* 91.2±8.6 mmHg, P = 0.21) between the patients and the controls.

Table 11. Baseline characteristics of the study population.

Characteristic	PAD patients (n = 39)	Controls (n = 34)	P value
Age (y)	57.6±7.4	54.9±7.0	0.13
BMI (kg/m^2)	23.3±3.4	25.4±2.9	0.006
ABPI	0.49±0.20	1.18±0.15	< 0.001
Total cholesterol (mmol/L)	5.37±0.81	5.16±1.06	0.37
HDL cholesterol (mmol/L)	1.29±0.32	1.37±0.33	0.28
LDL cholesterol (mmol/L)	3.88±1.06	3.60±0.71	0.20
Triglycerides (mmol/L)	1.37±0.27	1.22±0.53	0.15
Glucose (mmol/L)	5.13±0.62	5.23±0.48	0.48
CRP (mg/L)	3.07 (1.3–6.98)	1.07 (0.68–1.64)	< 0.001
ICAM-1 (ng/mL)	252 (209.5–314)	199 (185.8–224)	< 0.001
MPO (ng/mL)	15.5 (11.9–21.1)	10 (8.9–14.3)	< 0.001
F_2-IsoPs (ng/mL)	5.35±1.94	2.34±0.81	< 0.001
F_2-IsoPs (ng/mg creatinine)	9.52±5.60	4.28±2.51	< 0.001
OxLDL (U/L)	143.6±49.1	124.2±40.0	0.09
AIx (%)	36.4±8.0	26.8±7.8	< 0.001
AIx@75 (%)	31.5±8.0	18.9±7.9	< 0.001
Tr (ms)	132.9±13.3	149.2±10.1	< 0.001
EDV (%)	4.6±4.2	9.4±3.5	< 0.001
EIDV (%)	20.7±6.4	20.2±4.9	0.75
EFI	0.22±0.19	0.48±0.17	< 0.001
Current smoking, n (%)	36 (92.3)	5 (14.7)	< 0.001

All values are expressed as means (±SD), medians (with 25% and 75% percentiles) or prevalence (%).

However, there was a significant difference in BMI, CRP, ICAM-1, MPO, F_2-IsoPs (also in creatinine indexed F_2-IsoPs), ABPI, AIx, AIx@75, Tr, EDV, EFI and smoking status between the groups. Heart rate (65.0±8.7 beats/min *vs* 58.5±7.1 beats/min, P = 0.001), central systolic BP (123.0±14.6 mmHg *vs* 113.7±11.8 mmHg, P = 0.03) and PP amplification (1.20±0.09 *vs* 1.25 ± 0.11, P = 0.048) were also different for the patients and for the controls. In addition, plasma oxLDL level tended to be higher in the patients, but the difference was statistically not significant (P = 0.09). Differences occurred also in medication between the subgroups: all patients were on pentoxyfylline treatment, 30 patients (76.9%) received aspirin and 10 patients (25.6%) received calcium channel blockers, while the controls did not receive any medicaments.

Relationship between endothelial function and inflammation

Linear regression analysis was used to establish whether EF correlated with inflammation within each group separately. There was a significant inverse association between EFI and ICAM-1 only in the controls (Figure 9).

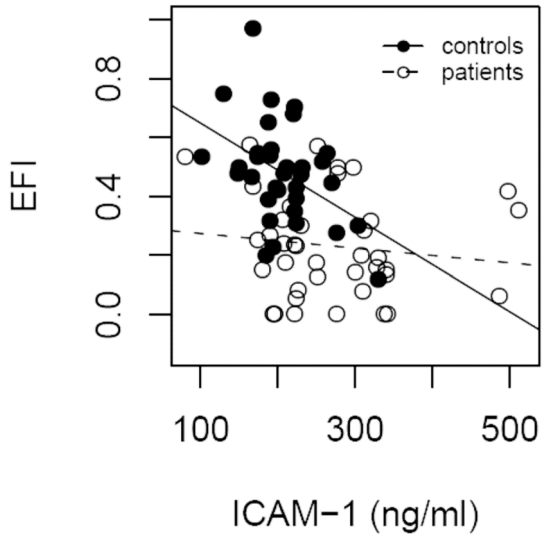

Figure 9. Scatterplot of EFI and ICAM-1 in 39 patients and 34 controls. The parameters were significantly correlated only in the controls (R = –0.44, P = 0.009), but not in the patients (R = –0.13, P = 0.43).

However, we found no relationship between EFI and ICAM-1 or the other inflammatory markers (CRP, MPO) in the patient group or between EFI and F_2-IsoPs or oxLDL in either group (data not shown). To investigate further the factors influencing independently EFI in the controls, a multiple linear regression model was developed with EFI as the dependent variable. In the multivariable regression model EFI was independently inversely correlated with ICAM-1, AIx@75 and BMI (Table 12).

Table 12. Multiple regression model for the controls with EFI as the dependent variable.

Variable	Regression coefficient	Standard error	P value
ICAM-1 (ng/mL)	–0.001	0.0005	0.01
AIx@75 (%)	–0.008	0.003	0.02
BMI (kg/m^2)	–0.02	0.009	0.03
LDL cholesterol (mmol/L)	–0.06	0.004	0.08

$R^2 = 0.41$, $P < 0.003$.

Association between arterial stiffness and oxidative stress

Significant associations were observed between AIx@75 and F_2-IsoPs (Figure 10), as well as between raw AIx and F_2-IsoPs ($R = 0.38$, $P = 0.02$) only in the patient group. There was also correlation between Tr and F_2-IsoPs in the patients but not in the controls (Figure 11). No significant relationship was found between arterial stiffness and the inflammatory markers (ICAM-1, CRP, MPO) in either group or between arterial stiffness and F_2-IsoPs or oxLDL in the controls (data not shown). To determine the factors influencing arterial stiffness, two multiple linear regression models were developed for the patient group with AIx and Tr as the dependent variables. The final model indicates that AIx was inversely associated with HR, positively correlated with F_2-IsoPs and MPO level, and clearly tended to associate with age (Table 13). The multiple regression model for aortic stiffness revealed that Tr was significantly inversely correlated with F_2-IsoPs, MPO and age (Table 14).

In addition, there occurred significant positive correlation between ICAM-1 and F_2-IsoPs only in the patient group but not in the healthy subjects (Figure 12).

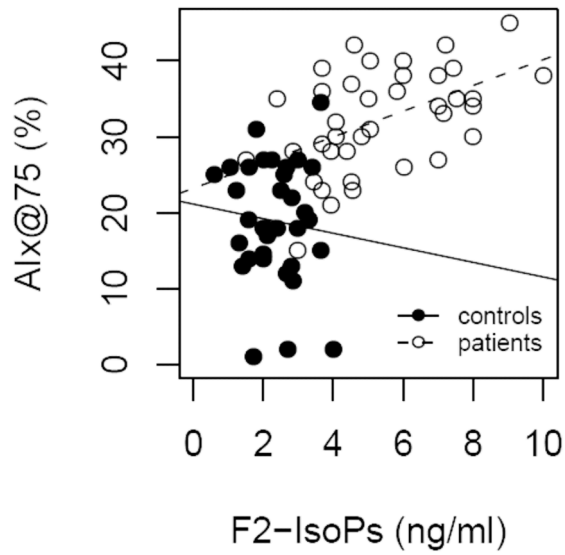

Figure 10. Scatterplot of AIx@75 and F_2-IsoPs in 39 patients and 34 controls. The parameters were significantly correlated only in the patients (R = 0.50, P = 0.001), but not in the controls (R = –0.10, P = 0.58).

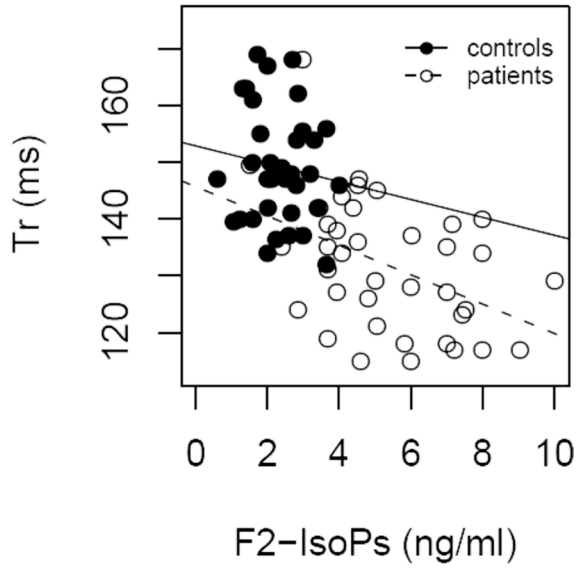

Figure 11. Scatterplot of Tr and F_2-IsoPs in 39 patients and 34 controls. The parameters were significantly correlated only in the patients (R = –0.43, P = 0.006), but not in the controls (R = –0.13, P = 0.47).

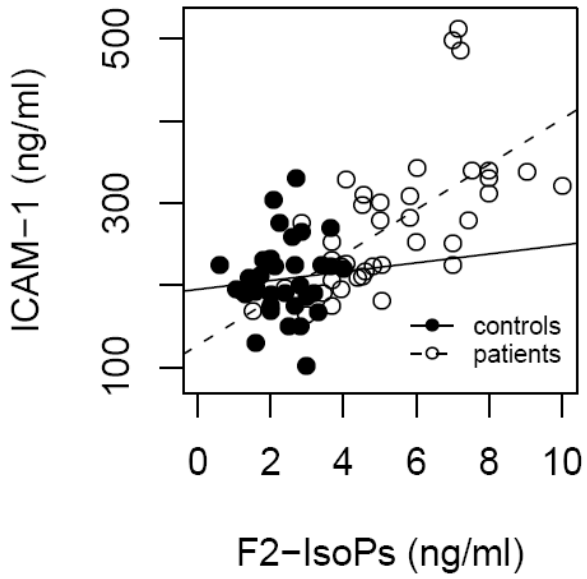

Figure 12. Scatterplot of ICAM-1 and F_2-IsoPs in 39 patients and 34 controls. The parameters were significantly correlated only in the patients (R = 0.63, P < 0.001), but not in the controls (R = 0.09, P = 0.60).

Table 13. Multiple regression model for the patients with AIx as the dependent variable.

Variable	Regression coefficient	Standard error	P value
F_2-IsoPs (ng/mL)	1.73	0.55	0.004
HR (beats/min)	–0.29	0.12	0.02
MPO (ng/mL)	0.31	0.15	0.04
Age (y)	0.26	0.13	0.05

$R^2 = 0.40$, $P < 0.001$.

Table 14. Multiple regression model for the patients with Tr as the dependent variable.

Variable	Regression coefficient	Standard error	P value
F_2-IsoPs (ng/mL)	–2.90	0.94	0.004
MPO (ng/mL)	–0.55	0.25	0.04
Age (y)	–0.48	0.22	0.04

$R^2 = 0.34$, $P < 0.002$.

6. DISCUSSION

6.1. Importance of testing vascular function in patients with peripheral arterial disease

Endothelial dysfunction and arterial stiffness play an important role in development and clinical course of atherosclerosis (Suwaidi *et al.* 2000; Gocke *et al.* 2003; Safar 2007a). Although severity of the symptoms of lower extremity PAD are determined in part by degree of conduit vessel obstruction, development of collateral vessels and derangement of skeletal muscle metabolism, other potential involved mechanisms might be endothelial dysfunction and arterial stiffness. It has been demonstrated that endothelial dysfunction is an important determinant of peripheral vascular resistance (Rizzoni *et al.* 2001), and increased arterial stiffness is associated with reduced arterial flow in the lower extremities (Tsuchiya *et al.* 2005). Degree of endothelial dysfunction (Sanada *et al.* 2005; Brevetti *et al.* 2003a; Silvestro *et al.* 2003), increased AIx (Khaleghi and Kullo in press) and reduced C2 (Duprez *et al.* 2001) are also related to severity of PAD. More importantly, low brachial artery EF adds to the prognostic value of ABPI (Brevetti *et al.* 2003b), the most powerful prognostic indicator of PAD (Newman *et al.* 1999). Additionally, brachial artery EF correlates with abnormal myocardial perfusion reserve (Perrone-Filardi *et al.* 2005) and AIx is associated with the reduced walking distance in patients with lower extremity PAD (Brewer *et al.* in press). These data point to the close association of endothelial dysfunction as well as arterial stiffness with lower extremity atherosclerosis, which could be an important explanation for linking PAD and increased CV risk.

Peripheral arterial disease is a manifestation of generalized atherosclerosis with an increased risk of MI and stroke (Ouriel *et al.* 2001). Therefore assessment of CV risk in these patients is a significant clinical challenge. We demonstrated, using the non-invasive PWA technique, that patients with lower extremity PAD had impaired bioavailability of NO to produce vasorelaxation (Papers I and IV), as well as increased arterial stiffness (Papers I, III and IV). In agreement with other authors (Wilkinson *et al.* 2002a; Hayward *et al.* 2002), our findings indicate that PWA is a useful method for detecting EF and arterial stiffness. However, current methods, especially those for endothelial measurement, are not suitable for inclusion in large-scale trials. The potential benefits of the PWA technique are that it is non-invasive, readily portable and is quickly learned. The use of Salb to induce endothelial NO release will allow global assessment of EF rather than assessment of a single vascular bed. In addition, the potential advantages of testing vascular function by PWA are the unique information gained about EF, central haemodynamics and arterial stiffness. In contrast, the other modalities largely provide information about the presence and

severity of fixed anatomic disease, which may be less relevant to the pathogenesis of CV events. Thus, we believe that a combination of non-invasive EF and arterial stiffness test with other clinical scoring systems could better determine individual CV risk in patients with PAD.

6.2. Endothelium-derived nitric oxide and regulation of arterial stiffness

Physiologically, stiffness of the large arteries depends on three main factors: structural elements within the arterial wall, such as elastin and collagen; distending pressure; and vascular smooth muscle tone. Changes in smooth muscle tone mediated by endothelium-derived NO provide functional regulation of arterial stiffness. Studies addressing the specific actions of NO have shown that endogenous NO regulates local arterial stiffness in the human brachial artery (Kinlay *et al.* 2001), in the sheep (Wilkinson *et al.* 2002b) and in the human iliac artery (Schmitt *et al.* 2005), as well as modulates both aortic stiffness and wave reflections in humans (Wilkinson *et al.* 2002c). It has been recently demonstrated that global (McEniery *et al.* 2006) as well as brachial artery EF (Nigam *et al.* 2003) correlate strongly with arterial stiffness. Conversely, other authors believe that MAP rather than any specific effect of NO is the most important determinant of short-term changes in aortic stiffness (Stewart *et al.* 2003).

In Paper I we evaluated changes in estimated aortic PWV following systemic stimulation of NO synthesis by Salb. We detected neither significant changes in MAP after Salb nor any correlation between estimated aortic PWV and distending pressure after Salb and NTG administration. These findings support the hypothesis that Salb modulates large artery stiffness, in part *via* a direct effect of NO on large-artery mechanics rather than changes in BP. In contrast, EIDV did not appear to play a role in explaining arterial stiffness.

We used an estimated measure of aortic stiffness (Tr) instead of carotid-femoral PWV, which is the "gold standard" for arterial stiffness (Laurent *et al.* 2006). Although there was significant association between Tr and carotid-femoral PWV (London *et al.* 1992; McEniery *et al.* 2005), these parameters are not absolutely identical: Tr is responsive, at least in part, to wave reflection, while carotid-femoral PWV characterizes more directly pulse wave transmission in the aorta. In agreement with a previous study (Wilkinson *et al.* 2001), we found linear relationship between the Salb- and NTG-produced changes in Tr and AIx. Both drugs resulted in a later return of the reflected wave to the aorta, indicating reduced aortic and systemic arterial stiffness. It seems likely that absence of correlation between Tr and MAP indicates that the decrease in Tr after Salb and NTG administration reflects slower wave

propagation in the aorta due to reduced aortic stiffness rather than changes in distending pressure.

In Paper II we demonstrated correlations of EF or endogenous NOS inhibitor ADMA with C1 and C2 in healthy subjects. The stronger association between both EF or ADMA and C2 compared with C1 can be explained by the fact that the smaller arteries, close to the arterial branching points, have a thinner media layer and NO therefore plays a more important role in producing dilation in them. Furthermore, it has been demonstrated that inhibition of NO synthesis with N^G-nitro-L-arginine methyl ester caused a significant reduction in C2, while subsequent L-arginine administration restored baseline (McVeigh *et al.* 2001). In addition, it has been shown previously in humans that infusion of ADMA decreases renal plasma flow (Kielstein *et al.* 2004) and increases pulmonary vascular resistance (Kielstein *et al.* 2005) as those vascular beds are characterized by a high density of thin-walled small arteries.

Our finding that C1 and C2 are associated with ADMA level suggests that ADMA (even within the normal range) plays a significant role in vascular stiffening. In agreement with our results, it has recently been shown that ADMA levels are associated independently with AIx (Weber *et al.* 2007). Furthermore, previously has been demonstrated significant correlation between AIx and C2, which suggests that C2 is, at least in part, measure of arterial wave reflectance (Rietzschel *et al.* 2001). All abovementioned results indicate that ADMA level is related to increased arterial wave reflections, most likely due to decreased endothelium-derived NO activity in the small arteries and arterioles.

Increased ADMA may affect vascular function and structure through various mechanisms. Evidence has accumulated to the effect that elevation in ADMA may at least partly cause endothelial NOS uncoupling, increase vascular superoxide level and contribute to OxS (Cooke *et al.* 2004; Toth *et al.* 2007). Increased levels of ADMA reduce bioavailability of NO *via* blocking all three isoforms of NOS, and enhance NO degradation due to endothelial NOS-mediated superoxide production. It has been demonstrated that ADMA causes vascular lesions also in an endothelial NOS-independent manner as well (Suda *et al.* 2004).

In conclusion, we found (1) stimulated NO release reduced aortic stiffness independently of changes in MAP, (2) significant association of arterial stiffness with EF and ADMA level. These findings suggest that arterial stiffness might be influenced specifically by endothelium-derived NO.

6.3. Association between arterial stiffness and atherosclerosis

The importance of assessment of arterial stiffness in patients with atherosclerosis is underlined by the strong positive correlation between aortic stiffness and degree of coronary atherosclerosis (Hirai *et al.* 1989; Haydar *et al.* 2004; Kullo *et al.* 2006; Venkitachalam *et al.* 2007). In addition, there has been found significant association of AIx (Fukui *et al.* 2003) as well as C2 (Duprez *et al.* 2000) with intima-media thickness. Moreover, aortic PWV is an independent predictor of CAD and stroke in healthy subjects (Mattace-Raso *et al.* 2006). The AIx is related to restenosis after coronary stenting (Ueda *et al.* 2004) as well as to presence and severity of CAD (Weber *et al.* 2004).

We demonstrated significantly increased AIx, AIx@75, aortic stiffness (Papers I and IV) and central systolic BP, and reduced and PP amplification (Paper IV), as well as C1 and C2 (Paper III) in the patients with lower extremity PAD compared with controls, despite that peripheral pressures and MAP did not differ significantly. Similarly, increased aortic PWV (van Popele *et al.* 2001), reduced C1 and C2 (Duprez *et al.* 2001) and impaired carotid and femoral visoelastic properties (Cheng *et al.* 2002) have been reported in patients with lower extremity PAD.

We believe that increased vascular stiffness in patients with lower extremity PAD characterizes a combination of atherosclerotic and arteriosclerotic alterations both in the aorta and in the peripheral arteries. Furthermore, wave reflections are an important mechanism of increased systolic BP in patients with PAD. In normal human subjects, the region of the terminal aorta acts as an important reflection site; whereas in subjects with lower extremity PAD, which involves gross arterial lesions of the lower part of the body, the terminal aorta may be a major site of reflections (Safar 2007a). Thus, pressure waves transverse the arterial system more quickly and lead to an increase in central systolic BP.

Several possibilities of the association between arterial stiffness and atherosclerosis can be hypothesized. One possibility is that the presence of atherosclerosis leads to stiffening of the arteries. In monkeys it has been shown that feeding with an atherogenic diet leads to an increase in PWV and feeding with an atherosclerosis regressing diet leads to a decrease in PWV (Farrar *et al.* 1991). An alternative explanation is that increased arterial stiffness leads to vessel wall damage and atherosclerosis. A raised PP due to increased vascular stiffness may induce arterial remodelling, increasing wall thickness, and promote development of plaques (Witteman *et al.* 1994). It has been shown that high PP is independently associated with arterial plaque ulceration, supporting the hypothesis that cyclical haemodynamic forces are important determinants of plaque rupture (Lovett *et al.* 2003; Demer 1991; Cheng *et al.* 1993). A third possibility is that

both mechanisms act and that atherosclerosis is not only a consequence of arterial stiffness but may by itself, in advanced stages, increase arterial stiffness as well. A fourth possibility is that arterial stiffness and atherosclerosis are independent processes that frequently occur at similar sites in the artery without existence of a causal relationship.

6.4. Oxidative stress, inflammation and vascular dysfunction

We demonstrated high-grade OxS (Papers III and IV) and augmented inflammation (Paper IV), increased arterial stiffness and impaired EF (Papers I, III and IV) in patients with lower extremity PAD in comparison with controls. The independent association of AIx@75, Tr and C2 with urinary F_2-IsoPs (Paper III and IV), but not with conventional metabolic parameters, indicates that novel integrated biomarkers may be more sensitive for predicting vascular abnormalities. There was no significant association between C1 and F_2-IsoPs in the patient or in the control group after adjustment for confounders (Paper III). However, recent studies have reported that the small arteries are particularly sensitive to CV disease-associated alterations (Cooke 1997) as well as to risk prediction (Grey *et al.* 2003).

The established significant correlations between urinary F_2-IsoPs and arterial stiffness indices (AIx@75, Tr, C1 and C2) in the patients with lower extremity PAD might emphasise the potential role of OxS in functional and structural stiffening of the arteries in atherosclerosis (Weber *et al.* 2004; Manzella *et al.* 2005). However, there is ample evidence that atheroclerosis represents a state of heightened OxS, which alters the bioavailability of NO (Stocker and Keaney 2004). As NO may contribute a functional component to arterial stiffness, endothelial dysfunction might be one potential mechanism underlying alterations in the elasticity of atheroclerotic vessels.

Despite the fact that there are several plausible linking mechanisms between OxS and arterial stiffness in atherogenesis, the exact mechanisms by which OxS may affect arterial stiffness are still undergoing investigation. Recent studies suggest that high-grade OxS could modulate the activity of matrix metalloproteinases (Rajagopalan *et al.* 1996), which may be involved in the process of arterial stiffening in humans (Yasmin *et al.* 2005). In addition, increased production of ROS may also influence vessel wall stiffness by many mechanisms as by enhancing smooth muscle tone (Peng *et al.* 2003), by promoting smooth muscle cell proliferation (Zieman *et al.* 2005), by damaging elastin directly (Paik *et al.* 1997) or by increasing Rho-associated kinase activity (Noma *et al.* 2007). Furthermore, oxLDL is also pro-inflammatory; recently association has been shown between the measures of inflammation and arterial

stiffness in healthy individuals (Kampus *et al.* 2004; Yasmin *et al.* 2004). However, because endothelial dysfunction, arterial stiffness and atherosclerosis often coexist, causality remains uncertain.

In Paper IV we found independent correlation between EFI and ICAM-1 in healthy subjects, which indicates potential association between vasodilatory action and the pro-inflammatory properties of the endothelium. The lack of association between EF and CRP in this study was somewhat surprising, as CRP is considered to be a powerful predictor of CV events (Pearson *et al.* 2003). However, many other researchers have not either found a significant relationship between EF and CRP (Holmlund *et al.* 2002; Prasad *et al.* 2002; Verma *et al.* 2004). We detected no association between ICAM-1 and EF in the patients. This finding indicates that soluble ICAM-1 cannot be used as a marker of EF in subjects with advanced atherosclerosis. The ICAM-1 is constitutively expressed at low levels by endothelial cells (Silvermann *et al.* 2001), whereas other adhesion molecules are predominantly expressed after stimulation with inflammatory cytokines. This might explain why EF in the present study was only related to ICAM-1 level in healthy subjects, in whom the degree of inflammation was significantly lower. Thus ICAM-1 appears to be more indicative for identifying CV risk in persons with no symptoms of PAD yet.

The strong association between EFI and ICAM-1 but not OxS markers in controls indicates that the vascular endothelium might be affected primarily by inflammatory reactions rather than OxS. However, the correlation between ICAM-1 and F_2-IsoPs in the patient group may suggest that inflammation and OxS are combined in atherosclerosis, and that OxS may amplify inflammation-initiated vascular remodelling. The mechanisms by which inflammation primarily may impair EF are not fully understood. It has been demonstrated that anti-tumor necrosis factor-α therapy reduced inflammation and improved EF in humans (Booth *et al.* 2004). Tumor necrosis factor-α could mediate endothelial dysfunction *via* diminished expression of endothelial NOS (Yoshizumi *et al.* 1993), altered degradation of ADMA (Sattar 2004), or can directly alter EF (Chia *et al.* 2003). An alternative possibility is that inflammatory molecules cause OxS (Clapp *et al.* 2004), in part by expression of the inducible NOS, and this high output of NO, coupled with generation of superoxide anion, causes endothelial dysfunction (Beckmann *et al.* 1996).

6.5. Limitations

The weaknesses of the present study (Papers I–IV) are its cross-sectional observational nature as well as the relatively small sample size. There exist limitations on the use of the indirect indices of arterial stiffness, particularly when elucidating the specific action of NO in regulating/modulating arterial stiffness (Paper I). Apart from characterizing aortic stiffness, Tr depends, in part, on

wave reflections and we cannot exclude the influence of release of endogenous vasoactive substances on peripheral arterial stiffness. However, in patients with PAD, direct PWV measurement was frequently complicated owing to the stenosis or occlusions of the iliac, femoral and/or carotid arteries.

Considering the design of present study, the causality of the biochemical mechanisms, responsible for alterations in the vascular wall, remains speculative (Papers III and IV). As such, these data provide primarily mechanistic insights into the relative contribution of biological pathways to EF and arterial stiffness. In order to distinguish between the causative role of inflammation and OxS in atherogenesis, it is necessary to undertake further clinical/experimental research.

An additional limitation of the study is the potential confounding long-term effects of medications and smoking. We were unable to withdraw chronically ill patients from their medications for extended periods of time. Therefore, a potential bias should be considered. However, to minimize the effect of different drugs and smoking on the results, medication and smoking were discontinued at least 12 hours before the study (Papers I–IV). Finally, considering the age composition of the study sample and the fact that the patients with lower extremity PAD were all men (Papers I, III and IV), the generalizability of our findings for younger individuals and females is questionable.

7. CONCLUSIONS

1. Aortic stiffness and wave reflections were increased in the patients with lower extremity peripheral arterial disease compared with the controls. Salbutamol-produced nitric oxide-mediated changes in aortic stiffness and wave reflections were reduced in the patients, while nitroglycerin-induced changes were not different in either group. These findings demonstrate that patients with atherosclerosis have stiffer arteries and a reduced capacity of the endothelium to produce nitric oxide.

2. Systemic stimulation of nitric oxide synthesis decreased aortic stiffness independently of changes in mean arterial pressure in whole study group consisting of patients with lower extremity peripheral arterial disease and healthy subjects. This finding would support the hypothesis that nitric oxide plays a crucial role in the regulation of large artery stiffness.

3. Large and small artery elasticity indices were significantly associated with endothelial function and plasma asymmetric dimethylarginine level in healthy subjects. This finding suggests that endothelial dysfunction and accumulation of asymmetric dimethylarginine may be important mechanisms underlying increased arterial stiffness in healthy subjects.

4. Large and small artery elasticity indices were reduced in the patients with lower extremity peripheral arterial disease. They had also increased plasma baseline diene conjugates of low-density lipoprotein and urinary 8-iso-prostaglandin F_{2a} concentrations compared with the controls. The negative significant correlation of large and small artery elasticity indices with 8-iso-prostaglandin F_{2a} indicates that oxidative stress may be involved in arterial stiffening in atherosclerosis.

5. Endothelial function was reduced, aortic stiffness and wave reflections were increased in patient with lower extremity peripheral arterial disease. The patients had also elevated plasma levels of C-reactive protein, intercellular adhesion molecule-1, myeloperoxidase and increased urinary 8-iso-prostaglandin F_{2a} level. Plasma concentration of intercellular adhesion molecule-1 was inversely associated with endothelial function in the healthy subjects, whereas urinary 8-iso-prostaglandin F_{2a} level was positively related to arterial stiffness in atherosclerosis. These findings suggest that inflammation and oxidative stress may be linked differently to endothelial function and arterial stiffening in in atherosclerosis as well as in healthy condition.

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SUMMARY IN ESTONIAN

Endoteeli funktsioon ja arterite jäikus ateroskleroosiga patsientidel ning tervetel inimestel

Kliinilis-biokeemiline uurimus

Kokkuvõte

Endoteelirakud vooderdavad veresoonkonda seestpoolt ning moodustavad bioloogiliselt aktiivse näärme (kaaluga umbes 1 kilogramm), millel on organismis mitmeid elutähtsaid ülesandeid. Endoteel toodab teiste ühendite hulgas lämmastikoksiidi, mis lisaks tugevale veresooni laiendavale toimele koordineerib endoteeli teiste ülesannete täitmist. Endoteeli funktsiooni kahjustudes langeb endoteelirakkude võime toota lämmastikoksiidi, mis on ka võimas antiaterogeenne molekul ja osaleb arterite jäikuse regulatsioonis. Endoteeli funktsiooni langus ja arterite suurenenud jäikus on varajased muutused südame ja veresoonkonna haiguste, sh. ateroskleroosi, patogeneesis, ennustavad iseseisvalt kardiovaskulaarset riski ning on spetsiifilised ravi sihtmärgid.

Endoteeli funktsiooni ja arterite jäikuse hindamise kaudu on võimalik saada laiapõhjalist teavet organismi aterogeensest seisundist, mis võimaldab sihipäraselt rakendada meetmeid, et haigusseisundi tekkimist ja progresseerumist aeglustada või ära hoida. Kuna nüüdisaja kliiniline meditsiin suundub üha enam patsiendisõbralike mitteinvasiivsete meetodite rakendamise poole, siis püütakse seda printsiipi arvestada ka endoteeli- ja arterite jäikuseuuringute puhul. Mitteinvasiivne ja kergesti läbiviidav pulsilaine analüüs on teaduslikult tunnustatud ning patsiendisõbralik meetod nii arterite jäikuse kui endoteeli funktsiooni uurimiseks.

Süsteemne põletikuline protsess ja tugev kestev oksüdatiivne stress on tähtsad patogeneesimehhanismid endoteeli funktsiooni kahjustuses ja arterite jäikuse suurenemises. Lisaks ennustavad põletik ja oksüdatiivne stress sõltumatult teistest riskifaktoritest südame- ja veresoonkonna haiguste tekkimist ja arenemist. Samas on veel lõplikult teadmata nende rollid ja eriti vahekord veresoonte kahjustuse tekkimises ja edasiarenemises. Arterite funktsiooni kahjustuse funktsionaalsete ja biokeemiliste mehhanismide parem tundmine võimaldab välja töötadada täpsemaid parameetreid veresoonte kahjustuse hindamiseks.

Alajäseme arterite oblitereeriv ateroskleroos on süsteemse ateroskleroosi ilming, mille korral on haigetel oluliselt suurenenud südame- ja veresoonkonna haiguste risk. Eelkõige on need haiged ohustatud müokardi- ja ajuinfarkti poolt. Lisaks ateroskleerotilise kahjustuse ulatusele määrab nende patsientide haiguse edasiarenemise ja tüsistuste tekkimise endoteeli funktsioon ja arterite jäikus. Kuna viimased muutused on instrumentaalselt kindlakstehtavad ja ka raviga

mõjustatavad, siis on neil haigetel endoteeli funktsiooni ja arterite jäikuse hindamine kliinilises mõttes väga oluline.

Eesmärgid

Antud töö eesmärgiks oli hinnata pulsilaine analüüsiga endoteeli funktsiooni ja arterite jäikust alajäseme arterite oblitereeriva ateroskleroosiga patsientidel ja tervetel inimestel. Lisaks uurida endoteeli funktsionaalsete ja biokeemiliste parameetrite seost arterite jäikusega ning selgitada oksüdatiivse stressi ja põletiku rolli veresoonte kahjustuses.

Uurimuse täpsed eesmärgid olid järgmised:

1. Hinnata aordi jäikust ja rõhulainete tagasipeegeldumist oblitereeriva ateroskleroosiga patsientidel ja tervetel inimestel. Selgitada muutuseid nii aordi jäikuses kui rõhulainete tagasipeegeldumises salbutamooli (stimuleerib lämmastikoksiidi sünteesi) ja nitroglütseriini manustamise järgselt.
2. Testida, kas lämmastikoksiidi sünteesi stimuleerimine vähendab aordi jäikust sõltumatult keskmise arteriaalse vererõhu muutusest oblitereeriva ateroskleroosiga patsientidel ja tervetel inimestel.
3. Uurida, kas suurte ja väikeste arterite elastsusindeksid on seotud plasma asümmeetrilise dimetüülarginiini kontsentratsiooniga ja endoteeli funktsiooniga tervetel inimestel.
4. Hinnata suurte ja väikeste arterite elastsusindekseid ja oksüdatiivse stressi taset oblitereeriva ateroskleroosiga patsientidel ja tervetel inimestel ning selgitada seoseid arterite jäikuse ja oksüdatiivse stressi vahel haigetel ja tervetel.
5. Hinnata endoteeli funktsiooni, aordi jäikust ja rõhulainete tagasipeegeldumist ning põletiku ja oksüdatiivse stressi taset oblitereeriva ateroskleroosiga patsientidel ja tervetel inimestel. Lisaks uurida, kas põletik ja oksüdatiivne stress on haigetel ja tervetel sarnaselt seotud endoteeli funktsiooni ja arterite jäikusega.

Patsiendid ja meetodid

Uuringualusteks olid 39 alajäseme oblitereeriva ateroskleroosiga patsienti (II-IV staadium Fontaine järgi) ja 63 tervet vabatahtlikku. Nii arteriaalset jäikust kui endoteeli funktsiooni hinnati pulsilaine analüüsiga Endoteeli Keskuses. Vereanalüüsid teostati Sihtasutus Tartu Ülikooli Kliinikumi Ühendlaboris ja Tartu Ülikooli Biokeemia Instituudis.

Tulemused ja järeldused

1. Aordi jäikus ja rõhulainete tagasipeegeldumine olid suurenenud oblitereeriva ateroskleroosiga patsientidel. Lämmastikoksiidi sünteesi stimuleerimine salbutamooliga ei suutnud mõjustada perifeerse ateroskleroosiga haigetel aordi jäikust ja rõhulainete tagasipeegeldumist. Seevastu kontrollgrupi patsientidel põhjustas salbutamooli manustamine statistiliselt olulise arterite jäikuse languse. Nitroglütseriini manustamise järgne arterite jäikuse langus ei erinenud gruppide vahel. Saadud tulemused näitavad, et ateroskleroosiga patsientide arterid on jäigad ning endoteeli võime toota lämmastikoksiidi kahjustunud.

2. Lämmastikoksiidi sünteesi stimuleerimine vähendas aordi jäikust sõltumatult keskmise arteriaalse vererõhu muutusest oblitereeriva ateroskleroosiga patsientidel ja tervetel inimestel. Saadud informatsioon toetab seisukohta, et lämmastikoksiid osaleb iseseisvalt suurte arterite jäikuse regulatsioonis.

3. Suurte ja väikeste arterite elastsusindeksid olid seotud nii plasma asümmeetrilise dimetüülarginiini kontsentratsiooniga kui endoteeli funktsiooniga tervetel inimestel. Antud tulemus viitab võimalusele, et endoteeli funktsiooni langus ja asümmeetrilise dimetüülarginiini kontsentratsiooni tõus suurendavad arterite jäikust.

4. Suurte ja väikeste arterite elastsusindeksid ja oksüdatiivse stressi tase olid suurenenud ateroskleroosiga patsientidel. Nii suurte kui väikeste arterite elastsusindeksid olid seotud oksüdatiivse stressiga vaid ateroskleroosiga patsientidel. Antud tulemus kinnitab oksüdatiivse stressi olulist rolli arterite jäikuse suurenemises ateroskleroosi korral.

5. Endoteeli funktsioon oli langenud, aordi jäikus, rõhulainete tagasipeegeldumine ning põletiku ja oksüdatiivse stressi tase olid suurenenud ateroskleroosiga patsientidel. Endoteeli funktsioon oli seotud põletikuga tervetel inimestel, seevastu kui arterite jäikus oli seotud oksüdatiivse stressiga patsientidel. Saadud informatsioon näitab, et põletikulise protsessi ja oksüdatiivne stressi artereid kahjustavates mehhanismides võib olla erinevusi.

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PUBLICATIONS

I

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II

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Arterial elasticity is associated with endothelial vasodilatory function
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III

Kals J, Kampus P, Kals M, Zilmer K, Kullisaar T, Teesalu R, Pulges A, Zilmer M. Impact of oxidative stress on arterial elasticity in patients with atherosclerosis. American Journal of Hypertension 2006; 19: 902–908.

IV

Kals J, Kampus P, Kals M, Pulges A, Teesalu R, Zilmer K, Kullisaar T, Salum T, Eha J, Zilmer M. Inflammation and oxidative stress are differently associated with endothelial function and arterial stiffness in healthy subjects and in patients with atherosclerosis (submitted).

INFLAMMATION AND OXIDATIVE STRESS ARE DIFFERENTLY ASSOCIATED WITH ENDOTHELIAL FUNCTION AND ARTERIAL STIFFNESS IN HEALTHY SUBJECTS AND IN PATIENTS WITH ATHEROSCLEROSIS

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ABSTRACT

Background: Inflammation and oxidative stress are considered to contribute to arterial dysfunction. However, studies designed to examine if endothelial function and arterial stiffness are determined by levels of inflammation and oxidative stress in a similar manner in atherosclerosis as well as in healthy condition are absent. The aim of this study was to evaluate possible relationships between vascular function, inflammation and oxidative stress in patients with atherosclerosis and in healthy subjects.
Methods: Endothelial function and arterial stiffness were assessed non-invasively by pulse wave analysis and blood/urinary samples were taken in 39 patients with peripheral arterial disease as well as in 34 controls.
Results: The patients showed significantly reduced endothelial function index and increased augmentation index, as well as higher estimated aortic pulse wave velocity and elevated values of the intercellular adhesion molecule-1 (ICAM-1), high sensitivity C-reactive protein, myeloperoxidase and urinary 8-iso-prostaglandin F_{2a} (F_2-IsoPs). There was inverse association between endothelial function index and the ICAM-1 ($R = –0.44$, $P = 0.009$) in the controls but not in the patients. Augmentation index and estimated aortic pulse wave velocity correlated with F_2-IsoPs only in the patients ($R = 0.5$, $P = 0.001$; $R = –0.43$, $P = 0.006$, respectively). After controlling for potential confounders, these associations remained significant.
Conclusions: The current study demonstrates that ICAM-1 is associated with endothelial dysfunction in healthy condition, whereas F_2-IsoPs is related to arte-

rial stiffening in atherosclerosis. These findings may prove useful to understanding better the possible biochemical mechanisms responsible for alterations in the vascular wall.

Key words: atherosclerosis, arterial stiffness, endothelium, inflammation, oxidative stress

1. INTRODUCTION

There is growing recognition that systemic inflammation [1] and high-grade oxidative stress (OxS) [2] are essential pathogenetic features of atherosclerosis, involving disease initiation and progression as well as development of complications. Inflammation and OxS may cause endothelial dysfunction [3] and arterial stiffening [4], which are both important characteristics of vascular health. These vascular parameters predict cardiovascular risk in different patient groups [5–7], e.g. in patients with peripheral arterial disease (PAD) [8].

The vascular endothelium is both affected by and contributes to the inflammatory processes that lead to atherosclerosis. Several pro-inflammatory factors activate endothelial cells to express adhesion molecules, which mediate transendothelial migration of leukocytes in atherogenesis [1,9]. It has been shown that the human intercellular adhesion molecule-1 (ICAM-1) correlates with vascular vasomotor reactivity [10], suggesting that it is a convenient biomarker for assessment of endothelial function. Moreover, elevated ICAM-1 not only indicates presence of vascular inflammation and endothelial dysfunction, but also independently predicts development of PAD [11] and cardiovascular complications [12].

Today there is substantial evidence that vascular dysfunction relates to increased production of reactive oxygen species (ROS) [2,3,9,13]. Studies directed to assessment of OxS suggest that 8-iso-prostaglandin F_{2a} (F_2-IsoPs) is a relevant marker for quantifying OxS in humans [14]. Interestingly, the importance of F_2-IsoPs in patients with PAD has been demonstrated only in a few studies [15,16], as well as data about associations between arterial elastic properties and OxS in humans are limited [17,18].

Although inflammation and OxS play key roles in atherogenesis, their causal relationship is incompletely understood. Much attention has been given to the possibility that inflammation is a primary process of atherosclerosis and oxidative events may be a consequence, rather than a cause, of the atherosclerosis [9]. If inflammation precedes high-grade OxS in atherogenesis, the markers of inflammation and OxS may be linked differently to endothelial function and arterial structural stiffening in atherosclerosis as well as in healthy condition. Accordingly, the aim of the study was to elucidate possible relationships

between endothelial function, arterial stiffness and several indices of inflammation and OxS in patients with PAD and in healthy volunteers.

2. METHODS

2.1. Study population

The study group consisted of 39 patients with PAD having stages of chronic ischaemia II or III as defined by Fontaine: stage II – intermittent claudication and stage III – leg pain at rest. All patients were recruited from the Clinic of Cardiovascular and Thoracic Surgery, University Clinics of Tartu, Estonia. The subjects were all male with angiographically proven PAD, i.e. with occlusion of the arteries of the lower extremities. Their ankle brachial pressure index (ABPI) was less than 0.78 (range 0.2–0.77). The patients' exclusion criteria were the following (based on clinical examination, ECG and blood tests): any concomitant acute or chronic inflammatory disease, myocardial infarction, coronary revascularization or cerebrovascular events during the last 6 months, earlier revascularization procedures at the lower limb, upper limb occlusive arterial disease, hypertension (blood pressure ≥ 140/90 mmHg), cardiac arrhythmias, or valve pathologies, diabetes mellitus (fasting serum glucose level > 6 mmol/L), malignancies and renal failure. Twelve (30.8 %) patients with coronary artery disease (CAD) as the co-morbidity were included in the study.

The control group of men (N = 34) was recruited from general population. The exclusion criteria for the control group were the following (based on clinical examination, ECG and blood tests): any acute or chronic inflammatory disease, CAD, cardiac arrhythmias, or valve pathologies, hypertension (blood pressure ≥ 140/90 mmHg), cerebral or peripheral atherosclerotic disease, diabetes mellitus (fasting serum glucose level > 6 mmol/L), malignancies, renal failure and regular use of any medications. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committee, University of Tartu. Informed written consent was obtained from each participant.

2.2. Study protocol

The subjects were studied and plasma samples were collected between 8:00 and 10:00 am after an overnight fast and abstinence from any medications, tobacco, alcohol and tea or coffee. After 15 minutes of rest in a quiet, temperature-controlled room, ABPI and blood pressure were measured and pulse wave analysis (PWA) was performed with provocative pharmacological tests. All haemodynamic and PWA recordings were made at least in duplicate for each time point. Thereafter, venous blood samples were drawn from the antecubital fossa, and urine samples were collected. The patients' height and weight were recorded, and body mass index (BMI) was calculated.

2.3. Laboratory methods

The plasma concentration of high sensitivity C-reactive protein (hsCRP) was determined by using a validated latex particle-enhanced immunoturbidimetric assay (CRP (Latex) HS, Roche Diagnostics GmPh®, Mannheim, Germany). The blood samples were centrifuged and plasma for ICAM-1, oxidized LDL (oxLDL), myeloperoxidase (MPO) as well as urine for F_2-IsoPs and creatinine were divided into aliquots and stored at −70°C until analysis.

Soluble ICAM-1 was measured by an enzyme-linked immunosorbentassay (ELISA) using a commercially available kit (Human soluble ICAM-1 Immunoassay, catalogue number BBE 1B, R&D Systems Inc.®, Minneapolis, USA). The intra- and inter-assay precision coefficients of variation for ICAM-1 were 4.8% and 7.4%, respectively. Enzyme-linked immunosorbentassay kits were also used to determine serum oxLDL (Mercodia, AB, Uppsala, Sweden) and MPO levels (BIOXYTECH® MPO-EIA, catalogue number 21013, *Oxis*Research®, Portland, USA), and the urinary content of F_2-IsoPs (BIOXYTECH® 8-Isoprostane Assay, catalogue number 21019, *Oxis*Research®, Portland, USA). All determination procedures were performed in accordance with the manufacturer's recommendation. The urinary concentrations of F_2-IsoPs were corrected by urinary creatinine concentrations to account for the differences in renal excretory function. Plasma glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride levels, as well as plasma and urinary creatinine concentrations were determined by standard laboratory methods using certified assays in the local clinical laboratory.

2.4. Assessment of haemodynamics, arterial stiffness and endothelial function

Peripheral blood pressure was measured in the dominant arm using a validated oscillometric technique (OMRON M4-I; Omron Healthcare Europe BV®, Hoofddorp, The Netherlands). Mean arterial pressure (MAP) was calculated from the integration of the radial pressure waveform using the Sphygmocor software (SCOR Px, 7.0; AtCor Medical®, Sydney, Australia).

Arterial stiffness and endothelial function were assessed by PWA using an Sphygmocor apparatus as described previously [19]. The peripheral pressure waveforms were recorded from the radial artery of the dominant arm at the wrist employing a high fidelity micromanometer (SPT-301B; Millar Instruments®, Texas, USA). Using a transfer function, the corresponding ascending aortic waveforms were then generated, from which central haemodynamics, augmentation index (AIx) and estimated aortic pulse wave velocity (Tr) were calculated. Augmentation index, a measure of systemic arterial stiffness, was calculated as the difference between the second and first systolic peaks of the central arterial waveform, expressed as the percentage of central pulse pressure. The AIx was corrected for a heart rate of 75 beats *per* minute (AIx@75). Estimated aortic pulse wave velocity represents the composite travel time of the

pulse wave to the periphery, the main reflectance site (aortic bifurcation) and its return to the ascending aorta, thus providing aortic stiffness.

After the baseline measurements of haemodynamics, a 500-µg tablet of nitroglycerin (NTG) (Nycomed®, Roskilde, Denmark) was placed under the tongue for 3 minutes and pulse waves were recorded 3, 5, 10, 15 and 20 minutes after NTG administration. Next, after restoration of haemodynamics, 400µg of the β_2-agonist salbutamol (Salb) (GlaxoWellcome Production®, Evreux, France) was given by inhalation and recordings were made 5, 10, 15 and 20 minutes after administration. A maximum improvement in AIx following Salb administration (due to stimulation of nitric oxide (NO) synthesis) was defined as endothelium-dependent vasodilatation (EDV), while an improvement in AIx after NTG was interpreted as a marker of endothelium-independent vasodilatation (EIDV). We used endothelial function index (EFI), defined as the EDV/EIDV ratio, to represent endothelial function. This index can evaluate vasodilatation depending of the activity of endothelial NO-synthase in relation to non-specific vasodilatation. All PWA recordings were performed consecutively by a single operator.

The ABPI was measured using Mini Dopplex D900 (Huntleigh Healtcare Ltd.®, Cardiff, UK). The recordings were taken from the (more) symptomatic lower limb in patients. An average of two closest measurements of all vascular parameters was included in statistical analysis.

2.5. Statistical analysis

All data were tested for normality. The continuous data were expressed as means ± SD if distributed normally, or otherwise by medians with 25% and 75% percentiles. The dichotomous variables are given as prevalence in number and percentage. Comparisons between the patients and the controls were assessed using unpaired 2-tailed Student's *t*-test (for the means) and Mann-Whitney *U* test (for the medians). Correlations between the variables were examined using linear regression analysis (software R, version 2.0.1 for Windows).

To determine the factors influencing endothelial function and arterial stiffness, multiple linear regression analyses were performed. Variables entered into the model were chosen from simple correlation analyses, and, from published observations, those variables known or likely to be associated with vascular function. For multiple regression model building, forward and backward stepwise variable selection procedures were applied. A $P < 0.05$ was considered significant.

3. RESULTS

3.1. Subject characteristics

The clinical characteristics of study groups are summarized in Table 1. There was no significant difference between the patients and the controls in age, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, glucose or EIDV. Nor occurred there differences in height (1.73±0.06 m *vs* 1.75±0.06 m, $P = 0.07$), peripheral systolic and diastolic BP (128.3±11.6 mmHg *vs* 122.4±11.4 mmHg, $P = 0.08$; 76.9±7.5 mmHg *vs* 73.9±7.0 mmHg, $P = 0.31$, respectively), central diastolic BP (77.8±7.7 mmHg *vs* 74.6±7.1 mmHg, $P = 0.26$) or MAP (95.8±10.2 mmHg *vs* 91.2±8.6 mmHg, $P = 0.21$) between the patients and the controls.

However, there was a significant difference in BMI, hsCRP, ICAM-1, MPO, F_2-IsoPs (also in creatinine indexed F_2-IsoPs), ABPI, AIx, AIx@75 (AIx, which was corrected for a HR of 75 beats per minute), Tr, EDV, EFI and smoking status between the groups. Heart rate (65.0±8.7 beats/min *vs* 58.5±7.1 beats/min, $P = 0.001$), central systolic BP (123.0±14.6 mmHg *vs* 113.7±11.8 mmHg, $P = 0.03$) and PP amplification (1.20±0.09 *vs* 1.25±0.11, $P = 0.048$) were also different for the patients and for the controls. In addition, there was noted an evident trend of difference in oxLDL between the groups. Differences occurred also in medication between the subgroups: all patients were on pentoxyfylline treatment, 30 patients (76.9%) received aspirin and 10 patients (25.6%) received calcium channel blockers, while controls did not receive any medicaments.

3.2. Relationship between endothelial function and inflammation

Linear regression analysis was used to establish whether endothelial function correlated with inflammation within each group separately. There was a significant inverse association between EFI and ICAM-1 only in the controls (Fig. 1). However, we found no relationship between EFI and ICAM-1 or other inflammatory markers (hsCRP, MPO) in the patient group or between EFI and F_2-IsoPs or oxLDL in either group (data not shown). To investigate further the factors influencing independently EFI in the controls, a multiple linear regression model was developed with EFI as the dependent variable. In multivariable regression model EFI was independently inversely correlated with ICAM-1, AIx@75 and BMI (Table 2).

3.3. Relationship between arterial stiffness and oxidative stress

Significant associations were observed between AIx@75 and F_2-IsoPs (Fig. 2), as well as between raw AIx and F_2-IsoPs ($R = 0.38$, $P = 0.02$) only in the patient group. There we also correlation between Tr and F_2-IsoPs in the patients but not in the controls (Fig. 3). No significant relationship was found between arterial stiffness and the inflammatory markers (ICAM-1, hsCRP, MPO) in either group

or between arterial stiffness and F_2-IsoPs or oxLDL in the controls (data not shown). To determine the factors influencing arterial stiffness, two multiple linear regression models were developed for the patient group with AIx and Tr as the dependent variables. The final model indicates that AIx was inversely associated with HR, positively correlated with F_2-IsoPs and MPO level, and clearly tended to associate with age (Table 3). The multiple regression model for aortic stiffness revealed that Tr was significantly inversely correlated with F_2-IsoPs, MPO and age (Table 4).

In addition, there occurred a significant linear correlation between ICAM-1 and F_2-IsoPs only in the patient group but not in the healthy subjects (Fig. 4).

4. DISCUSSION

The current study compares endothelial function and arterial stiffness, both measured non-invasively by PWA, as well as several indices of inflammation and OxS in patients with PAD and in healthy controls. Our main novel finding was that impairment of endothelial vasomotor capacity is related to the degree of inflammation in healthy conditions, whereas arterial stiffening is determined by the level of oxidative modifications in atherosclerosis.

Endothelial dysfunction is an initial step in atherogenesis and plays a crucial role in disease progression and in vascular complications [5,8]. Besides fulfilling several biofunctions, the endothelium regulates also vascular tone and structure [20], and impaired endothelial function contributes to progression of arterial stiffness [18,21]. As endothelial dysfunction and premature arterial stiffening are important parameters in stratification of cardiovascular risk (especially in PAD patients with increased cardiovascular burden) [8], non-invasive assessment of subclinical arterial disease is a subject of growing interest [19].

We found reduced EFI and increased inflammatory indices (ICAM-1, hsCRP, MPO) in the PAD patients in comparison with the controls. Nonetheless, endothelial function was significantly inversely associated with ICAM-1 level only in the healthy subjects (Fig. 1). In a multiple regression model, EFI was independently inversely determined by ICAM-1 level, AIx@75 and BMI (Table 2). It was expected that endothelial vasodilatory function was reduced in the subjects with higher BMI and stiffer arteries. Like other researchers [10], we found an independent correlation between EFI and ICAM-1 in the healthy volunteers, which exhibits potential association between vasodilatory action and the pro-inflammatory properties of the endothelium. The lack of association between EFI and hsCRP in this study was somewhat surprising, as hsCRP is considered to be powerful predictor of cardiovascular events [22]. However, other observers have not found relationship between endothelial function and hsCRP [10,23,24], which argue against a specific pathogenic role for hsCRP as an inducer of endothelial dysfunction.

We detected no association between ICAM-1 and vascular vasomotor reactivity in the patients. This finding indicates that soluble ICAM-1 cannot be used as a marker of endothelial function in subjects with advanced atherosclerosis. The intercellular adhesion molecule-1 is constitutively expressed at low levels by endothelial cells [25], whereas other adhesion molecules are predominantly expressed after stimulation with inflammatory cytokines. This might explain why endothelial vasodilatory function in the present study was only related to ICAM-1 level in healthy subjects with a low degree of inflammation. Thus, ICAM-1 appears more indicative for identifying asymptomatic persons with increased cardiovascular risk.

The F_2-IsoPs has vasoconstrictive and platelet activation properties and is supposed to be a candidate molecule for transducing, at least in part, the effects of lipid peroxidation and inflammation on vascular dysfunction [14]. In the present study we demonstrated high-grade OxS and increased arterial stiffness in patients, and established their correlations between F_2-IsoPs and AIx@75 (Fig. 2) as well as between F_2-IsoPs and Tr (Fig. 3). After controlling for potential confounders in a multiple regression model, the associations between AIx or Tr and F_2-IsoPs remained significant in the patient group (Tables 3 and 4). These findings support our previous work [26] and might indicate the potential superior role of OxS in functional and structural stiffening of the arteries in atherosclerosis [17,18].

The relationships considered by us support the hypothesis, set up by other authors [9], that inflammation is a primary process in atherogenesis producing oxidative events as a by-product. The strong association between EFI and ICAM-1 in the controls in the present study indicates that in subclinical condition vascular endothelium is primarily affected by inflammatory reactions. Low-grade chronic vascular inflammation promotes biochemical (e.g. peroxidative) changes in the vessel wall and reduces the bioavailability of NO. A decrease in NO production would favor an increased expression of adhesion molecules and induce inflammatory cells recruitment into the arterial wall [9,11,12]. The inflammatory process is central in development of atherosclerotic lesion but also leads to the production of ROS. However, cellular oxidative events can also inhibit the production of NO and consequent endothelial dysfunction appears a potential mechanism underlying alterations in the elasticity of atherosclerotic vessels [27].

The correlation between ICAM-1 and F_2-IsoPs in the patients with PAD (Fig. 4) may suggest that inflammation and OxS are combined in atherosclerosis, and that OxS may amplify inflammation-initiated vascular remodeling. Furthermore, MPO and F_2-IsoPs were independently related to arterial stiffness in the patients. The association between F_2-IsoPs, but not inflammation-related indices, and arterial stiffness in the patients indicates that oxidative modifications might mask inflammatory reactions during progression of atherosclerosis, predominantly contributing to vascular reorganization and stiffening. High-

grade OxS [28], as well as inflammatory reactions [29] could modulate the activity of matrix metalloproteinases, which are associated with destruction of the elastic laminae of the arteries and may be involved in the process of arterial stiffening. Increased production of ROS may also influence vessel wall elasticity by enhancing smooth muscle tone or by promoting smooth muscle cell proliferation [9].

Recently it has been pointed out that traditional risk factors explain less than 20 % of the variance in vascular dysfunction [30], intimating the emergence of novel surrogate risk markers for evaluation of subclinical cardiovascular pathologies [13]. Although inflammation related to OxS, their impact on progression of vascular alterations is not always similar. Therefore, measurement of the soluble biomarkers covering both pathogenetic cascades should be superior to assessment of either aspect alone. Besides, non-invasive assessment of the subclinical dysfunctional properties of the arteries and estimation of aortic pressures will facilitate identification of therapeutic targets for an individual at risk and will help monitor the success of treatment [19]. Thus, it remains to be seen if novel biomarkers in combination with pulse wave analysis will be used as part of routine risk factor assessment in both primary and secondary prevention.

4.1. Limitations

The weaknesses of the present study are its cross-sectional observational nature as well as the relatively small sample size. Considering the design of present study, the causality of the biochemical mechanisms responsible for alterations in the vascular wall remains speculative. As such, these data provide primarily mechanistic insights into the relative contribution of biological pathways to endothelial function and arterial stiffness. In order to distinguish between the causative role of inflammation and OxS in atherogenesis, it is necessary to undertake further clinical/experimental research.

An additional limitation of the study is the potential confounding long-term effects of medications and smoking. We were unable to withdraw chronically ill patients from their medications for extended periods of time. Therefore, a potential bias should be considered. However, to minimize the effect of different drugs and smoking on the results, medication and smoking were discontinued at least 12 h before the study. Finally, considering age composition of our sample and the fact that the patients with PAD are predominantly men, the generalizability of our findings for younger individuals and females is questionable.

4.2. Summary

Our data demonstrate that endothelial vasomotor function is associated with ICAM-1 level in healthy individuals, whereas arterial stiffness is determined by F_2-IsoPs concentration in patients with PAD. We believe that the findings of this study provide a better knowledge of the links between inflammation, OxS,

endothelial function and arterial stiffness as well as encourage use of a complex of biochemical and functional parameters for measuring vascular health.

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Table 1. Baseline characteristics of the study population.

Characteristic	PAD patients ($N = 39$)	Controls ($N = 34$)	P value
Age (years)	57.6±7.4	54.9±7.0	0.13
BMI (kg/m^2)	23.3±3.4	25.4±2.9	0.006
Ankle brachial pressure index	0.49±0.20	1.18±0.15	< 0.001
Total cholesterol (mmol/L)	5.37±0.81	5.16±1.06	0.37
HDL-cholesterol (mmol/L)	1.29±0.32	1.37±0.33	0.28
LDL-cholesterol (mmol/L)	3.88±1.06	3.6±0.71	0.2
Triglycerides (mmol/L)	1.37±0.27	1.22±0.53	0.15
Glucose (mmol/L)	5.13±0.62	5.23±0.48	0.48
hsCRP (mg/L)	3.07 (1.3–6.98)	1.07 (0.68–1.64)	< 0.001
ICAM-1 (ng/mL)	252 (209.5–314)	199 (185.8–224)	< 0.001
Myeloperoxidase (ng/mL)	15.5 (11.9–21.1)	10 (8.9–14.3)	< 0.001
F_2-IsoPs (ng/mL)	5.35±1.94	2.34±0.81	< 0.001
F_2-IsoPs (ng/mg creatinine)	9.52±5.60	4.28±2.51	< 0.001
Oxidized LDL (U/L)	143.6±49.1	124.2±40.0	0.09
Augmentation index (%)	36.4±8.0	26.8±7.8	< 0.001
AIx@75 (%)	31.5±8.0	18.9±7.9	< 0.001
Tr (ms)	132.9±13.3	149.2±10.1	< 0.001
EDV (%)	4.6±4.2	9.4±3.5	< 0.001
EIDV (%)	20.7±6.4	20.2±4.9	0.75
Endothelial function index	0.22±0.19	0.48±0.17	< 0.001
Current smoking, n (%)	36 (92.3)	5 (14.7)	< 0.001

Values are expressed as means (±SD), medians (with 25% and 75% percentiles) or prevalence (%). BMI, body mass index; hsCRP, high-sensitivity C-reactive protein; ICAM-1, intercellular adhesion molecule-1; F_2-IsoPs, 8-iso-prostaglandin F_{2a}, AIx@75, augmentation index, corrected for a heart rate of 75 beats per minute; Tr, estimated aortic pulse wave velocity; EDV, endothelium-dependent vasodilatation; EIDV, endothelium-independent vasodilatation.

Table 2. Multiple regression model for controls with endothelial function index as the dependent variable.

Variable	Regression coefficient	Standard error	P value
ICAM-1 (ng/mL)	–0.001	0.0005	0.01
AIx@75 (%)	–0.008	0.003	0.02
BMI (kg/m^2)	–0.02	0.009	0.03
LDL-cholesterol (mmol/L)	–0.06	0.004	0.08

$R^2 = 0.41$, $P < 0.003$. ICAM-1, intercellular adhesion molecule-1; AIx@75, augmentation index, corrected for the heart rate 75 beats per minute; BMI, body mass index.

Table 3. Multiple regression model for patients with augmentation index as the dependent variable.

Variable	Regression coefficient	Standard error	*P* value
F_2-IsoPs (ng/mL)	1.73	0.55	0.004
Heart rate (beats/min)	–0.29	0.12	0.02
Myeloperoxidase (ng/mL)	0.31	0.15	0.04
Age (years)	0.26	0.13	0.05

$R^2 = 0.40$, $P < 0.001$. F_2-IsoPs, 8-iso-prostaglandin F_{2a}.

Table 4. Multiple regression model for patients with estimated aortic pulse wave velocity as the dependent variable.

Variable	Regression coefficient	Standard error	*P* value
F_2-IsoPs (ng/mL)	–2.90	0.94	0.004
Myeloperoxidase (ng/mL)	–0.55	0.25	0.04
Age (years)	–0.48	0.22	0.04

$R^2 = 0.34$, $P < 0.002$. F_2-IsoPs, 8-iso-prostaglandin F_{2a}.

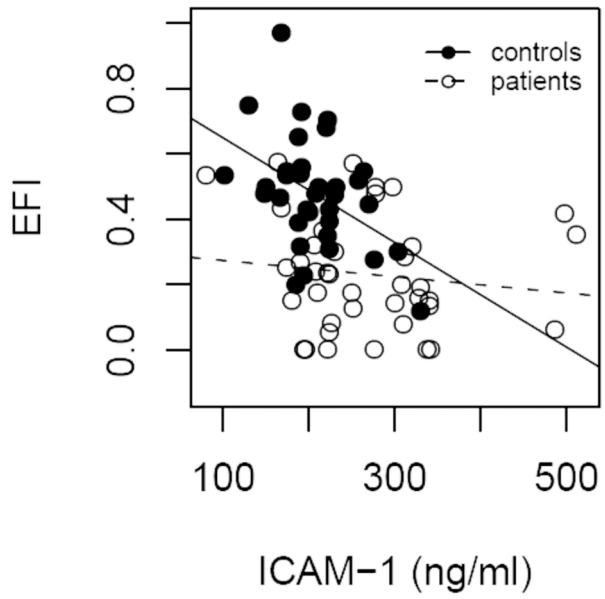

Figure 1. Scatterplot of endothelial function index (EFI) and intercellular adhesion molecule-1 (ICAM-1) in 39 patients and 34 controls. The parameters were significantly correlated only in the controls ($R = –0.44$, $P = 0.009$), but not in the patients ($R = –0.13$, $P = 0.43$).

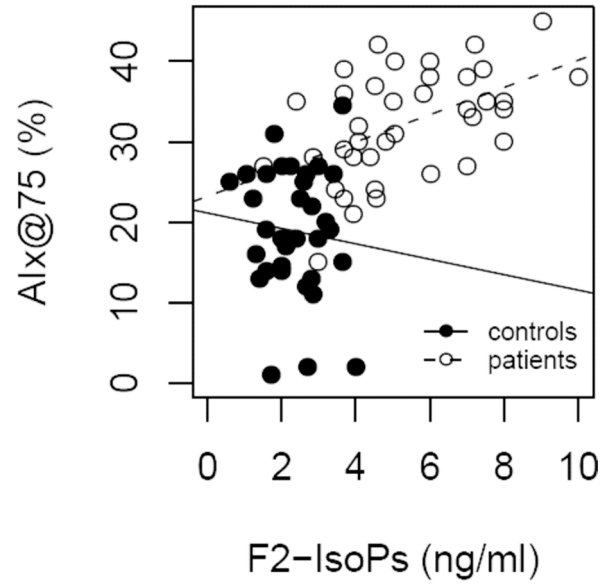

Figure 2. Scatterplot of augmentation index (AIx@75) and 8-iso-prostaglandin F_{2a} (F_2-IsoPs) in 39 patients and 34 controls. The parameters were significantly correlated only in the patients ($R = 0.50$, $P = 0.001$), but not in the controls ($R = –0.10$, $P = 0.58$).

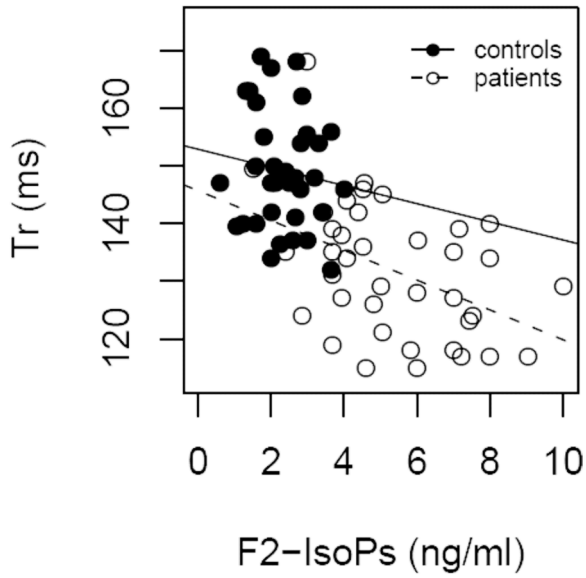

Figure 3. Scatterplot of estimated aortic pulse wave velocity (Tr) and 8-iso-prostaglandin F_{2a} (F_2-IsoPs) in 39 patients and 34 controls. The parameters were significantly correlated only in the patients (R = –0.43, P = 0.006), but not in the controls (R = –0.13, P = 0.47).

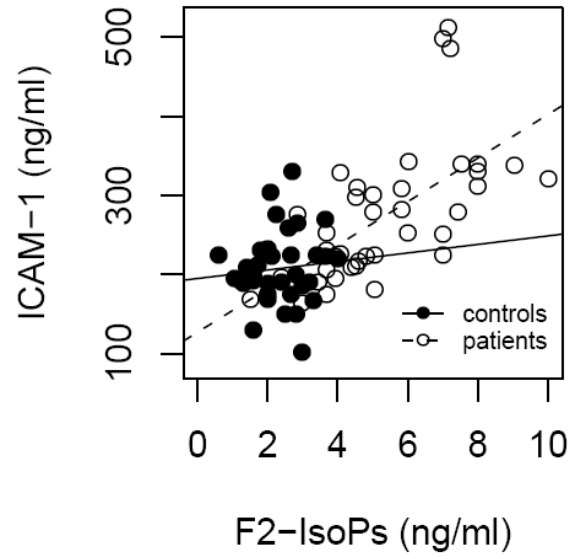

Figure 4. Scatterplot of intercellular adhesion molecule-1 (ICAM-1) and 8-iso-prostaglandin F_{2a} (F_2-IsoPs) in 39 patients and 34 controls. The parameters were significantly correlated only in the patients (R = 0.63, P < 0.001), but not in the controls (R = 0.09, P = 0.60).

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Professional employment

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2004	Signal Transduction in Cardiovascular System, Warsaw, Poland
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2005	FELASA C-category Competence Course of Laboratory Animal Science, Tartu, Estonia
2005	Arterial Stiffness: Theory and Practice, Cambridge, United Kingdom
2005	ARTERY 5: Forum for Discussion on Arterial Structure and Physiology, Paris, France
2006	Training Course on Arterial Stiffness. Budapest, Hungary
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Scientific work

Main research focuses on measurement of endothelial function and arterial stiffness in patients with cardiovascular pathologies as well as in animal models. 20 scientific articles (including 13 articles in international peer-review journals) and 16 presentations at international scientific conferences.

Membership: European Society for Cardiovascular Surgery, the Artery Society and the Estonian Medical Association.

Publications in peer-review journals

1. Kals J, Kampus P, Kals M, Teesalu R, Zilmer K, Pulges A, Zilmer M. Arterial elasticity is associated with endothelial vasodilatory function and asymmetric dimethylarginine level in healthy subjects. The Scandinavian Journal of Clinical and Laboratory Investigation (in press).
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Haridus

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2001–2002	Tartu Ülikooli arstiteaduskonna internatuur
2002–2007	Tartu Ülikooli biokeemia instituudi doktorantuur
2005	Biomeditsiini magister (MSc)(Tartu Ülikool)

Teenistuskäik

2004–	Tartu Ülikooli arstiteaduskonna biokeemia instituudi teadur
2005	Cardiffi Ülikooli (Suurbritannia) teadur (4 kuud)
2006–	Tartu Ülikooli arstiteaduskonna kardiovaskulaarkirurgia residentuur

Erialane eneseteäiendus

2003	The Human Circulation: Noninvasive Haemodynamic, Autonomic and Vascular Monitoring, Graz, Austria
2003	Poly-ADP-Ribosylation in Health and Disease, Debrecen, Ungari
2004	Theory and Applications of Pulse Wave Analysis, Cambridge, Suurbritannia
2004	Signal Transduction in Cardiovascular System, Varssav, Poola
2004	ARTERY 4: Forum for Discussion on Arterial Structure and Physiology, London, Suurbritannia
2005	FELASA C-category Competence Course of Laboratory Animal Science, Tartu, Eesti
2005	Arterial Stiffness: Theory and Practice, Cambridge, Suurbritannia
2005	ARTERY 5: Forum for Discussion on Arterial Structure and Physiology, Pariis, Prantsusmaa
2006	Arterial Stiffness, Budapest, Ungari
2006	ARTERY 6: Forum for Discussion on Arterial Structure and Physiology, Ateena, Kreeka

Teadustegevus

Teadustöö põhisuunaks on endoteeli funktsiooni ja arterite jäikuse uurimine erinevate kardiovaskulaarsüsteemi patoloogiaga haigetel ning loomkatsetes. Teadusartiklite üldarv on 20 (neist 13 rahvusvahelistes eelretsenseeritavates ajakirjades) ja lisaks 16 ettekannet rahvusvahelistel konverentsidel.

Kuulun Euroopa Kardiovaskulaarkirurgide Ühingusse, Artery Ühingusse ja Eesti Arstide Liitu.

Artiklid eelretsenseeritavates ajakirjades:

1. Kals J, Kampus P, Kals M, Teesalu R, Zilmer K, Pulges A, Zilmer M. Arterial elasticity is associated with endothelial vasodilatory function and asymmetric dimethylarginine level in healthy subjects. The Scandinavian Journal of Clinical and Laboratory Investigation (in press).
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