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MAKSIM ZAGURA

Biochemical, functional and structural profiling of arterial damage in atherosclerosis

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Department of Biochemistry, University of Tartu, Tartu, Estonia
Department of Cardiology, University of Tartu, Tartu, Estonia

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Supervisors: Professor Mihkel Zilmer, PhD
Department of Biochemistry, Faculty of Medicine,
University of Tartu, Tartu, Estonia

Professor Jaan Eha, MD, PhD
Department of Cardiology, Faculty of Medicine,
University of Tartu, Tartu, Estonia

Research Fellow Jaak Kals, MD, PhD
Department of Biochemistry, Faculty of Medicine,
University of Tartu, Tartu, Estonia

Reviewers: Professor Vallo Tillmann, MD, PhD
Department of Paediatrics, Faculty of Medicine,
University of Tartu, Tartu, Estonia

Senior Research Fellow Vallo Volke, MD, PhD
Department of Physiology, Faculty of Medicine,
University of Tartu, Tartu, Estonia

Opponent: Professor Toste Länne, MD, PhD
Division of Cardiovascular Medicine,
Department of Medical and Health
Sciences, Faculty of Health Sciences, Linköping University,
Linköping, Sweden

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To my parents

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–IV):

I Zagura M, Serg M, Kampus P, Zilmer M, Zilmer K, Eha J, Unt E, Lieberg J, Kals J. Association of osteoprotegerin with aortic stiffness in patients with symptomatic peripheral arterial disease and in healthy subjects. American Journal of Hypertension 2010; 23: 586–591.

II Kals J, Zagura M, Serg M, Kampus P, Zilmer K, Unt E, Lieberg J, Eha J, Peetsalu A, Zilmer M. β_2-microglobulin, a novel biomarker of peripheral arterial disease, independently predicts aortic stiffness in these patients. Scandinavian Journal of Clinical and Laboratory Investigation 2011; 71: 257–263.

III Zagura M, Serg M, Kampus P, Zilmer M, Eha J, Unt E, Lieberg J, Cockcroft J, Kals J. Aortic stiffness and vitamin D are independent markers of aortic calcification in patients with peripheral arterial disease and in healthy subjects. European Journal of Vascular and Endovascular Surgery 2011; 42: 689–695.

IV Zagura M, Kals J, Serg M, Kampus P, Zilmer M, Jakobson M, Unt E, Lieberg J, Eha J. Structural and biochemical characteristics of arterial stiffness in patients with atherosclerosis and in healthy subjects. Hypertension Research 2012 (in press).

Author's contribution:

I The author collected and analyzed the data. The author prepared the manuscript.

II The author collected the data. The author participated in data interpretation and in the preparation of the manuscript.

III The author was in charge of the collection and analysis of the data and in the preparation of the manuscript.

IV The author participated in data collection. The author analysed the data and prepared the manuscript.

ABBREVIATIONS

ABPI	ankle-brachial pressure index
ACS	aortic calcification score
AIx	augmentation index
AIx@75	augmentation index corrected for a heart rate of 75 beats per minute
aPWV	aortic pulse wave velocity
AngSc	angiographic score
baPWV	brachial-ankle pulse wave velocity
bkPWV	brachial-knee pulse wave velocity
β_2M	β_2-microglobulin
BMI	body mass index
BP	blood pressure
bPWV	brachial pulse wave velocity
CAD	coronary artery disease
CDBP	central diastolic blood pressure
CPP	central pulse pressure
CRP	C-reactive protein
CSBP	central systolic blood pressure
CT	computed tomography
CTA	computed tomography angiography
CV	cardiovascular
DSA	digital subtraction angiography
ECG	electrocardiography
eGFR	estimated glomerular filtration rate
hsCRP	high-sensitivity C-reactive protein
HDL	high-density lipoprotein
IL-6	interleukin-6
IMT	intima-media thickness
LDL	low-density lipoprotein
MAP	mean arterial pressure
MRA	magnetic resonance angiography
NO	nitric oxide
25(OH)D	25-hydroxyvitamin D
1,25$(OH)_2$D	1,25-dihydroxyvitamin D
OPG	osteoprotegerin
OPN	osteopontin
oxLDL	oxidized low-density lipoprotein
OxS	oxidative stress
PAD	peripheral arterial disease
PSBP	peripheral systolic blood pressure
PDBP	peripheral diastolic blood pressure

PPP	peripheral pulse pressure
PWA	pulse wave analysis
ROS	reactive oxygen species
sICAM	soluble intercellular adhesion molecule-1
Tr	travel time of the reflected wave
VCa	vascular calcification
VSMC	vascular smooth muscle cells

1. INTRODUCTION

Arterial stiffness describes the rigidity of arterial wall and is one of the earliest detectable manifestations of adverse structural and functional changes within vessel wall (Laurent *et al.* 2006). Arterial stiffening increases the left ventricular afterload and promotes development of left ventricular hypertrophy (Toprak *et al.* 2009). Moreover, arterial stiffening leads to reduction in diastolic blood pressure (BP), thus diminishing coronary perfusion (O'Rourke 2008, Milan *et al.* 2011) and causes unbalanced myocardial demand/coronary perfusion predisposing to ischaemia (Mottram *et al.* 2005). Arterial stiffness is also associated with atherosclerosis in different vascular beds (Van Popele *et al.* 2001; Boutouyrie *et al.* 2002; Agabiti-Rosei and Muiesan 2007b). Aortic pulse wave velocity (aPWV) is considered the „gold standard" parameter of arterial stiffness. The aPWV has an independent predictive value for cardiovascular (CV) events and all-cause mortality in high-risk patients and in general population (Laurent *et al.* 2003; Vlachopoulos *et al.* 2010). Current European guidelines for management of arterial hypertension introduce assessment of arterial stiffness by aPWV as an index of subclinical target organ damage (Mancia *et al.* 2007).

Vascular calcification (VCa) results from the deposition of calcium hydroxyapatite in the blood vessels (Demer and Tintut 2008). Experimental and clinical evidence indicate that VCa is an actively regulated process that involves a complex interplay between the promoters and inhibitors of calcification (Johnson *et al.* 2006). There is evidence that VCa might contribute to the increase in arterial stiffness (Sigrist *et al.* 2007), and vice versa (Dao *et al.* 2005). In chronic kidney disease, strong cross-sectional relationship has been demonstrated between VCa and aortic stiffness (Guerin *et al.* 2000; Verbeke *et al.* 2010). The VCa is an important contributor to CV morbidity and mortality in patients with advanced atherosclerosis (Alexopoulos and Raggi 2009), diabetes mellitus (Olson *et al.* 2000) and chronic kidney disease (Johnson *et al.* 2006).

Osteoprotegerin (OPG) is an inhibitor of calcification, which has been considered as a possible link between bone metabolism and vascular disease (Schoppet *et al.* 2002). Animal models indicate that OPG has disparate effects within the bones and the arteries. In genetic knockout animal models, OPG deficiency leads to medial calcification of the aorta and renal arteries (Bucay *et al.* 1998). In contrast, human studies have shown positive association between serum OPG levels and CV disease. Elevated serum OPG levels have been associated with coronary artery disease (CAD), diabetic complications, VCa and CV mortality (Kiechl *et al.* 2006; Anand *et al.* 2007; Van Campenhout and Golledge 2009).

Osteopontin (OPN) is also an inhibitor of VCa (Scatena *et al.* 2007). Serum OPN deficiency is related to ectopic calcification in animal models (Steitz *et al.* 2002). However, OPN levels are positively correlated with severity of aortic calcification in haemodialysis patients (Barreto *et al.* 2011). The OPN is

abundantly expressed in atherosclerotic plaques, and stimulates recruitment of macrophages and production of inflammatory cytokines (Scatena *et al.* 2007). Serum OPN level is related to presence and extent of CAD (Ohmori *et al.* 2003) and is an independent predictor of adverse CV events in patients with chronic stable angina (Minoretti *et al.* 2006).

Vitamin D is another important regulator of VCa. The effects of vitamin D on VCa appear to follow a biphasic pattern, with both excess and deficiency promoting its development (Shroff *et al.* 2008a). Low levels of 25-hydroxyvitamin D (25(OH)D) are associated with extensive VCa (Garcia-Canton *et al.* 2011) and injection of activated vitamin D increases survival in patients with end-stage renal disease (Teng *et al.* 2005). On the other hand, treatment with vitamin D increases aortic calcification in animal models (Niederhoffer *et al.* 1997). Moreover, recent data indicate that 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) downregulates the renin-angiotensin system (Li *et al.* 2002) and reduces proinflammatory cytokine secretion (Giulietti *et al.* 2007).

Inflammation plays a key role in development and progression of atherosclerosis. Several studies have demonstrated the association of various markers of inflammation, including acute phase reactants, cytokines and cellular adhesion molecules, with manifestations of atherosclerotic vascular disease. Elevated C-reactive protein (CRP) has been shown to be useful for predicting the risk of CV events in patients with established CAD (Heslop *et al.* 2010) and in asymptomatic individuals (Ridker *et al.* 1997). Soluble intercellular adhesion molecule-1 (sICAM) is a marker of presence and severity of atherosclerosis (Pradhan *et al.* 2002) and predicts myocardial infarction in apparently healthy men (Ridker *et al.* 1998). The β_2-microglobulin (β_2M) has recently emerged as a novel marker of inflammation (Wilson *et al.* 2007). Plasma β_2M is increased in patients with autoimmune diseases, infections and atherosclerosis (Wilson *et al.* 2007; Shinkai *et al.* 2008). A recent population-based study has demonstrated that β_2M is an independent predictor of all-cause mortality in elderly subjects (Shinkai *et al.* 2008).

High-grade oxidative stress (OxS) is another important contributor to atherosclerosis and CV mortality. Severe OxS promotes endothelial dysfunction (Heitzer *et al.* 2001), arterial stiffening (Kals *et al.* 2006b), proliferation and migration of vascular smooth muscle cells (VSMC) and activation of matrix metalloproteinases (Hulsmans *et al.* 2010). Oxidized low-density lipoprotein (oxLDL) is a marker of OxS, which is associated with presence of atherosclerotic lesions in the coronary arteries, the carotid artery and the arteries of the lower extremity (Madamanchi *et al.* 2005).

Peripheral arterial disease (PAD) is a highly prevalent public health problem associated with major detrimental effects on quality of life, being the main cause of limb amputation. Furthermore, patients with the diagnosis of PAD are at high risk of myocardial infarction, stroke and CV death (Hirsch *et al.* 2007). Calcified atherosclerotic arteries (Niskanen *et al.* 1990), increased arterial stiffness (Kals *et al.* 2006a), high-grade OxS (Haslacher *et al.* 2011) and

inflammation (Tzoulaki *et al.* 2005; Urbonaviciene *et al.* 2011) might influence the clinical course of PAD. However, data about the association between these parameters in patients with PAD are limited. The main aim of the present thesis was to investigate the relationship between arterial stiffness (functional profile), VCa and angiographic score (AngSc) (structural profile), OxS and inflammation (biochemical profile) in patients with symptomatic PAD and in clinically healthy subjects. We chose to investigate patients with PAD as it is a manifestation of systemic atherosclerosis that involves not only arteries of the lower extremities but also coronary, cerebral and renal vascular beds.

2. REVIEW OF THE LITERATURE

2.1. Arterial stiffness and cardiovascular disease

Arterial stiffness has been known as a sign of CV disease since the 19th century (Roy 1881). However, the clinical utility of arterial stiffness has only emerged in recent time. Nowadays, arterial stiffness can be accurately measured by a range of non-invasive techniques and it is established as a predictor of adverse CV outcome in high-risk patients (Boutouyrie *et al.* 2002; Cruickshank *et al.* 2002) and in general population (Laurent *et al.* 2006). Current European guidelines for the management of arterial hypertension indicate that increased aPWV indicates target organ damage and predicts high CV risk (Mancia *et al.* 2007). Recently, the reference values of aPWV have been published (Boutouyrie and Vermeersch 2010), which facilitates the use and interpretation of aPWV in clinical practice.

2.1.1. Determinants of arterial stiffness

Large arteries have two interrelated haemodynamic functions. First, they function as a conduit to deliver adequate blood supply from the heart to peripheral tissues. Second, large arteries dampen the pressure oscillations that result from intermittent ventricular ejection ensuring peripheral organ perfusion at steady flow and pressure (London and Pannier 2010). The efficiency of this function depends on the elastic properties of arterial wall.

Arterial wall consists of three layers: the intima (including the endothelium), the media and the adventitia. Each of these layers has specific roles in the regulation of arterial stiffness. Endothelial cells synthesize nitric oxide (NO) and endothelin-1, which are involved in the regulation of arterial stiffness (McEniery *et al.* 2003; Schulz *et al.* 2011). The NO reduces arterial stiffness via its vasodilatatory activity and inhibitory activity against VSMC growth (Cai and Harrison 2000; Simionescu 2007). The medial layer consists of elastic fibres, VSMC and the extracellular matrix. In aorta, elastic fibres are gathered together in sheets arranged in concentric layers throughout the thickness of the media (Shirwany and Zou 2010). The adventitia consists of collagen, extracellular matrix components, fibroblasts and vasa vasorum. Collagen fibres are approximately 500 times stiffer than elastic fibres; therefore, they are one of the major determinants of arterial stiffness (Nichols and O'Rourke 1998). Thus, arterial stiffness is influenced by endothelial function, VSMC tone and the relative content of elastin and collagen fibres (Astrand *et al.* 2011).

Increased arterial stiffness has been associated with a number of CV risk factors. Age is one of the most important determinants of arterial stiffness (Ahlgren *et al.* 1997; McEniery *et al.* 2005). Throughout life, arteries are subjected to a great number of cycles of stress, which leads to progressive degeneration of arterial wall. Age-related remodeling leads to gradual increase

in arterial stiffening through fatigue fracture and degradation of elastin fibres and increased loading on collagen fibres (McEniery *et al.* 2005; O'Rourke and Hashimoto 2007). The BP is another important determinant of arterial stiffness. Hypertension causes fractures and fragmentation of elastic lamellae, as well as increases collagen and calcium content in arterial wall (Benetos *et al.* 1993; Avolio *et al.* 1998). Diabetes mellitus also contributes to arterial stiffening (Heilman *et al.* 2009a; Llaurado *et al.* 2012). In diabetic patients, accumulation of advanced glycation end products in arterial wall promotes development of arterial stiffness through impairment of endothelial function (Rojas *et al.* 2000) and promotion of inflammation (Wendt *et al.* 2006). Other important determinants of arterial stiffness include dyslipidemia (Wilkinson *et al.* 2002), smoking (Mahmud and Feely 2003) and sedentary lifestyle (Tanaka *et al.* 1998).

2.1.2. Measures of arterial stiffness and their predictive value

Various methodologies and indices are used to assess arterial stiffness. Non-invasive methods for assessment of arterial stiffness are based on 1) change in vessel diameter or lumen area due to the change in distending pressure (local arterial stiffness), 2) estimation of PWV (regional arterial stiffness), 3) pulse wave analysis (PWA) (wave reflection) (Sakuragi and Abhayaratna 2010).

2.1.2.1. Local arterial stiffness

Local arterial stiffness is usually determined from the superficial arteries, mainly the brachial, femoral and the carotid arteries. Assessment of local stiffness is based on the measurement of change in vessel size in relation to the change in distending pressure. Local pulse pressure should be measured at the site of distension measurement. The use of brachial BP in calculation of these indices may introduce systematic errors, particularly in younger subjects in whom peripheral pulse pressure (PPP) is significantly higher than central pulse pressure (CPP). Local arterial stiffness can be measured using echo-tracking techniques, applanation tonometry or magnetic resonance imaging (Resnick *et al.* 1997; Turesson *et al.* 2005; Laurent *et al.* 2006). Evaluation of local arterial stiffness requires good technical experience and longer time to detect very small changes in arterial diameter, hence this method is unsuitable for large epidemiological studies.

2.1.2.2. Regional arterial stiffness (pulse wave velocity)

The PWV is a measure of regional arterial stiffness. Measurement of PWV is generally accepted as the most simple, robust and reproducible method to determine arterial stiffness (Laurent *et al.* 2006). The PWV is calculated as the distance travelled by the pulse wave divided by the time taken to travel the

distance (Laurent *et al.* 2006) (Figure 1). Accurate PWV quantification is dependent on accurate measurement of the distance between recording sites (Van Bortel *et al.* 2012). Readings from the two sites are taken simultaneously or separate readings are gated to a fixed point in the cardiac cycle, usually the R wave in electrocardiography (ECG) (Laurent *et al.* 2006). Pulse waves can be detected by applanation tonometry, Doppler ultrasound or by magnetic resonance imaging (Laurent *et al.* 2006; Redheuil *et al.* 2010). Transit time is usually measured from the foot of the wave using the point of intersecting tangents from the decline of the previous pulse and the sharp upstroke of the following pulse.

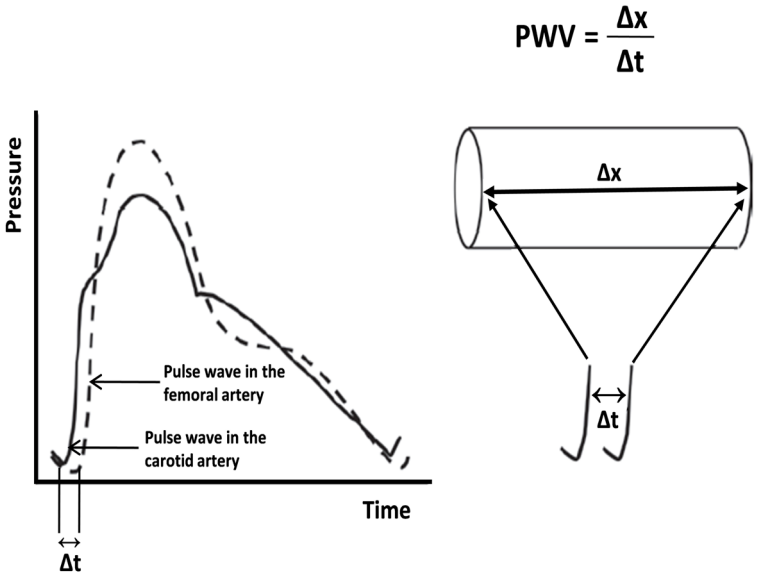

Figure 1. Measurement of aPWV with the foot to foot method (Adji *et al.* 2011, modified).

The PWV can be measured from the central and peripheral arteries. However, it is well known that aortic pressure is the major determinant of left ventricular afterload (Laurent *et al.* 2006). Therefore, aPWV is the most clinically relevant index of arterial stiffness, since it measures the propagation of pulse wave along the aorta and iliac artery. Accordingly, aPWV increases exponentially with aging and is a sensitive indicator of arterial stiffness after the age of 50 to 60 years, whereas brachial pulse wave velocity (bPWV) does not increase with aging (McEniery *et al.* 2005). Moreover, it has been demonstrated that bPWV or femoro-tibial PWV has no predictive value in patients with end-stage renal

disease. In contrast, aPWV was an independent predictor of CV mortality in these patients (Pannier *et al.* 2005).

An increasing number of studies have demonstrated the accuracy of PWV as an independent predictor of CV morbidity and mortality in patients with different co-morbidities and CV risk. Several longitudinal studies have shown that aPWV is an independent predictor of all-cause and CV mortality in high-risk patients, such as those with hypertension (Laurent *et al.* 2001), diabetes mellitus (Blacher *et al.* 2012) and end-stage renal disease (Blacher *et al.* 1999). In addition, increased aPWV predicts CV mortality in general population (Shokawa *et al.* 2005; Willum-Hansen *et al.* 2006; Inoue *et al.* 2009). The results of a recent meta-analysis indicate that aortic stiffness is strongly related to CV events, independent of age, BP and other risk factors of CV disease (Vlachopoulos *et al.* 2010).

2.1.2.3. Wave reflection (pulse wave analysis)

Central PWA provides information about wave reflection. Ventricular ejection generates the forward pressure wave that travels to the periphery along the arterial tree. The pressure wave is reflected from the periphery, mainly at branch points or sites of impedance mismatch (Laurent *et al.* 2006). The reflected wave is superimposed on the forward pressure wave. In elastic arteries, PWV is low and the reflected waves arrive at the aortic root during diastole, which improves coronary blood flow. In stiff arteries, PWV is increased and the reflected waves arrive at the ascending aorta earlier, adding to the forward wave and augmenting the systolic pressure (Mottram *et al.* 2005). The forward and reflected pressure waves can be quantified through the augmentation index (AIx), which is defined as the difference between the second and the first systolic peaks expressed as a percentage of pulse pressure (Figure 2) (Mackenzie *et al.* 2002). As AIx is influenced by the timing of the reflected wave, the distance to the reflecting sites and the amplitude of the reflected wave, it is an indirect measure of arterial stiffness (Nichols and O'Rourke 1998; Safar *et al.* 2003).

The main determinants of AIx include heart rate, height, age, mean arterial pressure (MAP) and PWV (Lemogoum *et al.* 2004; Agabiti-Rosei *et al.* 2007a). It is well established that AIx is inversely related to heart rate (Agabiti-Rosei *et al.* 2007a). With increase in heart rate, ejection time is shortened. Therefore, reflected waves can have less effect on late systolic pressure and more effect on the diastolic part of the pulse wave (Nichols and O'Rourke 1998). In addition, AIx is inversely associated with height. Hence, AIx is normally higher in women than in men (McEniery *et al.* 2005). Age is an important determinant of wave reflections. It has been shown that AIx increases almost linearly with age up to the age of 60 years; however, only minimal rise in AIx is observed after the age of 65 years (Mitchell *et al.* 2004; McEniery *et al.* 2005). As increased central arterial stiffness and peripheral vascular resistance are associated with an

increase in PWV and shifting of the reflection site in the proximal direction, they elevate AIx (Nichols 2005; Adji *et al.* 2011).

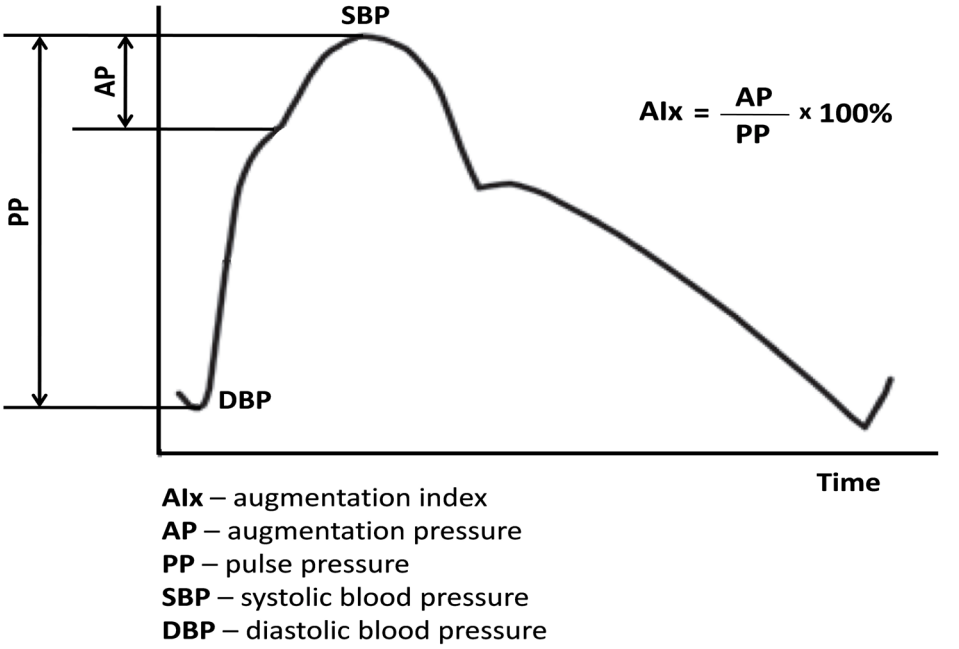

Figure 2. Pulse wave analysis (Adji *et al.* 2011, modified). Augmentation index is calculated from the ratio of augmentation pressure to pulse pressure and is expressed in percentages.

From PWA, also central BP can be derived. In young subjects, central systolic BP (CSBP) is significantly lower than peripheral systolic BP (PSBP), whereas diastolic BP remains approximately constant throughout the arterial tree (Agabiti-Rosei *et al.* 2007a). This phenomenon is called pulse pressure amplification. However, an age-related increase in arterial stiffness attenuates the difference between CPP and PPP (Wilkinson *et al.* 2001; McEniery *et al.* 2008). Assessment of central BP is of clinical importance, because central rather than peripheral BP affects directly the function of target organs (Agabiti-Rosei *et al.* 2007a; Kampus *et al.* 2011).

The prognostic value of central BP and AIx has been documented in several studies. Elevated AIx is independently associated with increased risk of restenosis, myocardial infarction and mortality in patients undergoing percutaneous coronary interventions (Weber *et al.* 2005). In patients with end-stage renal disease, AIx is a strong predictor of all-cause and CV mortality (London *et al.* 2001). The same study group has shown that CPP predicts independently all-cause mortality in haemodialysis patients. In contrast, PPP had no predictive

value for mortality after adjustment for CV risk factors (Safar *et al.* 2002). Similarly, higher CSBP but not measures of peripheral BP independently predicts CV mortality in elderly individuals (Pini *et al.* 2008).

2.1.3. Arterial stiffness and atherosclerosis

The relationship between arterial stiffness and atherosclerosis has been investigated in numerous studies. Animal experiments indicate that presence of atherosclerosis may increase arterial stiffness. Indeed, extensive atherosclerotic lesions in the aorta were associated with aortic stiffening in apolipoprotein E-deficient mice (Wang *et al.* 2000). Moreover, in monkeys, atherogenic diet significantly increased aPWV, whereas cholesterol regression diet led to a decrease in aPWV (Farrar *et al.* 1991).

Human studies confirm the results of animal experiments. Results from a longitudinal study demonstrate that aPWV is an independent predictor of coronary events in patients with essential hypertension (Boutouyrie *et al.* 2002). In addition, large artery stiffness contributes to exercise induced myocardial ischaemia (Kingwell *et al.* 2002) and predicts outcome after coronary interventions in patients with CAD (Duprez and Cohn 2007). In general population, aortic stiffness is strongly associated with presence of plaques in the carotid artery and in the aorta (Van Popele *et al.* 2001). It has been shown that patients with PAD have stiff arteries (Kals *et al.* 2006a). Moreover, arterial stiffening is related to decreased ankle-brachial pressure index (ABPI) in elderly subjects (Lind 2011). However, there is no data regarding the potential association of aortic stiffness with angiographic severity grade of atherosclerosis in patients with PAD. In Paper IV of the present thesis, we evaluated the relationship of aPWV with AngSc in patients with symptomatic PAD.

2.2. Vascular calcification

Ectopic calcification in the vasculature has been noted for many decades (Frink *et al.* 1970). There are two distinct patterns of VCa: calcification of the media (arteriosclerosis) and calcification of the intima (atherosclerosis). Previous studies indicate that in patients with atherosclerosis mostly intimal calcification is seen (Verbeke *et al.* 2010). Intimal calcification is associated with the infiltration of vessel wall with T cells and macrophages and shows the distinguishing features of endochondral ossification (Vattikuti and Towler 2004; Johnson *et al.* 2006). On the other hand, medial calcification is associated with diabetes mellitus, end-stage renal disease and aging (Sutliff *et al*. 2011). This type of calcification is characterized by intra-membranous ossification. The VCa could be assessed by biomarkers or by instrumental techniques (e.g. computed tomography (CT)).

2.2.1. Biomarkers of vascular calcification

Until recently, VCa was simply viewed as passive deposition of hydroxyapatite in arterial wall. Accumulating evidence now points to a tightly regulated process, with competition between the factors promoting and inhibiting calcification (Ketteler *et al.* 2011). The OPG, OPN and fetuin-A are important inhibitors of VCa that are implicated in the pathogenesis of atherosclerosis (Isoda *et al.* 2002; Jono *et al.* 2010; Ix *et al.* 2011).

2.2.1.1. Osteoprotegerin, osteopontin and fetuin-A

The OPG is a glycoprotein that belongs to the tumour necrosis factor receptor superfamily of cytokines. The OPG is a decoy receptor that inhibits osteoclast differentiation and activation (Lacey *et al.* 1998). Animal experiments indicate that OPG might be a possible link between bone metabolism and CV disease. In a mouse model, deficiency of OPG resulted in severe osteoporosis and VCa (Bucay *et al.* 1998). Moreover, OPG inhibits progression of atherosclerotic plaques by preventing an increase in lesion size and lesion calcification in mice (Bennett *et al.* 2006).

In contrast to the apparent protective role of OPG observed in animal models, observational studies in patients have demonstrated positive relationship between serum levels of OPG and CV disease (Kiechl *et al.* 2004; Anand *et al.* 2006; Rasmussen *et al.* 2006). Moreover, increased OPG levels have been reported in patients with CAD, carotid atherosclerosis, stroke, heart failure and hypertension (Browner *et al.* 2001; Kiechl *et al.* 2006; Blazquez-Medela *et al.* 2011). In patients with symptomatic PAD, serum levels of OPG are positively correlated with severity of atherosclerosis (Ziegler *et al.* 2005) and with endothelial dysfunction (Golledge *et al.* 2008).

Apart from influencing endothelial function, OPG is also implicated in arterial stiffening. It has been shown that OPG is positively correlated with brachial-ankle pulse wave velocity (baPWV) in haemodialysis patients (Talib *et al.* 2011; Nakashima *et al.* 2011) and in patients with type 2 diabetes mellitus (Kim *et al.* 2005; Jung *et al.* 2010). Moreover, several studies have shown that higher serum OPG levels are independently associated with aPWV in patients with chronic kidney disease (Scialla *et al.* 2011; Ford *et al.* 2012) and in general population (Schnabel *et al.* 2008).

The OPN is another important inhibitor of VCa. The OPN is a glycoprotein that is expressed in osteoblasts, osteocytes, VSMC and endothelial cells (Denhardt *et al.* 2001). It directly inhibits calcification by binding to hydroxyapatite (Wada *et al.* 1999) and by inducing decalcification (Asou *et al.* 2001). The OPN might be involved in regulation of inflammation (Giachelli and Steitz 2000). Indeed, OPN is produced in activated T cells and macrophages (Ramaiah and Rittling 2008) and its levels are elevated in inflammatory diseases (Wong *et al.* 2005; Agnholt *et al.* 2007). Moreover, OPN could contribute to progression of

atherosclerosis (Isoda *et al.* 2003). Expression of OPN correlates with amount of inflammatory cells (Isoda *et al.* 2002) and calcium deposits (Fitzpatrick *et al.* 1994) in atherosclerotic plaques. In addition, OPN has emerged as a marker of increased CV risk. In patients with CAD, plasma OPN levels are significantly associated with presence and extent of CV disease independently of traditional risk factors (Ohmori *et al.* 2003). Moreover, plasma OPN levels are independent predictors of restenosis after percutaneous coronary intervention (Kato *et al.* 2006).

Previous research suggests that OPN might be implicated in arterial stiffening. In mice, overexpression of OPN is associated with thickening of the aorta (Chiba *et al.* 2002). Moreover, elevated OPN levels are independently associated with increased aortic stiffness in patients with rheumatoid arthritis (Bazzichi *et al.* 2009).

Fetuin-A is a glycoprotein which acts as a potent inhibitor of VCa. Fetuin-A binds calcium phosphate and calcium carbonate and inhibits the process of matrix mineralization (Schinke *et al.* 1996). Both animal models and human studies indicate that fetuin-A deficiency is associated with increased VCa. Indeed, fetuin-A knockout mice develop higher soft tissue calcification than wild-type mice (Schäfer *et al.* 2003). Furthermore, lower levels of fetuin-A are significantly associated with greater extent of valvular (Wang *et al.* 2005) and coronary (Koos *et al.* 2009) calcification in patients with chronic kidney disease. In addition, fetuin-A levels are inversely related to severity of coronary artery calcification in subjects free from clinical CV disease (Ix *et al.* 2011).

Fetuin-A is not only a potent inhibitor of ectopic calcification but it also influences inflammatory response (Reynolds *et al.* 2005; Wang *et al.* 1997). It has been shown that fetuin-A inhibits the production of proinflammatory cytokines (Kelly and Smith 1997) and is inversely correlated with CRP levels in dialysis patients (Wang *et al.* 2005). The data regarding the association of fetuin-A with arterial stiffness is limited. Recent research indicates that fetuin-A is inversely related to baPWV in haemodialysis patients (Talib *et al.* 2011). In addition, decreased fetuin-A is independently associated with aortic stiffening in patients with chronic kidney disease (Ford *et al.* 2010) and in patients with normal kidney function (Roos *et al.* 2009).

In the present thesis, we investigated the possible relationship of aPWV with OPG (Paper I), fetuin-A (Paper II) and OPN (Paper IV) in PAD patients and in clinically healthy subjects.

2.2.1.2. Vitamin D

Vitamin D is classically known for its crucial role in calcium and bone metabolism (Bouillon *et al.* 2008). There exist two major forms of vitamin D: vitamin D_3, which is mainly derived by way of synthesis in the skin and from animal sources, and vitamin D_2 (ergocalciferol), which is derived from plants (Zilmer *et al.* 2010). Vitamin D as a precursor exerts no significant biological

activity. First, vitamin D is hydroxylated to 25(OH)D in the liver. Then, the enzyme 1α-hydroxylase converts 25(OH)D to $1,25(OH)_2D$. The kidney is the major site for production of circulating $1,25(OH)_2D$ (Zilmer *et al.* 2010).

An accumulating body of evidence suggests that vitamin D may be implicated in the pathogenesis of CV disease (Zittermann *et al.* 2005; Norman and Powell 2005; Brewer *et al.* 2011). Vitamin D receptors are present in VSMC, endothelial cells, myocardiocytes, macrophages and lymphocytes (Landry *et al.* 2011). The influence of vitamin D in these tissues could have important implications for vascular function and disease. Several studies have reported lower 25(OH)D levels in patients with cerebrovascular disease (Cigolini *et al.* 2006; Poole *et al.* 2006), CAD (Scragg *et al.* 1990), heart failure (Zittermann *et al.* 2003), hypertension (Forman *et al.* 2007) and diabetes mellitus (Martins *et al.* 2007). Moreover, low vitamin D status has also been associated with subclinical (Melamed *et al.* 2008b) and clinical PAD (Fahrleitner *et al.* 2002).

The relationship between vitamin D and VCa remains controversial. On the one hand, deficiency of vitamin D is associated with VCa in patients with chronic kidney disease (Garcia-Canton *et al.* 2011). On the other hand, vitamin D toxicity is associated with increased VCa in animal models (Henrion *et al.* 1991; Norman and Powell 2005).

There have been relatively few studies examining the association between vitamin D and arterial stiffness. Low 25(OH)D levels are significantly associated with increased baPWV in patients with type 2 diabetes mellitus (Lee *et al.* 2012). In addition, several studies have demonstrated that serum 25(OH)D is inversely related to aPWV in apparently healthy subjects (Al Mheid *et al.* 2011; Salum *et al.* 2011) and in general population (Mayer *et al.* 2011). In a longitudinal study, supplementation of 25(OH)D significantly decreased aPWV in adolescents (Dong *et al.* 2010).

In the present thesis, we investigated the possible relationship between 25(OH)D and aortic calcification in PAD patients and in clinically healthy subjects (Paper III). Moreover, we evaluated the association between ABPI and the composite measure of aPWV and 25(OH)D in PAD patients (Paper III).

2.2.2. Measurement of vascular calcification by computed tomography

Several imaging techniques have been developed for visualization and compositional characterization of VCa. Electron beam CT and multi-slice CT are presently considered the „gold standard" for assessing the extent of VCa and its progression (Bellasi and Raggi 2007). The CT is a non-invasive, sensitive method of detecting VCa (Rumberger *et al.* 1995). The advantages of CT techniques include rapid acquisition of images, prevention of image blurring and accurate visualisation of small calcific deposits without utilising contrast media (Fuseini *et al.* 2003) (Figure 3). However, there are also substantial

limitations to these technologies. The CT techniques cannot differentiate intimal and medial calcification. Moreover, CT technologies are expensive and provide substantial exposure to ionized radiation (Bellasi and Raggi 2007).

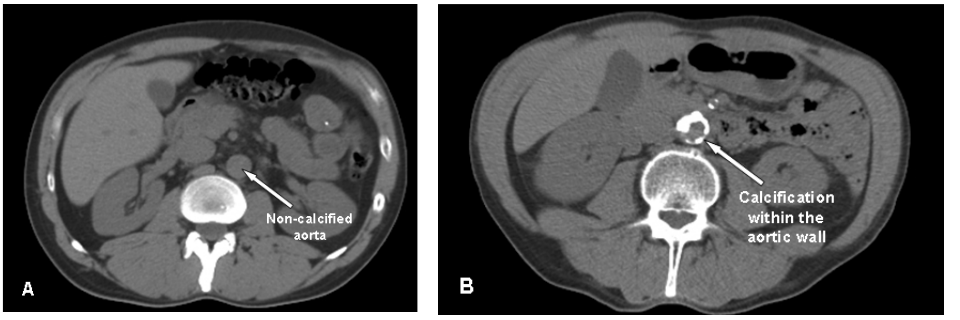

Figure 3. A cross-section of the abdomen illustrating (A) non-calcified aortic wall, (B) calcification within the aortic wall as detected by CT (Paper III).

2.2.3. Vascular calcification and arterial stiffness

Previous research suggests that intimal and medial calcification are differently associated with arterial stiffness. The relationship between intimal calcification (atherosclerosis) and arterial stiffness remains unclear (Mackey *et al.* 2007). In contrast, several studies have demonstrated that arterial stiffness is independently associated with medial artery calcification (arteriosclerosis), which is common in diabetes, chronic kidney disease, hypertension and aging (Iribarren *et al.* 2000; Davies and Hruska 2001; Guerin *et al.* 2008). Thus, strong cross-sectional relationship has been demonstrated between VCa and aortic stiffness in patients with chronic kidney disease (Guerin *et al.* 2000; Verbeke *et al.* 2010). Moreover, rapid progression of VCa is associated with arterial stiffening and increased mortality in patients with impaired renal function (Sigrist *et al.* 2007). Furthermore, aortic calcification is independently related to aortic stiffening in patients with isolated systolic hypertension (McEniery *et al.* 2009). However, the relationship between aortic calcification and stiffness in patients with PAD has not been studied. In Paper III of the present thesis, we aimed to investigate the association of aortic calcification with aPWV in subjects with symptomatic PAD and in clinically healthy individuals.

2.2.4. Vascular calcification and cardiovascular outcome

Both calcification within atherosclerotic plaque and diffuse medial calcification increase the risk of adverse CV events. It has been shown that atherosclerotic calcification may contribute to dissection after balloon angioplasty (Fitzgerald *et al.* 1992). The role of calcification in the process of atherosclerotic plaque

rupture is unclear. On the one hand, plaque calcification destabilizes a plaque, specifically in the areas of interface between hard and soft tissues (Virmani *et al.* 1998). On the other hand, other investigations have suggested that calcified plaques are more stable and less prone to rupture (Ge *et al.* 1999).

Medial artery calcification is also an important determinant of CV outcome. Indeed, abdominal aortic calcification is an independent predictor of mortality and nonfatal CV events in dialysis patients (London *et al.* 2003; Verbeke *et al.* 2011). Furthermore, in patients with diabetes, medial artery calcification is strongly correlated with CAD and adverse CV events (Niskanen *et al.* 1994; Olson *et al.* 2000). It has also been demonstrated that the magnitude of medial artery calcification of the femoral artery is a risk factor for amputation in patients with PAD (Lehto *et al.* 1996).

2.3. Inflammation

Inflammation has been recognised as a crucial factor for development and progression of atherosclerosis (Ridker *et al.* 1997; Ross 1999). At early stages of atherosclerosis endothelial cells begin to express adhesion molecules on their surface, which facilitates the recruitment of monocytes and T-lymphocytes and their attachment to the endothelium. (Badimon *et al.* 2011). Monocytes and T-lymphocytes that had adhered to the endothelium penetrate into the intima. Local inflammatory response stimulates the transformation of monocytes into macrophages. Increase in the permeability of the endothelial layer promotes accumulation of low-density lipoprotein (LDL) particles in the extracellular matrix where they become targets for oxidative modifications. The oxLDL enhances leukocyte attachment to the endothelial layer and stimulates trans-migration of leukocytes into the intima. Macrophages express scavenger receptors for modified lipoproteins, ingest LDL particles and become foam cells (Moore and Freeman 2006). Accumulation of foam cells leads to formation of the lipid core. T-lymphocytes produce γ-interferon and lymphotoxin, which stimulate macrophages and endothelial cells. Moreover, activated leukocytes secrete fibrogenic mediators, which promote migration and proliferation of VSMC (Libby *et al.* 2002). Proliferating VSMC produce extracellular matrix proteins, which form fibrous caps found on atherosclerotic plaques. Inflammation does not only promote formation of atheroma but it also contributes to acute thrombotic complications of atherosclerosis (Cimmino *et al.* 2011). Activated macrophages produce proteolytic enzymes, which stimulate plaque destabilization and rupture (Peeters *et al.* 2011). In addition, macrophages release tissue factor that, upon plaque rupture, contributes to thrombus formation (Badimon *et al.* 2011).

Recognition of the central role of inflammation in atherogenesis has stimulated the evaluation of different inflammatory markers as potential predictors of CV risk. Increased circulating levels of cytokines, chemokines, cell-adhesion

molecules and high-sensitivity C-reactive protein (hsCRP) have been shown to be involved in the pathogenesis of atherosclerosis (Szmitko *et al.* 2003; Ridker *et al.* 2005) and they also predict adverse CV events (Blankenberg *et al.* 2001; Danesh *et al.* 2004).

2.3.1. Soluble intercellular adhesion molecule-1

Structurally, sICAM belongs to the immunoglobulin superfamily. The sICAM promotes adhesion of monocytes to the endothelium and facilitates their migration into subendothelial space (Witkowska and Borawska 2004). Consistent with these data, sICAM is related to endothelial dysfunction in apparently healthy subjects (Kals *et al.* 2008). Moreover, sICAM is an established marker of CV disease (Witowska and Borawska 2004). Previous studies have shown that circulating sICAM levels are elevated in patients with hypertension (Rohde *et al.* 1999), unstable angina pectoris (Ghaisas *et al.* 1997), carotid artery atherosclerosis (Gross *et al.* 2012) and diabetes mellitus (Heilman *et al.* 2009b). It has been demonstrated that sICAM predicts development of PAD in middle-aged men (Pradhan *et al.* 2002) and correlates with treadmill walking distance in patients with intermittent claudication (Nylaende *et al.* 2006).

2.3.2. Interleukin-6 and C-reactive protein

Interleukin-6 (IL-6) is a multifunctional pro-inflammatory cytokine with a variety of biological activities, including the ability to stimulate differentiation of B- and T-cells as well as activate macrophages (Kanda and Takahashi 2004). Elevated IL-6 levels have been associated with increased CV risk among apparently healthy men (Ridker *et al.* 2000). Moreover, IL-6 is an important stimulus to the production of acute phase proteins, such as CRP (Yudkin *et al.* 2000). Elevated CRP has been widely accepted as a potent indicator of CV risk. It has been shown that elevated CRP has a clear prognostic value for major CV events and mortality (Ridker *et al.* 1997), whereas lowering of CRP is associated with a reduction of CV risk (Ridker *et al.* 2009). Besides its role as a „bystander", CRP has been shown to exert a wide array of pro-atherogenic effects, including impairment of endothelial function (Venugopal *et al.* 2002), stimulation of plaque remodelling (Montero *et al.* 2006) and activation of coagulation (Cermak *et al.* 1993).

2.3.3. β_2-microglobulin

The β_2M is a nonglycosylated protein that is synthesized by all nucleated cells and it forms a light chain subunit of the major histocompatibility complex (MHC) class I antigen (Saijo *et al.* 2005). Because it is noncovalently asso-

ciated with the α-chain of MHC class I molecules and has no direct attachment to cell membrane, β_2M can exchange with free soluble β_2M on cell surface (Shi *et al.* 2009). Serum β2M level is associated with carotid artery intima-media thickness (IMT) (Zumrutdal *et al.* 2005) and independently predicts total mortality in a general population of older adults (Shinkai *et al.* 2008). As the surfaces of lymphocytes and monocytes are rich in β_2M and as it is associated with autoimmune and infectious diseases (Shi *et al.* 2009), β_2M might be implicated in the regulation of inflammatory response (Xie and Yi 2003).

2.3.4. Inflammation and arterial stiffness

The association between inflammation and arterial stiffness is well recognized. It has been shown that aPWV (Yasmin *et al.* 2004) and AIx (Kampus *et al.* 2004) are positively associated with CRP in apparently healthy individuals and in patients with acute ischaemic stroke (Tuttolomondo *et al.* 2010). Similarly, aPWV is related to serum IL-6 in general population (Schnabel *et al.* 2008). In addition, increased baPWV is related to the combination of high β_2M and high CRP in clinically healthy subjects (Saijo *et al.* 2005). However, there are limited data exist on associations between inflammatory markers and aortic stiffness in patients with PAD. In the present thesis, we investigated the possible relationship of aPWV with CRP (Paper I), β_2M (Paper II), IL-6 (Paper III) and sICAM (Paper III) in PAD patients and in apparently healthy subjects.

2.4. Oxidative stress

A net of pro-oxidants and the antioxidant defence system are normally balanced in the body (Zilmer *et al.* 2010). Principal pro-oxidants are reactive species (including free radicals). Reactive species, which are divided into reactive oxygen species (ROS) and reactive nitrogen species, mediate the main effects of other pro-oxidative factors (Sies 1991; Halliwell 1999). In the human body, the most important ROS include superoxide radical, hydroxyl radical, lipid peroxyl radical and non-radical hydrogen peroxide (the latter is produced from superoxide by superoxide dismutase). The principal reactive nitrogen species are NO radical and non-radical peroxynitrite (Zilmer *et al.* 2010). Most of the mentioned reactive species originate from endogenous sources as by-products of normal essential metabolic processes, while exogenous sources involve exposure to cigarette smoke, environmental pollutants, radiation, drugs, bacterial infections, excess of food iron and dysbalanced intestinal microflora (Zilmer *et al.* 2010). Thus, abnormal formation of reactive species can occur *in vivo* and it leads to the damage of lipids, proteins, nucleic acids and carbohydrates in cells and tissues. Excessive production of reactive species causes an imbalance in the system of pro- and antioxidants. Any imbalance in favour of pro-oxidants can potentially lead to damage, which is termed as OxS (Tsutsui *et al.* 2011).

Recently, an additional adapted concept of OxS has been advanced. According to the novel concept, OxS is defined as „a disruption of redox signalling and control" (Jones and Go 2010), emphasizing the impact of the redox ratio as a good tool for quantification of OxS. The physiological grade of OxS is needed for numerous biofunctions, such as intracellular messaging, growth, cellular differentiation, phagocytosis and immune response (Elahi *et al.* 2009). However, prolonged high-grade OxS is one of the key players in the pathogenesis of CV diseases (Drummond *et al.* 2011).

2.4.1. Oxidized low-density lipoprotein

Oxidative modification of LDL is a key step in initiation and progression of atherosclerosis (Stocker and Keaney 2004; Huang *et al.* 2011). Previous *in vitro* studies have shown that macrophages (Parthasarathy *et al.* 1986) and lymphocytes (Lamb *et al.* 1992) are capable of oxidizing LDL. The oxLDL leads to endothelial dysfunction, which is the initial step in the formation of an atheroma (Ahmadi *et al.* 2010; Zilmer *et al.* 2010). In addition, oxLDL promotes the growth and migration of VSMC, monocytes and fibroblasts (Holvoet and Collen 1994). Moreover, previous studies have suggested that oxLDL may also play a role in triggering thrombosis by inducing platelet adhesion and by decreasing the fibrinolytic capacities of endothelial cells (Holvoet and Collen 1994; Matsuura *et al.* 2003).

Recent research suggests that increased oxLDL levels might have a destabilizing effect on plaque composition by enhancing the inflammatory processes and surface thrombosis (Ehara *et al.* 2001). It has been demonstrated that the surface area containing oxLDL-positive macrophages is significantly higher in patients with unstable angina than in those with stable angina (Ehara *et al.* 2001). Furthermore, oxLDL levels are independently related to IMT (Kampus *et al.* 2007) and plaque size (Andersson *et al.* 2009) in the carotid artery. In addition, oxLDL levels are significantly associated with presence and extent of carotid atherosclerosis (Tsimikas *et al.* 2006) and predict CV events in clinically healthy subjects (Lobbes *et al.* 2006).

Previous studies suggest that oxLDL could contribute to VCa. It has been shown that oxLDL stimulates VCa (Tang *et al.* 2006), whereas inhibition of oxLDL production attenuates VCa (Tang *et al.* 2007) in rats. The results of *in vitro* studies indicate that oxLDL decreases the gene expression of OPG (Liu *et al.* 2011) and increases the activity of alkaline phosphatase (Lomashvili *et al.* 2004). However, no studies have assessed possible association between the serum levels of OPG and oxLDL in patients with atherosclerosis. In Paper I, we investigated the potential relationship between OPG and oxLDL levels in patients with PAD and in healthy subjects.

2.4.2. Oxidative stress and arterial stiffness

The oxLDL may be implicated in arterial stiffening. Recent research indicates that serum oxLDL is associated with baPWV in newly-diagnosed diabetes patients (Ha *et al.* 2011). Furthermore, the results of a large cross-sectional study show that oxLDL levels are independently associated with aPWV in community-dwelling older adults (Brinkley *et al.* 2009). However, only limited data are available regarding the relationship between serum oxLDL and aortic stiffness in subjects with clinically evident PAD. In Paper IV, we investigated the possible association between aPWV and serum oxLDL in PAD patients and in apparently healthy subjects.

2.5. Patients with peripheral arterial disease

Lower-extremity PAD is a manifestation of systemic atherosclerosis, which predominantly affects the distal aorta and the lower-extremity arteries (Hirsch *et al.* 2006). The PAD affects about 5% of the population aged over 55 years, in the Western world (Fowkes *et al.* 1991). The prevalence of PAD is age-dependent, reaching 10% in people aged over 60 years (Criqui 2001) and 20% in those aged over 75 (Selvin and Erlinger 2004).

2.5.1. Assessment of severity of peripheral arterial disease

The main symptoms of PAD include intermittent claudication, ischaemic rest pain, ulceration and gangrene (Beard 2000). However, PAD is often under-diagnosed, which may be partly due to the fact that only 10–30% of all PAD patients present with classic clinical symptoms (Hirsch *et al.* 2007). Coexisting musculoskeletal disease or neuropathy may confound the clinical picture, and almost half of patients are so sedentary as to be asymptomatic (McDermott *et al.* 2004). Nonetheless, PAD patients are still at a significant risk of developing serious CV complications, such as myocardial infarction and stroke (Criqui *et al.* 1997; Selvin and Erlinger 2004). Therefore, early diagnosis of PAD would trigger intensive risk factor modification and therapy.

Several tests and imaging techniques have been designed for the detection of PAD in clinical practice. The ABPI is a noninvasive test of choice when evaluating a patient for PAD (Hirsch *et al.* 2006). Previous research demonstrates that with an ABPI diagnostic threshold of 0.9, the sensitivity of the ABPI was 95% and the specificity was 100% for detection of haemodynamically significant stenoses (Fowkes 1988). Duplex sonography is a noninvasive test to determine the location of PAD and to delineate stenotic versus occlusive lesions (Moneta *et al.* 1992). For detection of haemodynamically significant stenoses, the sensitivity of the duplex sonography was 88% and the specificity was 96% (Collins *et al.* 2007). Digital subtraction angiography (DSA) is a well-estab-

lished technique for evaluating severity, location and extent of PAD (Norgren *et al.* 2007) and allows endovascular treatment of haemodynamically significant arterial stenoses or occlusions. However, in many centres, magnetic resonance angiography (MRA) and computed tomography angiography (CTA) are widely used for the initial diagnostic evaluation and treatment planning of patients with PAD. For detection of haemodynamically significant stenoses in the lower-extremity arteries, MRA had the sensitivity and the specificity of 95% and 97%, respectively, whereas both the sensitivity and the specificity of CTA were 91% (Collins *et al.* 2007).

2.5.1.1. Ankle-brachial pressure index

Measurement of ABPI is widely used for screening and estimation of progression rate of PAD. The ABPI represents a ratio of ankle systolic BP to brachial systolic BP. The ABPI less than 0.9 is considered suggestive of PAD (Hirsch *et al.* 2006). Decreased ABPI is related to presence of CV risk factors, subclinical CV disease or development of CV events (Newman *et al.* 1993; Leng *et al.* 1996; Heald *et al.* 2006). Recent meta-analysis has shown that subjects with low ABPI but without prior history of CAD have increased all-cause mortality and a trend for increased CV mortality (Fowkes *et al.* 2008). However, ABPI greater than 1.4 might indicate poor compressibility of the lower-extremity arteries (usually in patients with diabetes) and is related to presence of medial artery calcification (Kennedy *et al.* 2005). Accordingly, ABPI greater than 1.4 is associated with a higher risk of CV mortality as compared to normal ABPI (Resnick *et al.* 2004).

The relationship between ABPI and arterial stiffness remains controversial. In non-diabetic PAD patients, baPWV and brachial-knee pulse wave velocity (bkPWV) are positively associated with ABPI (Khandapour *et al.* 2009). In contrast, stiffness of the carotid artery is inversely related to ABPI in elderly subjects (Lind 2011). Moreover, recent research indicates that ABPI is not associated with aPWV in the clinically healthy subjects (Rabkin *et al.* 2012). However, there are no data about the association between ABPI and aPWV in the patients with PAD. In Paper IV of the present thesis, we assessed the possible relationship between aPWV and ABPI in the PAD patients and in apparently healthy subjects.

2.5.1.2. Angiographic score

The semiquantitative method for assessment of lower-extremity atherosclerosis from routine angiographic images was introduced by Bollinger in 1981 (Bollinger *et al.* 1981). The Bollinger scoring system assigns different scores to plaques, stenoses and occlusions. The scores for each of the arterial segments are then added up to obtain AngSc for each patient. Thus, evaluation of AngSc provides information about the site and severity of atherosclerotic lesions in

patients with PAD (Nylaende *et al.* 2006). Several studies have demonstrated that AngSc is inversely related to ABPI (Delius and Erikson 1969; Kiekara and Riekkinen 1985; Muller-Buhl *et al.* 1999) and it is an independent risk factor for major amputation in diabetic subjects (Faglia *et al.* 1998). Moreover, AngSc is negatively correlated with maximum treadmill walking distance (Nylaende *et al.* 2006) and positively correlated with duration of claudication (Muller-Buhl *et al.* 1999). However, there are no data regarding the possible association between aortic stiffness and severity grade of PAD as assessed by DSA. In Paper IV we investigated the relationship between aPWV and AngSc in patients with symptomatic PAD.

3. AIMS OF THE STUDY

Overall aim

To measure aortic stiffness (functional profiling), aortic calcification (structural profiling), angiographic score (structural profiling), and to assess levels of biomarkers of calcification, inflammation and oxidative stress (biochemical profiling) in patients with peripheral arterial disease and in clinically healthy subjects. To evaluate the relationships between functional, biochemical and structural profiles of the arterial damage and to estimate their potential roles in determining arterial dysfunction in atherosclerosis.

Specific aims

1. To evaluate aortic pulse wave velocity (functional profiling) and serum levels of osteoprotegerin and oxidized low-density lipoprotein (biochemical profiling) and to assess their relationship in patients with symptomatic peripheral arterial disease in comparison with clinically healthy individuals.
2. To measure aortic pulse wave velocity (functional profiling), plasma β_2-microglobulin and fetuin-A levels (biochemical profiling) in patients with symptomatic peripheral arterial disease and in clinically healthy subjects and to test if elevated plasma β_2-microglobulin is associated with increased aortic stiffness and plasma fetuin-A levels.
3. To assess aortic calcification score (structural profiling), aortic pulse wave velocity, ankle-brachial pressure index (functional profiling) and serum vitamin D (biochemical profiling) and to evaluate the relationship between these parameters in patients with symptomatic peripheral arterial disease as well as in clinically healthy subjects.
4. To measure aortic pulse wave velocity (functional profiling), serum osteopontin and oxidized low-density lipoprotein (biochemical profiling) and to examine the relationship between the functional and biochemical profiles in patients with symptomatic peripheral arterial disease and in clinically healthy individuals.
5. To investigate the relationship of aortic pulse wave velocity (functional profiling) with angiographic score (structural profiling) in patients with atherosclerosis.

4. SUBJECTS AND METHODS

4.1. Study subjects

4.1.1. Clinically healthy subjects

The total number of the participants in the control group was 84. The control group consisted of 68 subjects in Paper I, 66 subjects in Paper II, 74 subjects in Paper III and 84 subjects in Paper IV (Table 1). All participants were men. Clinically healthy subjects were recruited by a family physician and by a specialist of sports medicine. The exclusion criteria for the control group were the following (based on clinical examination, ECG and blood tests): CAD, cardiac arrhythmias or valve pathologies, cerebral atherosclerotic disease, PAD, diabetes mellitus (fasting serum glucose level >7 mmol/L), malignancies, renal failure (estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2) and known inflammatory conditions. The control subjects did not use any medications on a regular basis.

4.1.2. Patients with peripheral arterial disease

The study population comprised 79 men with symptomatic PAD. The patients' group consisted of 69 subjects in Paper I, 66 subjects in Paper II, 78 subjects in Paper III and 79 subjects in Paper IV (Table 1). The patients were recruited from the Department of Vascular Surgery, Tartu University Hospital, Estonia. The diagnosis of PAD required (1) clinical symptoms of PAD, (2) ABPI <0.9 and (3) significant stenoses or occlusions of arteries confirmed by angiography (DSA or CT). The patients had stages II–IV of chronic ischaemia as defined by Fontaine: stage II = intermittent claudication (n=48, n=48, n=53 and n=54 in Papers I–IV, respectively); stage III = leg pain at rest (n=14, n=13, n=17 and n=17 in Papers I–IV, respectively); stage IV = focal tissue necrosis or gangrene (n=7, n=5, n=8 and n=8 in Papers I–IV, respectively). Patients were excluded in case they had had myocardial infarction, coronary revascularization or cerebrovascular events during the previous 6 months, earlier revascularization procedures at the lower limb, upper limb occlusive arterial disease, atrial fibrillation, valve pathologies, known inflammatory conditions, diabetes mellitus, malignancies and renal failure (eGFR <60 ml/min/1.73 m^2). Overall, 21 (30.4%) patients with hypertension and 7 (10.1%) patients with CAD were included in Paper I; 21 (31.8 %) patients with hypertension and 7 (10.6%) patients with CAD were included in Paper II; 30 (38.5%) patients with hypertension and 12 (15.4%) patients with CAD were included in Paper III; 31 (39%) patients with hypertension and 12 (15%) patients with CAD were included in Paper IV.

4.2. Study design

The subjects were studied in the morning between 8 and 10 AM. They had abstained from smoking and intake of caffeine-containing food and beverages for the previous 12 hours. Studies were conducted in a quiet temperature-controlled room. First, height and weight were assessed and, next, body mass index (BMI) was calculated. After the subjects had spent 15 minutes resting in the supine position, brachial BP and radial artery waveforms were recorded. Further, aPWV, bPWV, augmentation index corrected for a heart rate of 75 beats per minute (AIx@75) and ABPI were measured (Table 1). All measurements were made in duplicate at each time point and the average of the two measurements was used in the analysis. Twenty millilitres of blood were drawn from the antecubital fossa into plain tubes. The subjects then underwent CT (Paper III). The next day, the patients underwent routine DSA of the aorta and the arteries of the lower extremities (Paper IV).

The study complies with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University of Tartu. All subjects gave their written informed consent.

Table 1. Study participants and vascular parameters.

	Study participants				**Vascular parameters**					
Paper	Patients		Controls		aPWV	bPWV	AIx@75	ABPI	ACS	AngSc
	Number	Age	Number	Age						
I	69	63±7	68	54±8	+	+	+	+	–	–
II	66	63±7	66	55±6	+	+	+	+	–	–
III	78	63±7	74	61±10	+	+	+	+	+	–
IV	79	64±7	84	63±8	+	+	+	+	–	+

4.3. Methods

4.3.1. Measurement of biomarkers

Plasma glucose as well as serum LDL, high-density lipoprotein (HDL), triglycerides and creatinine were measured according to standardized protocols in the local clinical laboratory. Serum lipid levels were measured by the enzymatic colorimetric method on the Hitachi 912 analyser (Roche Diagnostics®, Basel, Switzerland). Plasma glucose was assessed by the hexose kinase method on a Hitachi 912 analyser (Roche Diagnostics®, Basel, Switzerland). Kinetic colorimetric method was used for determination of serum creatinine. Measurements were made using the Hitachi 912 analyser (Roche Diagnostics®, Basel, Switzerland). Calculation of eGFR was performed using the Modification of Diet in Renal Disease formula, equation MDRD 1 (Brosius *et al.* 2006).

4.3.1.1. Biomarkers of calcification

Biomarkers of calcification (OPG, OPN, fetuin-A and 25(OH)D) were measured from serum, which was collected and stored at –70ºC until analysis.

4.3.1.1.1. Serum level of osteoprotegerin

Serum OPG was measured by an enzyme-linked immunosorbent assay using a commercially available kit (Human Osteoprotegerin ELISA; Biovendor, Heidelberg, Germany) (Paper I). Samples were incubated in microplate wells pre-coated with monoclonal anti-human OPG antibody. After incubation and washing, biotin labelled polyclonal anti-human OPG antibody was added and incubated with captured OPG. After another washing, Streptavidin-horseradish peroxidase conjugate was added. After incubation and washing, a substrate solution was added. The reaction was stopped by addition of an acidic solution and the absorbance of the resulting yellow product was proportional to the concentration of OPG. The intra- and inter-assay coefficients of variation for OPG were 3.5% and 5.8%, respectively.

4.3.1.1.2. Serum level of osteopontin

Serum OPN levels were measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota, USA) (Paper IV). A monoclonal antibody specific for OPN was pre-coated onto a microplate. Then the standards and the samples were pipetted into the wells and any OPN present was bound by the immobilized antibody. After incubation and washing, an enzyme-linked polyclonal antibody specific for OPN was added to the wells. After another washing, a substrate solution was added to the wells and colour developed in proportion to the amount of OPG bound in the initial step. Colour development was stopped and the intensity of the colour was measured. The intra- and inter-assay coefficients of variation for OPN were 4.0% and 6.6%, respectively.

4.3.1.1.3. Serum level of fetuin-A

Fetuin-A was measured by an enzyme-linked immunoabsorbent assay using a commercially available kit (BioVendor Laboratory Medicine, Inc.® Brno, Czech Republic) (Paper II). Samples were incubated in microplate wells pre-coated with polyclonal antibody specific for fetuin-A. After incubation and washing, polyclonal anti-human fetuin-A antibody, conjugated with horseradish peroxidase, was added to the wells and incubated with captured fetuin-A. The reaction was stopped by addition of an acidic solution and the absorbance of the resulting yellow product was measured. The absorbance was proportional to the

concentration of fetuin-A. The intra- and inter-assay coefficients of variation for fetuin-A were 3.9% and 5.4%, respectively.

4.3.1.1.4. Serum level of 25-hydroxyvitamin D

Serum 25(OH)D level was measured using a radioimmune assay (25-Hydroxyvitamin D, ^{125}I Ria Kit, DiaSorin Corporation, Minnesota, USA) (Paper III). The assay consisted of two steps. The first step involved an extraction of 25(OH)D from serum with acetonitrile. The extraction was followed by competitive radioimmune assay using ^{125}I-labelled 25(OH)D and antibody to 25(OH)D. The sample, antibody and tracer were incubated for 90 minutes. Phase separation was accomplished after 20-minute incubation with a second antibody precipitating complex. Then a buffer was added to reduce non-specific binding. This was followed by centrifugation. Intra- and inter-assay coefficients of variation were 8.1% and 10.2%, respectively.

4.3.1.2. Biomarkers of inflammation

The biomarkers of inflammation (sICAM, IL-6, hsCRP and β_2M) were determined from serum or plasma, which was collected and kept frozen at –70ºC until analysis.

4.3.1.2.1. Plasma level of soluble intercellular adhesion molecule-1

The plasma level of sICAM was measured by an enzyme-linked immunosorbent assay using a commercially available kit (Human soluble intercellular adhesion molecule-1 Immunoassay; R&D Systems; Minneapolis, USA) (Papers I and III). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody, specific for sICAM, was pre-coated onto a microplate. Standards, samples, controls and the conjugate were pipetted into wells and any sICAM present was sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sICAM. Following a wash to remove the unbound substances or the antibody-enzyme reagent, a substrate solution was added and colour developed in proportion to the amount of bound sICAM. Colour development was stopped and the intensity of colour was 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 coefficients of variation for sICAM were 4.8% and 7.4%, respectively.

4.3.1.2.2. Serum level of interleukin-6

Serum levels of IL-6 were determined using a chemiluminescent immunoassay (Immulite; Diagnostic Products Corporation, Los Angeles, USA) (Paper III). This assay employed the quantitative sandwich enzyme immunoassay technique.

A monoclonal antibody specific for IL-6 was pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution was added to the wells and light was produced in proportion to the amount of IL-6 bound in the initial step. A microplate luminometer was used to measure the intensity of emitted light. The minimum detectable value of the IL-6 assay was 2 pg/mL.

4.3.1.2.3. Plasma level of C-reactive protein

The plasma level of CRP was measured by a validated latex particle-enchanced high-sensitivity immunoturbidimetric assay (CRP (Latex) HS, Roche Diagostics Gmbh®, Mannheim, Germany), and analysed by the Hitachi 912 analyser (Roche Diagostics®, Basel, Switzerland) (Papers I–IV). Latex particles coated with an antibody specific for human CRP aggregated in the presence of CRP from the sample forming immune complexes. The immune complexes caused an increase in light scattering which was proportional to the concentration of CRP in the serum. Light scattering was measured by reading turbidity at 570 nm. The CRP concentration curve was determined from a calibration curve developed from CRP standards of known concentration. The intra- and inter-assay coefficients of variation for CRP were 1.8% and 2.9%, respectively.

4.3.1.2.4. Plasma level of β_2-microglobulin

Plasma β_2M concentration was measured by a chemiluminescent immunoassay using a commercially available kit (L2KBM2, Siemens Medical Solutions Diagnostics®, California, USA) in the IMMULITE 2000 automated analyser (Siemens Medical Solutions Diagnostics®, California, USA) (Paper II). This assay employed the quantitative sandwich enzyme immunoassay technique. The first monoclonal antibody was coated on the surface of the microtiter wells and the second monoclonal antibody was used as the tracer. The β_2M molecules present in the standard solution or serum were bound to both antibodies. Following the formation of the coated antibody-antigen-antibody-enzyme complex, the unbound antibody-enzyme labels were removed by washing. The horseradish peroxidase activity bound in the wells was then assayed by adding the substrate reagents and by producing chemiluminescent reactions. The intensity of the light emitted from the associated well was proportional to the amount of the enzyme present and was directly related to the amount of β_2M in the sample. By reference to a series of β_2M standards assayed in the same way, the concentration of β_2M in the unknown sample was quantified. The intra- and inter-assay coefficients of variation for β_2M were 4.2% and 11%, respectively.

4.3.1.3. Plasma level of oxidized low-density lipoprotein

Serum oxLDL levels were determined by an enzyme-linked immunosorbent assay (Mercodia AB, Uppsala, Sweden) (Papers I and IV). This 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 removed non-reactive plasma components, a peroxidase conjugated anti-apoprotein B antibody recognized oxLDL bound to the solid phase. After a second incubation and a simple washing step that removed the unbound enzyme-labelled antibody, the bound conjugate was detected with 3,3',5,5'-tetramethylbenzidine. The reaction was stopped by adding an acid to obtain a colorimetric endpoint that was read spectrophotometrically at 450 nm by a photometer Sunrise (Tecan Austria GmbH®, Salzburg, Austria). The intra- and inter-assay coefficients of variation for oxLDL were 5.5% and 6.2%, respectively.

4.3.2. Measurement of blood pressure

The BP was measured in the supine position from the left arm with an automated digital oscillometric BP monitor (OMRON M4-I; Omron Healthcare Europe, Hoofdorp, the Netherlands). The MAP was obtained by integration of the radial pressure waveform using the Sphygmocor software (SCOR® Px, 7.0; AtCor Medical, Sydney, Australia). The PPP was calculated as the difference between PSBP and peripheral diastolic blood pressure (PDBP). All measurements of BP were made in duplicate and the mean values were used in subsequent analysis.

4.3.3. Assessment of arterial stiffness

4.3.3.1. Pulse wave velocity

The PWV was measured by the foot-to-foot method, using the Sphygmocor device. The aPWV was determined by sequentially recording ECG-gated carotid and femoral artery waveforms using the SphygmoCor software (DeLoach and Townsend 2008). Wave transit time was calculated as the time delay between the arrival of the pulse wave at the common carotid artery and the common femoral artery using the R wave of a simultaneously recorded ECG as the reference frame. The surface distance between the two recording sites was measured as the distance from the suprasternal notch to the common femoral artery minus the distance from the suprasternal notch to the common carotid artery (DeLoach and Townsend 2008). The within- and between-observer coefficients of variation for aPWV were 2.3% and 6.2%, respectively. The bPWV was measured from the carotid and radial waveforms. The surface

distance between the recording sites was estimated as the distance from the suprasternal notch to the radial artery minus the distance from the suprasternal notch to the carotid artery (Wilkinson *et al.* 1998). The within- and between-observer coefficients of variation for bPWV were 2.9% and 5.5%, respectively. All measurements were made in duplicate by two trained investigators, and mean values were used in subsequent analysis.

4.3.3.2. Pulse wave analysis

Radial artery pressure waveforms were obtained with a high-fidelity applanation tonometer (SPT-301B; Millar Instruments, Houston, Texas, USA) from the wrist of the left hand. After 20 sequential waveforms had been acquired, the integral software (SCOR Px, 7.0; AtCor Medical, Sydney, Australia) was used to generate a corresponding central (ascending aortic) waveform using a generalized transfer function, which has been prospectively validated for assessment of central BP (Sharman *et al.* 2006). The AIx, travel time of the reflected wave (Tr), CSBP, central diastolic blood pressure (CDBP) and CPP were determined by PWA. The AIx was calculated as the difference between the second and the first systolic peaks, divided by CPP and expressed in percentages (Wilkinson *et al.* 1998). The AIx values were adjusted to a heart rate of 75 beats/min using a SphygmoCor built-in algorithm. The within- and between-observer coefficients of variation for AIx@75 were 3.4% and 7.1%, respectively.

4.3.4. Measurement of ankle-brachial pressure index

The ABPI was measured using the Bidirectional Doppler MD 6 (D.E, Hokanson, Bellevue, Washington, USA). Systolic BP was assessed bilaterally over the brachial, tibialis posterior and dorsalis pedis arteries. The higher systolic BP of the dorsalis pedis or the posterior tibial artery was used for calculation of the ABPI (Hirsch *et al.* 2006). Two readings of the ABPI were obtained and the mean was calculated. The lower ABPI of the two legs was included in statistical analysis. The within- and between observer coefficients of variation for ABPI were 4.1% and 8.6%, respectively.

4.3.5. Measurement of calcification score

The CT scans of the aorta were performed in 52 patients with PAD and in 60 clinically healthy subjects. All study participants were informed about the purpose, methods, radiation dose and risks associated with radiation exposure. We could not perform CT scans in all of the subjects due to the lack of consent and considering also the fact that some patients had several CT scans in their medical history. The entire aorta (from the aortic valve to bifurcation) was visualized by obtaining 5 mm thick slices through the thorax, abdomen, and

pelvis using non-contrast helical CT (GE LightSpeed 16, General Electrical Medical Systems, Milwaukee, Wisconsin, USA; total dose: <8 mSv). Analysis was conducted using a Siemens Syngo Multimodality Workplace workstation. Aortic calcification score (ACS) was measured by an independent observer. The degree of calcification was determined by the volume scoring method. The number of voxels of 130 or greater Hounsfield units within the wall of the aorta yielded a calcification score in cubic centimetres. This is a validated and accurate technique that compares favourably with electron beam CT (Hopper *et al.* 2002).

4.3.6. Measurement of angiographic score

All patients were examined with DSA (Axiom Artis, Siemens Medical Solutions, Forchheim, Germany) of the aorta and the arteries of the lower extremities using the standard technique via the femoral approach at the Department of Radiology, Tartu University Hospital, Estonia. In the majority of the PAD patients DSA was followed by percutaneous transluminal angioplasty with stenting or by bypass surgery. In some patients conservative treatment was chosen due to anatomical considerations, concomitant diseases and the high risk of surgery. The following arterial segments were evaluated: abdominal aorta, common iliac artery, external iliac artery, common femoral artery, profunda femoris artery, superficial femoral artery, popliteal artery, tibio-peroneal trunk, anterior tibial artery, posterior tibial artery and peroneal artery. Severity of stenosis was assessed according to the following validated grading system: 0 = normal; 1 = stenosis < 50%; 2 = stenosis > 50%; 3 = occlusion (Nylaende *et al.* 2006). Different scores for each of the 21 segments were then added up to obtain AngSc for each patient, 63 being the maximal score. The AngSc was evaluated independently by an experienced radiologist, who was blinded to the results of haemodynamic and laboratory measurements. The within-observer coefficient of variation for AngSc was 5.4%.

4.3.7. Statistical analysis

The software R (version 2.8.1 for Windows; The R Foundation for Statistical Computing, Vienna, Austria) (Papers I and II) and the software STATISTICA (version 10.0 for Windows; StatSoft, Tulsa, Oklahoma, USA) (Papers III and IV) were used for all statistical analyses. Continuous variables are shown as a mean ± standard deviation or medians with interquartile ranges. Categorical variables are presented in percentages. The Kolmogorov-Smirnov test was performed to prove variables for a normal distribution (Papers I–IV). Skewed data were log-transformed to obtain a normal distribution and then analysed. As there was a significant difference in BP between the study groups, aPWV and bPWV were adjusted for MAP before analysis (Papers III and IV). Correlations

between continuous variables were quantified using Pearson's correlation coefficient. Multiple regression analysis was performed to investigate the independent determinants of aPWV (Papers I, II and IV), OPG (Paper I), β_2M (Paper II) and ACS (Paper III). Comparisons between the patients and the controls were performed using the unpaired t-tests for parametric data and the Mann-Whitney U-tests for non-parametrically distributed data. Comparison between multiple unpaired groups was performed using the one-way analysis of variance (ANOVA) (Paper III). P values of < 0.05 were considered statistically significant.

5. RESULTS

5.1. Association between arterial calcification and stiffness in patients with symptomatic peripheral arterial disease and in healthy subjects (Papers I, III and IV)

Association between osteoprotegerin and aortic stiffness (Paper I)

Characteristics of the study population

The baseline characteristics of 69 patients and 68 healthy subjects are presented in Table 2. The groups did not differ with respect to HDL, eGFR or brachial PWV. Our data revealed that OPG level and aPWV were different for the patients and for the healthy individuals. There occurred a significant difference in median ABPI and hsCRP between the groups. Age, height, BMI, the values of peripheral and central BP, heart rate, LDL, triglycerides, oxLDL, sICAM, Tr, AIx, AIx@75, prevalence of smoking and use of medications were also different for the patients and for the healthy subjects (Table 2).

Relationship between arterial stiffness and osteoprotegerin

Univariate analysis revealed that OPG level correlated significantly with aPWV for the patients with PAD ($r=0.37$, $p=0.003$) and for the healthy individuals ($r=0.40$, $p=0.001$) (Figures 4A and 4B, respectively). In multiple regression analysis, independent association between aPWV and serum OPG was found for the patients and for the healthy subjects (Table 3). In a multiple regression model containing only statistically significant variables, OPG, MAP and ABPI explained 38% of the variance in aPWV ($p<0.0001$) for the PAD patients. There was no correlation between serum OPG and bPWV in neither of the groups (data not shown).

The OPG level was positively correlated with AIx@75 ($r=0.25$, $p=0.04$) and negatively with Tr ($r=–0.34$, $p=0.005$) for the healthy subjects, but not for the patients (data not shown). There did not exist any independent association between AIx@75 and OPG level either for the patients or for the healthy subjects (data not shown).

Table 2. Baseline characteristics of the study subjects (mean ± standard deviation or prevalence (%)).

Variable	PAD patients (n=69)	Controls (n=68)	P value
Age (years)	63.2±7.1	53.9±7.5	<0.01
BMI (kg/m^2)	25.5±3.9	26.9±3.4	0.03
Height (m)	174.4±6.1	179.2±7.4	<0.01
PSBP (mmHg)	148±20	128±15	<0.01
PDBP (mmHg)	82±10	77±9	<0.01
CSBP (mmHg)	136±19	118±16	<0.01
CDBP (mmHg)	83±11	79±10	0.02
MAP (mmHg)	104±14	95±12	0.3
Heart rate (bpm)	67.2±12.1	58.2±10.2	<0.01
ABPI[a]	0.5 (0.2–0.6)	1.1 (1.1–1.2)	<0.01
Total cholesterol (mmol/L)	5.9±1.3	5.2±1.0	<0.01
HDL (mmol/L)	1.3±0.4	1.3±0.3	0.7
LDL (mmol/L)	4.1±1.1	3.6±0.9	<0.01
Triglycerides (mmol/L)	1.7±0.7	1.1±0.8	<0.01
Glucose (mmol/L)	5.6±1.0	5.4±0.5	0.2
eGFR (ml/min/1.73 m^2)	101.8±26.1	98.1±18.0	0.33
hsCRP (mg/L)[a]	4.0 (1.4–7.9)	0.9 (0.5–1.4)	<0.01
sICAM (ng/ml)	278.6±73.1	183.5±42.4	<0.01
OPG (pmol/L)	5.4±1.7	4.4±1.1	<0.01
oxLDL (U/L)	72.5±27.0	56.0±22.4	<0.01
AIx (%)	33.1±14.6	21.7±10.7	<0.01
AIx@75 (%)	28.1±8.1	13.6±11.1	<0.01
aPWV (m/s)	10.1±2.5	7.6±1.6	<0.01
bPWV (m/s)	8.9±1.5	8.9±1.2	0.70
Current smoking, n (%)	69 (100)	2 (2.9)	<0.01
Medication, n (%)			
Calcium channel blockers	7 (10.1)	0 (0)	<0.01
ACE inhibitors	7 (10.1)	0 (0)	<0.01
Angiotensin receptor blockers	3 (4.3)	0 (0)	0.03
Beta-blockers	2 (2.9)	0 (0)	0.16
Diuretics	1 (1.4)	0 (0)	0.25
Pentoxyfylline	21 (30.0)	0 (0)	<0.01
Aspirin	10 (14.3)	0 (0)	<0.01
Statins	2 (2.9)	0 (0)	0.16

[a] indicates medians and interquartile ranges.

Table 3. Multiple regression model for the patients and for the control subjects with aPWV as the dependent variable.

	Regression coefficient	Standard error	P value
Patients[a]			
OPG (pmol/L)	0.61	0.20	0.005
MAP (mmHg)	0.06	0.02	0.02
ABPI	–2.10	0.98	0.04
eGFR (ml/min/1.73m^2)	–0.02	0.01	0.10
Age (years)	0.05	0.05	0.24
Antihypertensive treatment	0.72	0.62	0.25
Controls[b]			
Age (years)	0.10	0.03	<0.001
MAP (mmHg)	0.05	0.01	0.001
OPG (pmol/L)	0.33	0.15	0.04

[a] R^2 value=0.47, p<0.0001, n=69; [b] R^2 value=0.44, p<0.0001, n=68.

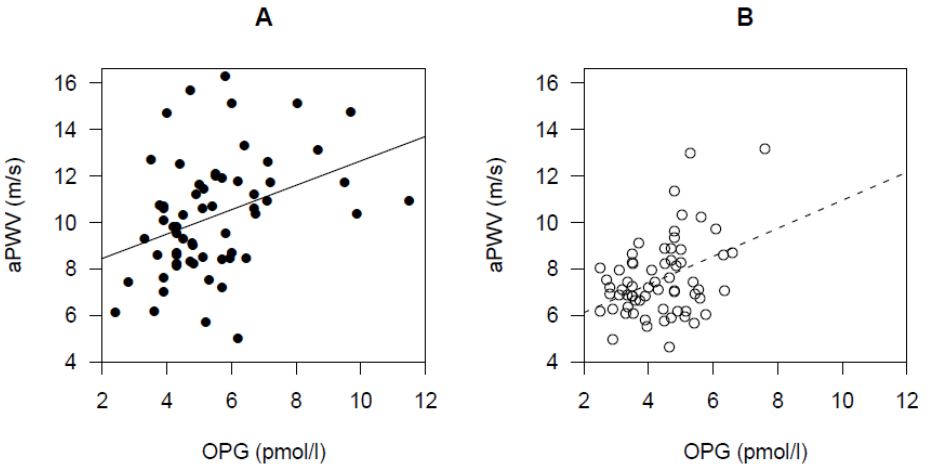

Figure 4. Correlation between aPWV and OPG for the PAD patients (Figure 4A, r=0.37, p=0.003) and for the healthy individuals (Figure 4B, r=0.40, p=0.001).

Association between aortic calcification, vitamin D and aortic stiffness (Paper III)

Participant characteristics

The characteristics of 78 patients with PAD and of 74 healthy subjects are summarized in Table 4. The patients with PAD had higher ACS, aPWV, PSBP, PDBP, PPP, CSBP and CPP compared to the controls. In contrast, 25(OH)D levels were significantly higher among the healthy subjects. Biomarkers of inflammation, such as hsCRP, sICAM and IL-6, were higher in the patient group.

Relationship between aortic calcification, arterial stiffness and vitamin D

The log-ACS was significantly correlated with aPWV for the PAD patients ($r=0.28$, $p=0.03$) and in the controls ($r=0.57$, $p<0.001$) (Figure 5). The log-ACS showed positive correlation with 25(OH)D levels for the PAD patients ($r=0.33$, $p=0.01$) and negative correlation for the control subjects ($r=–0.47$, $p<0.001$) (Figure 6). The log-ACS was positively correlated with log-hsCRP ($r=0.29$, $p=0.03$) and log-IL-6 ($r=0.28$, $p=0.03$) only for the PAD patients. Multivariate analysis revealed that log-ACS was independently associated with 25(OH)D, age, aPWV, calcium and eGFR for the PAD patients and with 25(OH)D, aPWV, cholesterol/HDL ratio and age for the clinically healthy subjects (Table 5). In a multiple regression model containing only statistically significant variables, 25(OH)D, age, aPWV, calcium and eGFR explained 47% of the variance in ACS ($p<0.001$) in the PAD patients. Adjustment for seasonal variation of vitamin D, antihypertensive and vasodilator treatment, systolic, diastolic and pulse pressure, glucose, LDL, triglycerides, height and weight did not alter the associations (data not shown). The log-ACS was significantly correlated with AIx@75 ($r=0.48$, $p<0.001$) only in the control subjects. However, this correlation was not significant after adjustment for confounders.

Vitamin D correlated significantly with AIx@75 for the controls ($r=–0.26$, $p=0.03$). There was a trend for emergence of correlation between 25(OH)D level and aPWV ($r=–0.23$, $p=0.057$) only for the control subjects.

Table 4. Baseline characteristics of the study subjects (mean ± standard deviation or prevalence (%)).

Parameter	PAD patients (n=78)	Controls (n=74)	P value
Age (years)	63±7	61±10	0.1
BMI (kg/m^2)	25.4±3.8	27.0±3.5	<0.01
ABPI	0.43±0.3	1.2±0.1	<0.01
ACS (cm^3)[a]	4.9 (2.3–8.9)	0.2 (0.03–1.6)	<0.01
Heart rate (beats/min)	66.6±12.1	58.9±9.2	<0.01
MAP (mmHg)	103.9±13.8	97.0±11.2	<0.01
PSBP (mmHg)	148.4±20.8	131.9±15.2	<0.01
PDBP (mmHg)	81.3±10.4	77.9±8.6	0.03
PPP (mmHg)	66.1±14.6	54.1±10.8	<0.01
CSBP (mmHg)	135.7±19.2	122.3±16.3	<0.01
CDBP (mmHg)	82.3±10.9	79.3±9.3	0.07
CPP (mmHg)	53.4±12.9	43.0±11.2	<0.01
aPWV (m/s)[b]	9.8±2.4	8.2±1.6	<0.01
bPWV (m/s)[b]	8.7±1.3	8.8±1.2	0.73
AIx@75 (%)	28.2±8.0	17.1±9.9	<0.01
Glucose (mmol/L)	5.6±1.0	5.4±0.5	0.13
Total cholesterol (mmol/L)	5.9±1.2	5.3±1.1	<0.01
LDL (mmol/L)	4.2±1.1	3.7±1.1	<0.01
HDL (mmol/L)	1.3±0.4	1.3±0.3	0.49
Triglycerides (mmol/L)	1.7±0.7	1.1±0.7	<0.01
eGFR (mL/min/1.73m^2)	99.7±25.8	95.1±20.7	0.2
hsCRP (mg/L)[a]	4.1 (1.4–8.5)	0.9 (0.5–1.6)	<0.01
Calcium (mmol/L)	2.4±0.2	2.2±0.2	<0.01
25(OH)D (ng/mL)	15.1±5.4	19.0±5.9	<0.01
sICAM (ng/mL)	265.5±65.5	174.9±36.3	<0.01
IL-6 (ng/L)[a]	4.0 (2.1–7.0)	2.0 (1.9–3.0)	<0.01
Current smoking, n (%)	78 (100)	18 (24)	<0.01
Framingham risk score (%)[c]	ND	11.4±7.3	ND
Medication, n (%)			
Calcium channel blockers	14 (17.9%)	0 (0)	<0.01
ACE inhibitors	15 (19.2%)	0 (0)	<0.01
Angiotensin receptor blockers	7 (9%)	0 (0)	<0.01
Beta-blockers	5 (6.4%)	0 (0)	<0.01
Diuretics	3 (3.8%)	0 (0)	0.03
Pentoxyfylline	31 (39.7%)	0 (0)	<0.01
Aspirin	20 (25.6%)	0 (0)	<0.01
Statins	9 (11.5%)	0 (0)	<0.01

[a] indicates medians and interquartile ranges; [b] aPWV and bPWV have been adjusted for MAP; [c] risk was not assessed for the PAD patients since they had established vascular disease, which indicates high CV risk.

Table 5. Multiple regression analysis for the PAD patients and for the control subjects with the logarithm of ACS as the dependent variable.

	Regression coefficient	Standard error	P value
Patients[a]			
25(OH)D (ng/mL)	0.04	0.01	0.008
Age (years)	0.04	0.01	0.01
aPWV (m/s)[c]	0.09	0.04	0.02
Calcium (mmol/L)	1.23	0.53	0.02
eGFR (mL/min/1.73m^2)	0.008	0.003	0.03
sICAM (ng/mL)	0.002	0.001	0.13
Controls[b]			
25(OH)D (ng/mL)	–0.05	0.02	0.003
aPWV (m/s)[c]	0.19	0.07	0.01
Cholesterol/HDL	0.15	0.07	0.03
Age (years)	0.02	0.01	0.03

[a] R^2 value=0.49, p<0.001, n=46; [b] R^2 value=0.55, p<0.001, n=55; [c] aPWV has been adjusted for MAP.

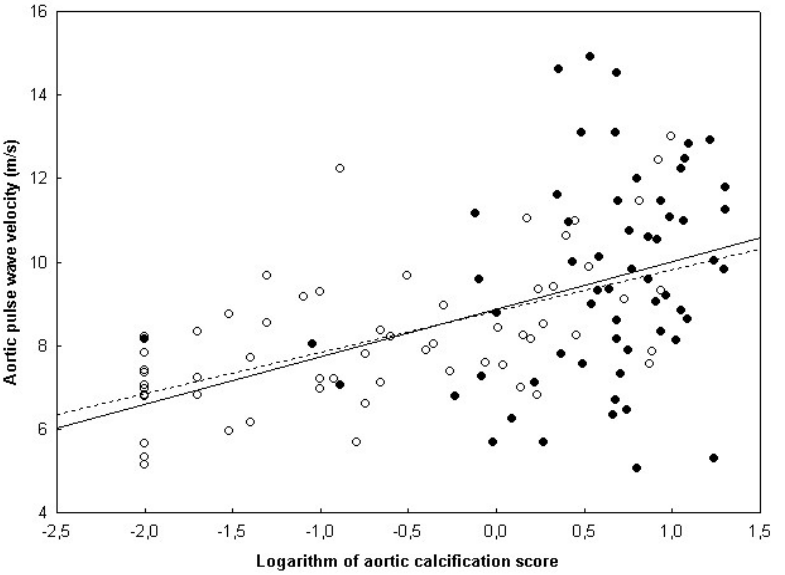

Figure 5. Correlation between aPWV and log-ACS for the patients (r=0.28, p=0.03) and for the control subjects (r=0.57, p<0.001). The aPWV has been adjusted for MAP. Filled dots represent patients; empty dots represent controls. Continuous line represents the regression line through the patient data; interrupted line represents the regression line through the control data.

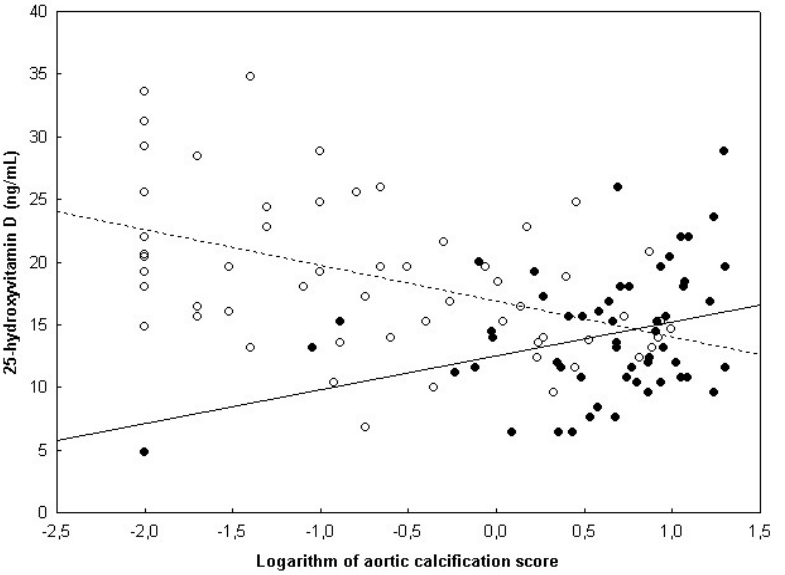

Figure 6. Correlation between 25(OH)D and log-ACS for the patients (r=0.33, p=0.01) and for the control subjects (r=–0.47, p<0.001). Filled dots represent the patients; empty dots represent the controls. Continuous line represents the regression line through the patient data; interrupted line represents the regression line through the control data.

Association between osteopontin and aortic stiffness (Paper IV)

Characteristics of the study subjects

The baseline characteristics of the study subjects are shown in Table 6. The groups did not differ with respect to age or BMI. The patients with PAD had higher aPWV, AIx@75, heart rate as well as measures of peripheral and central BP. In contrast, ABPI was significantly higher among the controls. Biomarkers of inflammation and oxidative stress, such as hsCRP, OPN and oxLDL, were higher in the patient group.

Relationship between osteopontin and aortic stiffness

The aPWV correlated significantly with log-OPN (Figure 7) for the patients with PAD and for the controls. In multiple regression analysis, aPWV was independently associated with AngSc, log-OPN, log-oxLDL and eGFR for the patients and with age, log-oxLDL, heart rate, and log-OPN in the controls (Table 7). In a multiple regression model containing only statistically significant variables, age, log-oxLDL, log-OPN and heart rate explained 37% of the variance in aPWV (p<0.001) in the controls. These associations persisted after correction for pack-years of smoking, ABPI, BMI, LDL, HDL, triglycerides, glucose, heart rate and log-hsCRP.

Table 6. Baseline characteristics of the study subjects (mean ± standard deviation or prevalence (%)).

Characteristic	PAD patients (n=79)	Controls (n=84)	P value
Age (years)	64±7	63±8	0.54
Body mass index (kg/m^2)	25.4±3.8	25.6±3.7	0.73
MAP (mmHg)	103.9±13.8	94.6±9.7	<0.001
PSBP (mmHg)	148.4±20.8	127.6±10.8	<0.001
PDBP (mmHg)	81.3±10.4	76.9±8.2	0.003
PPP (mmHg)	66.1±14.6	50.7±8.8	<0.001
CSBP (mmHg)	135.7±19.2	117.8±12.6	<0.001
CDBP (mmHg)	82.3±10.9	78.2±9.1	0.01
CPP (mmHg)	53.4±12.9	39.5±9.3	<0.001
Heart rate (beats/min)	66.1±12	60.0±9.8	<0.001
AIx@75 (%)	27.9±8.2	18.5±10.9	<0.001
aPWV (m/s)[b]	10±2.4	8.4±1.7	<0.001
bPWV (m/s)[b]	8.7±1.2	8.9±1.4	0.58
Angiographic score (AU)	28.3±7.6	ND	ND
ABPI	0.41±0.28	1.19±0.12	<0.001
Glucose (mmol/L)	5.6±1	5.4±0.5	0.08
Total cholesterol (mmol/L)	5.9±1.2	5.2±1.1	<0.001
LDL (mmol/L)	4.2±1.1	3.7±1.1	0.003
HDL (mmol/L)	1.3±0.4	1.3±0.3	0.7
Triglycerides (mmol/L)	1.7±0.7	1.2±0.6	<0.001
hsCRP (mg/L)[a]	3.9 (1.4–7.6)	0.9 (0.5–1.7)	<0.001
eGFR (ml/min/1.73m^2)	99.7±25.8	96.5±19	0.38
OPN (ng/mL)[a]	75 (62.3–85.8)	54.8 (47.7–67.9)	<0.001
oxLDL (U/L)[a]	67 (52.5–93.5)	47.5 (37–65.5)	<0.001
Current smoking, n (%)	79 (100)	0	<0.001
Pack-years of smoking	37±16	0	<0.001
Medication, n (%)			
Calcium channel blockers	15 (18.9)	0 (0)	<0.001
ACE inhibitors	15 (18.9)	0 (0)	<0.001
Angiotensin receptor blockers	8 (10.1)	0 (0)	<0.001
Beta-blockers	6 (7.6)	0 (0)	<0.001
Diuretics	3 (3.8)	0 (0)	0.2
Pentoxyfylline	32 (40.5)	0 (0)	<0.001
Aspirin	21 (26.6)	0 (0)	<0.001
Statins	9 (11.4)	0 (0)	<0.001

[a] indicates medians and interquartile ranges. [b] aPWV and bPWV have been adjusted for MAP.

Table 7. Multiple regression analysis for the PAD patients and for the control subjects with aPWV adjusted for MAP as the dependent variable.

	Regression coefficient	Standard error	P value
Patients[a]			
Angiographic score (AU)	0.08	0.03	0.01
Log-oxLDL	3.71	1.56	0.02
Log-OPN	4.36	1.84	0.02
eGFR (mL/min/1.73m^2)	–0.02	0.01	0.02
Age (years)	0.07	0.04	0.06
Controls[b]			
Age (years)	0.07	0.02	0.002
Log-oxLDL	2.46	0.98	0.01
Log-OPN	3.36	1.4	0.02
Heart rate (beats/min)	0.04	0.02	0.02
BMI (kg/m^2)	0.06	0.04	0.15

[a] R^2 value=0.44; p<0.001; n=72; [b] R^2 value=0.38; p<0.001; n=83.

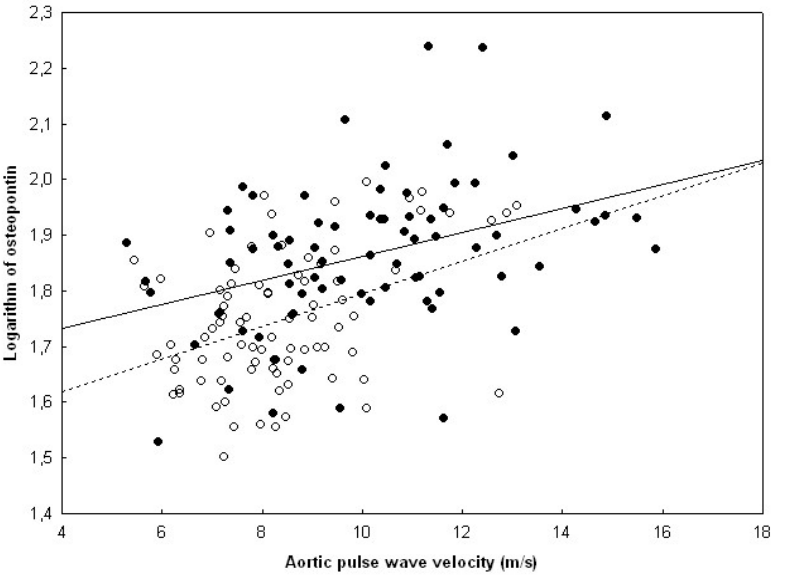

Figure 7. Correlation between aPWV and log-OPN for the patients (r=0.39, p<0.001) and for the control subjects (r=0.41, p<0.001). The aPWV has been adjusted for MAP. Filled dots represent the patients; empty dots represent the controls. Continuous line represents the regression line through the patient data; interrupted line represents the regression line through the control data.

5.2. β_2-microglobulin independently predicts aortic stiffness in patients with symptomatic peripheral arterial disease (Paper II)

Subject characteristics

The clinical characteristics of 66 patients with PAD and in 66 apparently healthy subjects are summarized in Table 8. There was no significant difference between the patients and the controls regarding glucose, HDL, GFR or fetuin-A levels (Table 8). However, there was a significant difference in age, BMI, ABPI, total cholesterol, LDL, triglycerides, hsCRP, β_2M, AIx, aPWV and smoking status between the groups. Heart rate, MAP, PSBP, PDBP and CSBP were also different for the patients and for the controls.

Relationship between aortic stiffness and other variables

Linear regression analysis was used to establish whether aPWV correlated with other variables within each group separately. A significant relationship was found between aPWV and eGFR or age for the patients ($r=-0.34$, $p=0.005$; $r=0.42$, $p<0.001$, respectively) and for the control group ($r=-0.26$, $p=0.03$; $r=0.53$, $p<0.001$, respectively). There was a significant positive association between β_2M and aPWV only in the patients (Figure 8). We did not find significant correlation between β_2M and aPWV in the control group or between β_2M and AIx for either group (data not shown). To determine whether the association between β_2M and aPWV was independent of traditional CV risk factors or specific factors influencing arterial stiffness, multiple linear regression models were developed with aPWV as the dependent variable for each group separately. The aPWV was independently associated only with β_2M and age for the patient group (Table 9). In a multiple regression model containing only statistically significant variables, β_2M and age explained 27% of the variance in aPWV ($p<0.001$) for the PAD patients. In contrast, there did not exist any significant association between β_2M and aPWV for the controls (data not shown). However, aPWV was independently correlated with MAP and age for the control subjects ($R^2=0.41$, $p<0.001$).

Relationship between β2-microglobulin and other variables

Univariate analysis revealed that β_2M level was correlated with age both in the PAD patients ($r=0.48$, $p<0.001$) and in the control subjects ($r=0.31$, $p=0.01$). Moreover, β_2M was inversely correlated with GFR in the patients ($r=-0.45$, $p<0.001$) and in the controls ($r=-0.43$, $p<0.001$). However, plasma β_2M was significantly correlated with fetuin-A level only for the patient group (Figure 9). To determine which biomarkers associated independently with β_2M, multiple linear regression models were developed for each group with β_2M as the

dependent variable. The final models indicated that β_2M was positively associated with fetuin-A, aPWV, age and GFR for the patients group (Table 10), while β_2M was significantly correlated with age and GFR among the controls (R^2=0.24, p<0.001).

Table 8. Baseline characteristics of the study subjects (mean ± standard deviation or prevalence (%)).

Characteristic	PAD patients (n=66)	Controls (n=66)	P value
Age (years)	63±7.2	54.7±6.3	<0.001
BMI (kg/m^2)	25.5±4	26.8±3.4	0.04
MAP (mmHg)	104±13	95±12	<0.001
PSBP (mmHg)	146±20	127±12	<0.001
PDBP (mmHg)	81±10	77±9	0.012
PPP (mmHg)	66±15	51±10	<0.001
CSBP (mmHg)	134±19	118±17	<0.001
CDBP (mmHg)	82±11	79±10	0.09
CPP (mmHg)	53±13	39±10	<0.001
Heart rate (beats/min)	67±12	58±10	<0.001
ABPI	0.42±0.3	1.12±0.14	<0.001
Total cholesterol (mmol/L)	5.9±1.29	5.2±0.98	<0.001
HDL (mmol/L)	1.28±0.41	1.35±0.32	0.26
LDL (mmol/L)	4.19±1.18	3.62±0.94	0.003
Triglycerides (mmol/L)	1.79±0.73	1.06±0.74	<0.001
Glucose (mmol/L)	5.61±1.04	5.44±0.49	0.11
hsCRP (mg/L)[a]	3.57 (1.2–7.12)	1.09 (0.58–2.04)	<0.001
GFR (mL/min/1.73 m^2)	102.5±26.5	97.9±18.2	0.25
β2M (μg/L)	1858.1±472.8	1554.5±277.9	<0.001
Fetuin-A (μg/mL)	260.2±106	254.8±104.9	0.77
AIx (%)	28±8	14±11	<0.001
aPWV (m/s)	9.9±2.2	7.6±1.6	<0.001
Current smoking, n (%)	64 (97)	2 (3)	<0.001
Medication, n (%)			
Calcium channel blockers	10 (15.2)	0 (0)	<0.001
ACE inhibitors	8 (12.1)	0 (0)	<0.001
Angiotensin receptor blockers	5 (7.6)	0 (0)	0.006
Beta-blockers	2 (3)	0 (0)	0.16
Diuretics	2 (3)	0 (0)	0.16
Pentoxyfylline	25 (37.9%)	0 (0)	<0.001
Aspirin	14 (21.2%)	0 (0)	<0.001
Statins	6 (9.1%)	0 (0)	0.002

[a] indicates medians and interquartile ranges.

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

Variable	Regression coefficient	Standard error	P value
β_2M (µg/L)	0.002	<0.001	0.005
Age (years)	0.084	0.038	0.031
MAP (mmHg)	0.025	0.018	0.168

$R^2 = 0.3$, $p<0.001$, n=66.

Table 10. Multiple regression model for the patients with β_2M as the dependent variable.

Variable	Regression coefficient	Standard error	P value
Fetuin-A (µg/mL)	1.655	0.409	<0.001
aPWV (m/s)	51.729	21.485	0.019
Age (years)	16.496	6.883	0.02
GFR (mL/min/1.73m^2)	–3.772	1.809	0.041

$R^2 = 0.5$, $p<0.001$, n=66.

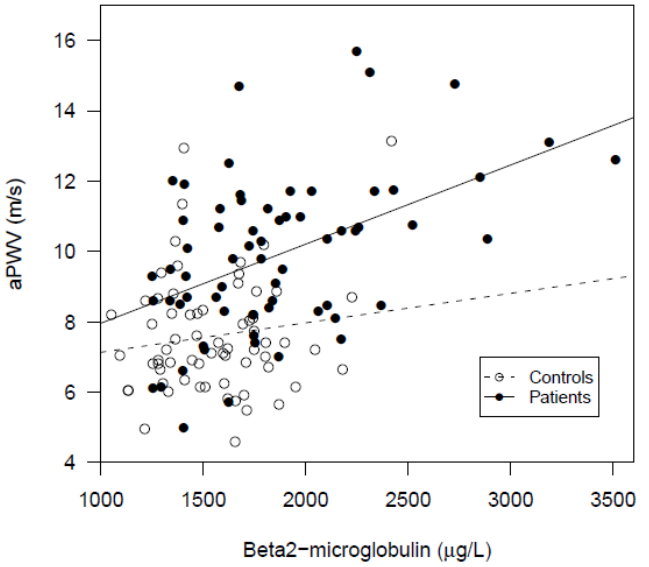

Figure 8. Scatterplots of the functional and biochemical parameters for 66 patients and 66 controls. The aPWV and β_2M were significantly correlated for the patients (r=0.47, p<0.001) but not for the controls (r=0.14, p=0.26).

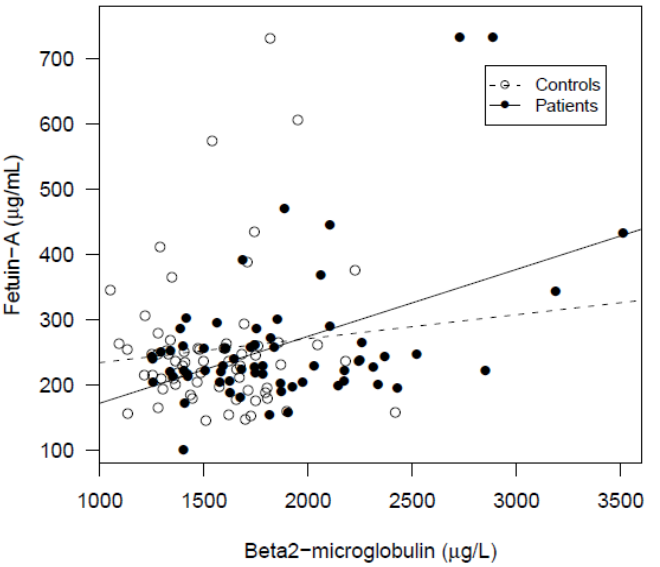

Figure 9. Scatterplots of the functional and biochemical parameters for 66 patients and 66 controls. The β_2M and fetuin-A levels were significantly correlated for the patients ($r=0.46$, $p<0.001$) but not for the controls ($r=0.1$, $p=0.44$).

5.3. Association between aortic stiffness and severity of peripheral arterial disease (Papers III and IV)

Relationship between ankle-brachial pressure index, aortic stiffness and vitamin D (Paper III)

The characteristics of the patients with atherosclerosis are summarized in Table 4. We tested the hypothesis about whether increased arterial stiffness and decreased vitamin D levels are associated with more advanced atherosclerotic disease. In univariate analysis, there was almost significant correlation between ABPI and 25(OH)D ($r=0.26$, $p=0.051$). We evaluated the relationship between ABPI and the composite measure of aPWV and 25(OH)D. The patients were divided into 3 groups according to aPWV and 25(OH)D values. The first group comprised patients with aPWV below the median (9.7 m/s) and 25(OH)D above the median (15.2 ng/mL) ($n=12$). The second group consisted of patients with either aPWV>9.7 m/s and 25(OH)D>15.2 ng/mL or aPWV≤9.7 m/s and 25(OH)D≤15.2 ng/mL ($n=35$). Patients in the third group had aPWV>9.7 m/s and 25(OH)D≤15.2 ng/mL ($n=19$). Twelve patients were omitted from the analysis, since the data on ABPI, aPWV or 25(OH)D were missing. There was a significant inverse association between ABPI and the composite measure of aPWV and 25(OH)D ($p=0.03$ by ANOVA). To illustrate this association, we

divided PAD patients into tertiles according to aPWV and 25(OH)D values. As shown in Figure 10, higher aPWV and lower 25(OH)D levels were related to lower ABPI.

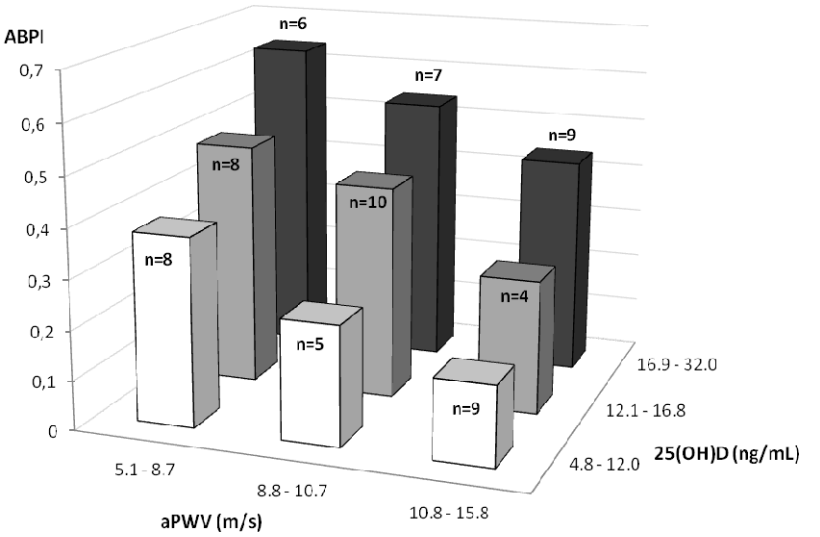

Figure 10. Relationship between ABPI and the tertiles of aPWV and 25(OH)D for the PAD patients (n=66). The aPWV has been adjusted for MAP.

Relationship of aortic stiffness with angiographic score and ankle-brachial pressure index (Paper IV)

The aPWV was positively correlated with AngSc (Figure 11) and negatively with ABPI (Figure 12) for the patient group. In multiple regression analysis, aPWV was independently associated with AngSc, log-OPN, log-oxLDL and eGFR for the patients and with age, log-oxLDL, heart rate and log-OPN in the controls (Table 7). In a multiple regression model containing only statistically significant variables, age, log-oxLDL, log-OPN and heart rate explained 37% of the variance in aPWV ($p<0.001$) for the controls. These associations persisted after correction for pack-years of smoking, ABPI, BMI, LDL, HDL, triglycerides, glucose, heart rate and log-hsCRP.

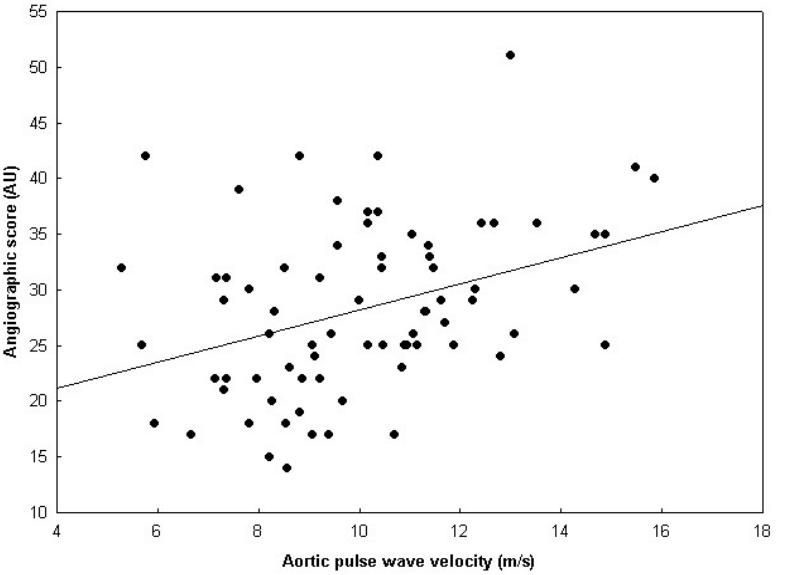

Figure 11. Correlation between aPWV and AngSc (r=0.37, p=0.001) for the PAD patients. The aPWV has been adjusted for MAP.

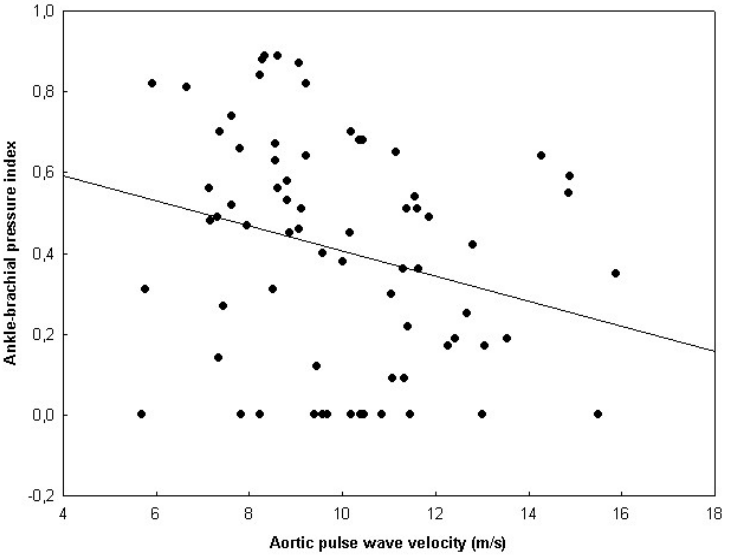

Figure 12. Correlation between aPWV and ABPI (r=–0.26, p=0.03) for the PAD patients. The aPWV has been adjusted for MAP.

5.4. Relationship of oxidative stress with arterial calcification and stiffness (Papers I and IV)

Relationship between oxidative stress and arterial calcification (Paper I)

The baseline characteristics of 69 patients and 68 healthy subjects are presented in Table 2. In univariate analysis, OPG was correlated with oxLDL (r=0.34, p=0.01) (Figure 13) for the patient group but not for the healthy subjects (data not shown). In multiple regression analysis, OPG correlated independently with aPWV and oxLDL for the patients (Table 11). In a multiple regression model containing only statistically significant variables, aPWV and oxLDL explained 31% of the variance in OPG (p<0.001) in the PAD patients.

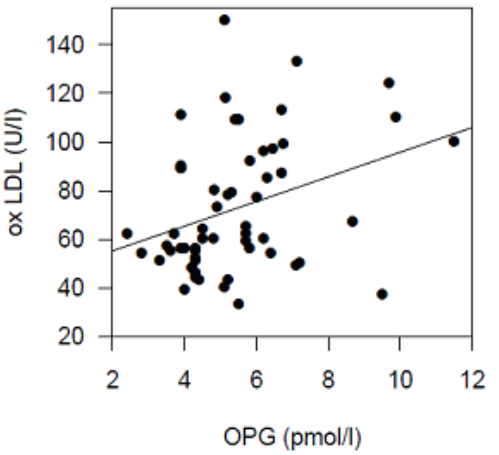

Figure 13. Correlation between OPG and oxLDL for the PAD patients (r=0.34, p=0.01).

Table 11. Multiple regression model for the patients and for the healthy subjects with OPG as the dependent variable.

	Regression coefficient	Standard error	P value
Patients[a]			
aPWV (m/s)	0.37	0.12	0.004
oxLDL (U/L)	0.02	0.01	0.03
HDL (mmol/L)	0.93	0.60	0.13
Age (years)	0.04	0.03	0.29
ABPI	0.88	0.82	0.29
Controls[b]			
aPWV (m/s)	0.26	0.11	0.02
HDL (mmol/L)	–0.96	0.56	0.09
Age (years)	0.04	0.03	0.2
oxLDL (U/L)	–0.01	0.008	0.22

[a] R^2 value=0.48, p <0.00001, n=69. [b] R^2 value=0.35; p <0.002, n=68.

Relationship between oxidative stress and aortic stiffness (Paper IV)

The baseline characteristics of 79 PAD patients and 84 clinically healthy subjects are shown in Table 6. The aPWV correlated significantly with oxLDL (Figure 14) for the patients with PAD and for the controls. In multiple regression analysis, aPWV was independently associated with AngSc, log-OPN, log-oxLDL, eGFR and age for the patients and with age, log-oxLDL, log-OPN, and heart rate for the controls (Table 7). As shown in Figures 15 and 16, higher levels of OPN and oxLDL were related to higher aPWV values for both study groups.

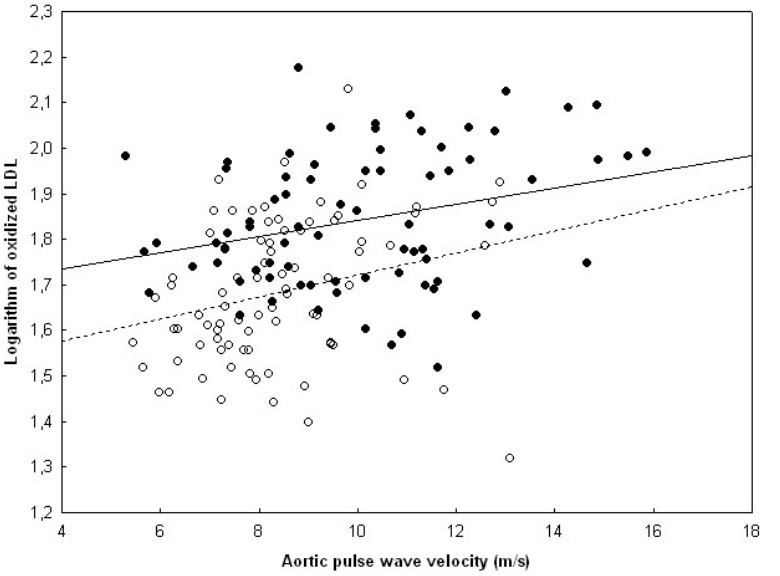

Figure 14. Correlation between aPWV and log-oxLDL for the patients (r=0.28, p=0.01) and for the control subjects (r=0.26, p=0.02). The aPWV has been adjusted for MAP. Filled dots represent the patients; empty dots represent the controls. Continuous line represents the regression line through the patient data; interrupted line represents the regression line through the control data.

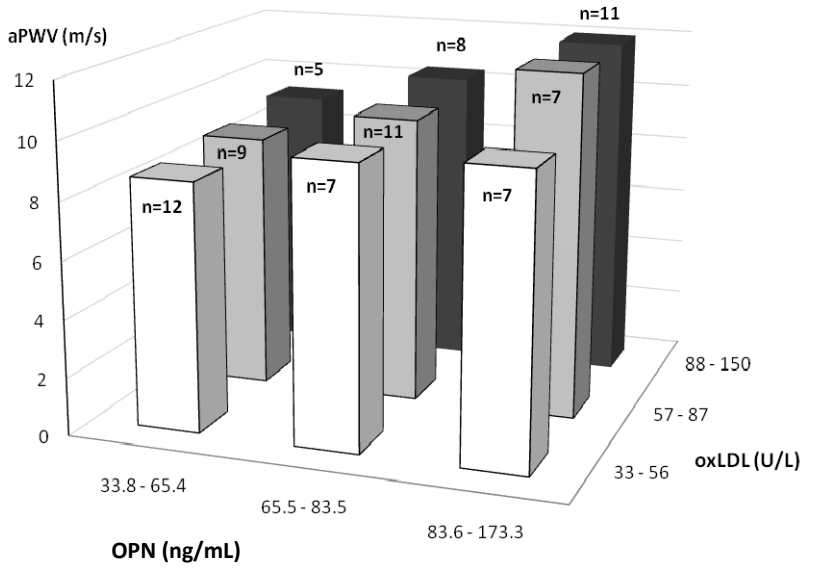

Figure 15. Relationship between aPWV and the tertiles of OPN and oxLDL for the PAD patients. The aPWV has been adjusted for MAP.

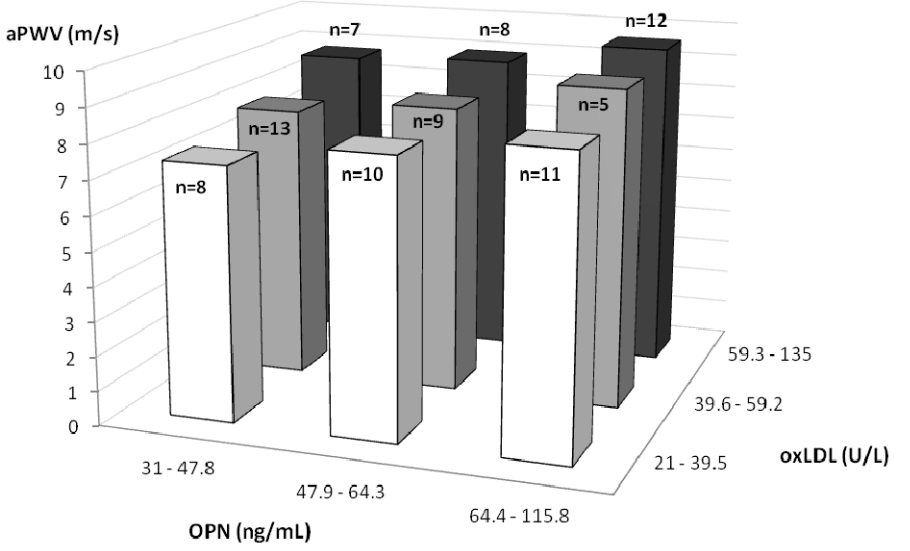

Figure 16. Relationship between aPWV and the tertiles of OPN and oxLDL for the controls. The aPWV has been adjusted for MAP.

6. DISCUSSION

6.1. The role of vascular calcification in aortic stiffening

The VCa may influence the clinical course of PAD (Guzman *et al.* 2008). The VCa leads to an increase in arterial wall thickness and reduction in conduit artery lumen area, which may promote arterial stiffening and impair distal tissue perfusion (Greenwald 2007). The presence and extent of VCa are predictors of critical limb ischaemia requiring amputation (Guzman *et al.* 2008) and subsequent vascular morbidity and mortality (Wilson *et al.* 2001; Rennenberg *et al.* 2009). Recent research suggests that VCa is an active and complex process that involves numerous mechanisms responsible for calcium deposition in arterial wall (Jayalath *et al.* 2005). A growing body of evidence suggests that regulators of bone remodelling, such as OPG, OPN and vitamin D might play a major role in VCa and arterial stiffening.

We have demonstrated that the patients with PAD had higher values of OPG as compared to the controls (Paper I). Similarly, several clinical studies have identified the relationship between elevated serum OPG and CAD, diabetic complications, heart failure, abdominal aortic aneurysm and CV mortality (Kiechl *et al.* 2006). In contrast, a recent cross-sectional study reported that OPG levels were not statistically different between patients with PAD and subjects without PAD (Koshikawa *et al.* 2009). However, Koshikawa and co-authors included subjects with hypertension and diabetes in the control group, whereas the control group in our study comprised clinically healthy individuals. Increased levels of OPG in patients with atherosclerosis may be explained by several mechanisms. As calcification is common within atherosclerotic plaque, elevated OPG levels could indicate an increased burden of atherosclerosis. Alternatively, increased OPG may represent a compensatory response to mitigate further vascular injury (Morony *et al.* 2008). Consistent with this hypothesis, OPG is known to inhibit apoptosis (Vitovski *et al.* 2007). As apoptotic debris may promote calcium deposition in the vascular wall, expression of OPG in the endothelium might be upregulated to inhibit apoptosis (Shroff *et al.* 2008b).

The present study has also revealed that serum OPG is independently related to aPWV in the PAD patients and in apparently healthy subjects. Similarly, the association between circulating levels of OPG and aortic stiffness has been shown in patients with chronic kidney disease (Scialla *et al.* 2011) and in postmenopausal women (Frost *et al.* 2008). The relationship between aortic stiffening and elevated serum OPG might be influenced by endothelial dysfunction. Previous research demonstrates that impaired endothelial function is associated with elevated serum OPG (Golledge *et al.* 2008) and increased arterial stiffness (Kals *et al.* 2006a). It might be that endothelial injury leads to arterial stiffening and upregulates the expression of OPG in endothelial cells (Shroff *et al.* 2008b).

Serum OPN is another important inhibitor of VCa that has been implicated in the pathogenesis of atherosclerosis (Momiyama *et al.* 2010; Waller *et al.* 2010). Recent research indicates that plasma OPN levels are higher in patients with unstable angina than in those with stable angina or in control subjects (Soejima *et al.* 2006). Moreover, circulating OPN levels are elevated in patients with carotid atherosclerosis (Kurata *et al.* 2006). In Paper IV, we showed that patients with PAD had higher OPN as compared to controls, which is consistent with the results of a previous study (Koshikawa *et al.* 2009). However, the results of our study expand the findings of Koshikawa and co-authors, as the patients in our study were younger and free of diabetes (Koshikawa *et al.* 2009). Increased circulating OPN might have prognostic significance, as it has been demonstrated that OPN levels predict CV death, myocardial infarction, stroke and endovascular interventions in patients undergoing carotid surgery (de Kleijn *et al.* 2010).

It is intriguing to hypothesize that OPN might be involved in the pathogenesis of PAD. We have shown that aortic stiffness is independently associated with serum levels of OPN in the patients with PAD as well as in the healthy subjects (Paper IV). This mechanistic insight is consistent with a previous observation that elevated OPN levels are independently associated with increased aortic stiffness in patients with rheumatoid arthritis (Bazzichi *et al.* 2009). The OPN might modulate aortic stiffness by stimulating proliferation of the VSMC and thickening of the media (Isoda *et al.* 2002). Moreover, animal models have highlighted the importance of OPN as an inducible inhibitor of vascular mineralization (Speer *et al.* 2002). Thus, the positive association between OPN and aortic stiffness may represent a protective increase in OPN aimed at limiting vascular damage.

Previous research suggests that VCa is involved in the development of aortic stiffness and the subsequent pathogenesis of isolated systolic hypertension (Blacher *et al.* 2001; McEniery *et al.* 2009). Moreover, aortic stiffness is associated with the extent of coronary artery, aortic and valvular calcifications in patients with chronic kidney disease (Haydar *et al.* 2004; Raggi *et al.* 2007). In Paper III, we have demonstrated that aortic calcification is associated with aortic stiffness in patients with PAD and in healthy subjects. It could be that deposition of calcium in the aorta leads to aortic stiffening (Sigrist *et al.* 2007). Alternatively, increased arterial stiffness could lead to vessel wall damage and calcification (McEniery *et al.* 2009). Finally, it might be that both VCa (Wong *et al.* 2011) and arterial stiffness (McEniery *et al.* 2010; Astrand *et al.* 2011) are the consequences of age-related degenerative processes in the vasculature.

Recently it has been reported that vitamin D is an important determinant of all-cause and CV mortality in high-risk patients (Pilz *et al.* 2011; Schierbeck *et al.* 2011) and in general population (Holick 2007; Melamed *et al.* 2008a). We have demonstrated that 25(OH)D levels are significantly lower in the PAD patients than in the controls (Paper III), which is consistent with the results of a

previous study (Reis *et al.* 2008). It could be that patients with PAD are less mobile and are therefore less affected by sun exposure (Melamed *et al.* 2008b).

The role of vitamin D in the development of VCa is controversial. The effects of vitamin D on VCa appear to follow a biphasic pattern, with both excess and deficiency promoting its development (Shroff *et al.* 2008a). In our study, serum levels of 25(OH)D correlated positively with ACS in the PAD patients and negatively in the controls (Paper III). It could be that different localizations of calcium deposits in aortic wall were responsible for different patterns of correlation between aortic calcification and vitamin D seen among the study groups. Previous studies indicate that in patients with atherosclerosis mostly intimal calcification is seen (Verbeke *et al.* 2010). In contrast, medial calcification is associated with diabetes mellitus, end-stage renal disease and aging (Verbeke *et al.* 2010). Based on these observations, we hypothesized that a combination of intimal and medial calcification occurred in PAD patients, whereas age-related medial calcification was predominant in healthy subjects.

Vitamin D could be involved in the regulation of arterial stiffness. Lower levels of 25(OH)D are independently associated with increased arterial stiffness in patients with chronic kidney disease (Patange *et al.* 2012) and in healthy subjects (Al Mheid *et al.* 2011). Vitamin D could influence arterial stiffness by modulating VSMC tone (Shan *et al.* 1993), inhibiting the renin-angiotensin system (Li *et al.* 2002), or by regulating the expression of a number of proteins present in the arterial wall, such as elastin, collagen, myosin and matrix metallo-proteinase (Norman *et al.* 2005). We have demonstrated borderline association between vitamin D and aortic stiffness in clinically healthy subjects (Paper III). The lack of statistically significant association between these parameters might be attributable to small sample size.

In conclusion, we have shown that aortic stiffness is independently associated with serum levels of OPG, OPN and 25(OH)D as well as aortic calcification in patients with symptomatic PAD and in apparently healthy subjects. Assessment of aortic stiffness as well as of the biochemical and structural markers of VCa could provide additional insight into the pathogenesis of PAD.

6.2. β_2-microglobulin as a predictor of aortic stiffness in patients with peripheral arterial disease

The β_2M has recently emerged as a biomarker of atherosclerosis (Amighi *et al.* 2011). It has been shown that circulating β_2M levels are increased in PAD patients and correlate with severity of disease as assessed by ABPI or treadmill testing (Wilson *et al.* 2007; Fung *et al.* 2008). Importantly, in patients with CAD, β_2M levels were higher in the patients who also had PAD as compared to subjects without PAD (Wilson *et al.* 2007). This finding indicates that β_2M predicts the presence of PAD even in patients with high CV risk. Consistently

with these data, we demonstrated that plasma levels of β_2M are elevated in patients with PAD as compared with controls (Paper II). However, we did not find significant association between β_2M levels and ABPI in either of the study groups. The discrepancy in the results between our study and the previous one (Wilson *et al.* 2007) may be explained by the fact that Wilson and the co-authors studied both men and women, whereas all participants in our study were men. Moreover, the PAD patients in the study by Wilson and the co-authors had significantly lower eGFR compared to the patients in our study. As β2M is mainly eliminated by glomerular filtration (Donadio *et al.* 2001), the difference in eGFR between the studies may have affected the association between β2M and ABPI.

Aortic stiffness is another important determinant of CV risk (Mattace-Raso *et al.* 2006; Vlachopoulos *et al.* 2010). We have demonstrated increased aortic stiffness and wave reflection in patients with PAD compared with controls (Papers I–IV). This finding supports the results of previous studies (Cheng *et al.* 2002; Kals *et al.* 2006a; Tsuchikura *et al.* 2010). Research data suggest that aortic stiffness is a major contributor to atherosclerosis. Aortic stiffening leads to an increase in systolic BP and a widening of pulse pressure, which promotes arterial wall damage and accelerates progression of the atherosclerotic process (Boutouyrie *et al.* 2002). Moreover, increased aortic stiffness decreases diastolic BP and is related to impaired blood flow in the arteries of the lower extremities (Tsuchiya *et al.* 2005). Finally, aortic stiffness influences functional capacity in patients with atherosclerosis. In PAD patients, increased aPWV is independently associated with slower gait speed (Watson *et al.* 2011), whereas reduction of PWV by an ACE inhibitor is significantly related to improved performance on the treadmill walk test (Ahimastos *et al.* 2008).

We found independent association between aortic stiffness and plasma β_2M in patients with PAD (Paper II). Accordingly, β_2M is independently associated with arterial stiffness in general population (Saijo *et al.* 2005). However, we did not find significant association between β_2M and aortic stiffness in apparently healthy subjects. Several explanations could be given for the discrepancies between the above study (Saijo *et al.* 2005) and the present one. Saijo and co-authors assessed arterial stiffness by baPWV, which describes the properties of both the aorta and the lower limb arteries. However, we used aPWV as a „gold standard" measure of arterial stiffness (Laurent *et al.* 2006). Moreover, both men and women participated in the above study, whereas all the participants of our study were men. As gender is an important determinant of aortic stiffness (Sonesson *et al.* 1993), the gender difference could account for the discrepancy between the results of the studies.

There are several possible linking mechanisms between β_2M and aortic stiffness. Chronic inflammation is involved in the pathogenesis of vascular remodelling and atherosclerosis (Hassoun *et al.* 2009; Recio-Mayoral *et al.* 2011). Previous studies have shown that CRP is related to aortic stiffness (Yasmin *et al.* 2004) and wave reflection (Kampus *et al.* 2006) in apparently

healthy individuals. Moreover, a combination of increased CRP and β2M is significantly associated with elevated arterial stiffness in healthy subjects (Saijo *et al.* 2005). Thus, inflammation may be a potential link between β_2M and aortic stiffness. In contrast, neither β_2M nor aPWV was associated with hsCRP in our study, which might be explained by relatively small sample size. Alternatively, renal function could modulate the association of β_2M with aortic stiffness. It has been reported that both β_2M (Jovanovic *et al.* 2003) and aPWV (Mourad *et al.* 2001; Kawamoto *et al.* 2008) are related to eGFR. Moreover, aPWV and β_2M are independent predictors of all-cause mortality in patients with end-stage renal disease (Blacher *et al.* 1999; Okuno *et al.* 2009). Consistently with these data, we demonstrated that eGFR is inversely related to β_2M and aPWV in patients with PAD and in controls. These findings suggest that β_2M might be important in renal-induced vascular stiffening. Finally, VCa may provide a mechanistic link between β_2M and aortic stiffness. We have shown that fetuin-A correlates independently with β_2M in the patient group (Paper II). Fetuin-A is an important inhibitor of ectopic calcification and is inversely related to coronary (Jung *et al.* 2011) and valvular (El-Shehaby *et al.* 2010) calcification in haemodialysis patients. Furthermore, accumulation of β_2M amyloid is associated with mitral valve calcification in haemodialysis patients (Takayama *et al.* 2001). It would be intriguing to hypothesize that VCa increases aortic stiffness and elevates circulating levels of β_2M and fetuin-A.

To summarise, we have demonstrated that β_2M is independently associated with aortic stiffness in patients with PAD. This finding suggests that β_2M may be related to the stiffening of the aorta in advanced atherosclerosis. Our study supports the use of β_2M as a biomarker of atherosclerosis.

6.3. Aortic stiffness is a determinant of severity of peripheral arterial disease

The PAD is complicated by high rate of coronary and cerebral events (McDermott *et al.* 2001). However, as the majority of PAD patients do not manifest the classic symptomatology, early diagnosis and assessment of CV risk in these patients is a significant clinical challenge (Hirsch *et al.* 2007).

The ABPI is an established clinical tool for assessing presence and severity of PAD (Guo *et al.* 2008). We have demonstrated that increased aPWV and lower levels of 25(OH)D are related to lower ABPI values in patients with PAD (Paper III). Similarly, a recent study has shown that reduced arterial compliance is related to decreased ABPI in elderly subjects (Lind 2011). However, Lind assessed arterial compliance by ultrasound as the distensibility of the carotid artery, whereas we evaluated aortic stiffness by aPWV. We did not find any association between ABPI and aPWV in clinically healthy subjects, which is consistent with the results of a previous study (Rabkin *et al.* 2012). Interestingly, a recent study has reported that ABPI is positively associated with

baPWV and bkPWV in non-diabetic PAD patients (Khandapour *et al.* 2009). However, baPWV and bkPWV characterize wave propagation not only along the aorta but also along the lower-extremity arteries. Therefore, presence of stenoses or occlusions in the lower-extremity arteries affects the propagation of pulse wave (Motobe *et al.* 2005), and hence modulates the relationship between aPWV and ABPI.

There are several lines of evidence suggesting that vitamin D may have a protective role in CV disease. Vitamin D improves endothelial function in patients with diabetes (Sugden *et al.* 2008). Moreover, vitamin D has been shown to be an inhibitor of the renin-angiotensin system (Li *et al.* 2002). A large population-based study has reported that ABPI is positively associated with 25(OH)D levels in apparently healthy Caucasian subjects (Reis *et al.* 2008). Furthermore, lower serum 25(OH)D levels are associated with higher prevalence of PAD (Melamed *et al.* 2008b). In our study, a borderline correlation was noted between ABPI and 25(OH)D in patients with PAD, which did not reach statistical significance, probably because of the small number of subjects.

The AngSc provides information about the severity and distribution of atherosclerotic lesions in the lower-extremity arteries (Van der Feen *et al.* 2002). In Paper IV, we have demonstrated that aPWV is independently related to AngSc in PAD patients, suggesting that aortic stiffness is related to the grade of atherosclerosis in the lower extremity arteries. There are several possible explanations for the relationship between arterial stiffness and atherosclerosis.

It could be hypothesized that VCa influences the association between arterial stiffening and atherosclerosis. In our study, serum levels of calcification inhibitor OPN were positively correlated with AngSc and aPWV in patients with atherosclerosis (Paper IV). As the expression of OPN is increased at sites of calcification in atherosclerotic plaques (Fitzpatrick *et al.* 1994) and predicts aortic stiffening in patients with rheumatoid arthritis (Bazzichi *et al.* 2009), it might provide a mechanistic link between arterial stiffening and atherosclerosis.

Inflammation is an important determinant of the formation of atherosclerotic plaques (Derlin *et al.* 2011) and has been implicated in the process of arterial stiffening (De Silva *et al.* 2008). Previous studies have reported that CRP levels are associated with arterial stiffness in patients with rheumatoid arthritis (Provan *et al.* 2011), in women with systemic lupus erythematosus (Bjarnegård *et al.* 2006) and in apparently healthy individuals (Kampus *et al.* 2004; Yasmin *et al.* 2004). Furthermore, CRP is predictive of disease progression, all-cause and CV mortality in patients with PAD (Van Der Meer *et al.* 2002; Vidula *et al.* 2008). Therefore, inflammation may influence the relationship between aortic stiffening and atherosclerosis.

Endothelial dysfunction is another potential link between arterial stiffening and atherosclerosis (Halcox *et al.* 2002; Figueiredo *et al.* 2012). Endothelial dysfunction has been found to be associated with severity of PAD (Silvestro *et al.* 2003) and predicts adverse CV events in these patients (Brevetti *et al.* 2003).

Impaired endothelial function could contribute to progression of PAD by impairing blood flow responses to ischaemia and by promoting vasospasm, plaque rupture and thrombosis (Vita and Hamburg 2010). Although we did not assess endothelial function in the present study, we have previously reported that endothelial dysfunction is related to increased arterial stiffness in patients with symptomatic PAD (Kals *et al.* 2006a).

Alternatively, both aortic stiffening (Astrand *et al.* 2011) and atherosclerosis (Van Popele *et al.* 2001) might be the consequences of aging. In our study, aortic stiffness was significantly correlated with age in both study groups. However, an independent association between AngSc and aPWV remained significant after adjustment for age.

To summarise, increased aortic stiffness and lower vitamin D levels are related to decreased ABPI in the PAD patients. Moreover, aPWV is independently associated with AngSc in the patients with PAD. Given that both vitamin D (Schierbeck *et al.* 2011) and aortic stiffness (Laurent *et al.* 2001) have been associated with an increased risk of CV events, assessment of these parameters may be used in risk stratification and in assessment of the rate of progression of PAD.

6.4. Aortic stiffness, vascular calcification and oxidative stress

Pathological OxS is a key factor in the pathogenesis of atherosclerosis. Both oxLDL (Maziere *et al.* 2010; Parthasarathy *et al.* 2010) and 8-iso-prostaglandin $F_{2\alpha}$ (Mueller *et al.* 2004; Schwedhelm *et al.* 2004) have been recognized as reliable markers of OxS. The oxLDL is involved in the initiation and progression of atherosclerosis, and contributes to functional and structural alterations of large arteries (Ehara *et al.* 2001; Andersson *et al.* 2009). We have shown that serum oxLDL is significantly higher in PAD patients than in controls (Papers I and IV), which is in accordance with the results of previous research (Mueller *et al.* 2004; Kals *et al.* 2006b). However, in these studies, OxS was assessed by 8-iso-prostaglandin $F_{2\alpha}$, whereas oxLDL was measured in the present study. The oxLDL is not only a marker of OxS but may also promote progression of atherosclerosis. It has been shown that unstable carotid plaques have a much greater content of oxLDL than stable carotid plaques (Nishi *et al.* 2002). Furthermore, plasma oxLDL are higher in patients with unstable angina and correlate with the incidence of angiographically complex coronary plaques (Anselmi *et al.* 2006).

We detected no association between serum oxLDL and ABPI in either of the study groups (Paper IV). Similarly, a prior study did not find any association between circulating oxLDL and severity of atherosclerosis in patients with symptomatic PAD (Rosoky *et al.* 2010). There are several possible explanations for the lack of association between oxLDL and ABPI. It could be that

circulating oxLDL has a better predictive value at early stages of atherosclerosis rather than in patients with established atherosclerosis (Rosoky *et al.* 2010). Alternatively, circulating oxLDL levels increase in the acute phase of ischaemia and might not reflect disease severity in patients with stable chronic atherosclerosis. Consistent with this hypothesis, oxLDL levels increase rapidly in patients suffering myocardial infarction and decrease towards baseline levels over the following 7 months (Tsimikas *et al.* 2003).

Profound OxS has also been implicated in arterial stiffening. An earlier study has established that urinary 8-iso-prostaglandin F_{2a} concentration is related to the indices of arterial elasticity in patients with PAD (Kals *et al.* 2008). In the present study (Paper IV), we expanded previous research by demonstrating an independent association between serum oxLDL and aortic stiffness in PAD patients and in healthy subjects. Similarly, cystine, which is a non-free radical marker of OxS, has been associated with aortic stiffness in apparently healthy subjects (Patel *et al.* 2011).

Aortic stiffness and OxS might be linked by different pathophysiological mechanisms. First, endothelial dysfunction may influence the relationship between increased production of reactive species and aortic stiffening. Prior studies indicate that oxLDL contributes to endothelial dysfunction (Wang *et al.* 2011) and activates apoptosis in the endothelial cells (Dimmeler and Zeiher 2000). Decreased bioavailability of NO could lead to vasoconstriction and aortic stiffening (Kals *et al.* 2006a; Loffredo *et al.* 2007). Second, profound OxS may promote degradation of elastin by matrix metalloproteinases and reduce the elasticity of the aortic wall (Rajagopalan *et al.* 1996; Galis and Khatri 2002). Accordingly, matrix metalloproteinase-2 is positively associated with aPWV in hypertensive patients (Yasmin *et al.* 2005). Moreover, experimental research indicates that OxS enhances collagen secretion by aortic smooth muscle cells, and increases therefore aortic stiffness (Zhou *et al.* 2011).

Apart from influencing aortic stiffness, profound OxS may also contribute to VCa (Mody *et al.* 2001). We have demonstrated that oxLDL levels are independently related to serum OPG in patients with atherosclerosis (Paper I). Thus, OxS might be related to VCa in PAD patients. Accordingly, elevated OPG levels are associated with surrogate markers of OxS in patients with chronic kidney disease (Matsubara *et al.* 2009). In addition, a recent animal study has reported that increased production of reactive species is related to overexpression of OPG in the vascular tissue (Aydin *et al.* 2011). It has been suggested that high levels of OPG may represent an inadequate compensatory response aiming at limiting vascular injury in patients with atherosclerosis (Van Campenhout and Golledge 2009). Thus, our finding that OPG is associated with oxLDL in PAD patients could suggest that high-grade OxS is related to VCa in advanced atherosclerosis.

The association between serum OPG and oxLDL levels can be explained by several mechanisms. As OPG is highly expressed in VSMC (Zhang *et al.* 2002), the fact that reactive species stimulate hypertrophy and proliferation of VSMC

(Dimmeler and Zeiher 2000) may provide a mechanistic link between elevated levels of OPG and oxLDL. An alternative possibility is that the relationship between OxS and VCa can be modulated by apoptosis. The ROS are known to induce apoptotic cell death in endothelial cells (Dimmeler and Zeiher 2000). Apoptotic bodies of endothelial and foam cells might provide a suitable microenvironment for VCa (Shao *et al.* 2006).

The association of oxLDL levels with aPWV and OPG in PAD patients indicates that oxLDL may be related to the pathogenesis of aortic stiffening and calcification in advanced atherosclerosis. In addition, serum oxLDL is associated with aPWV in controls, suggesting that OxS might be implicated in aortic stiffening in apparently healthy subjects.

7. CONCLUSIONS

1. Aortic pulse wave velocity and serum levels of osteoprotegerin and oxidized low-density lipoprotein were increased in the patients with symptomatic peripheral arterial disease as compared to the healthy subjects. Serum osteoprotegerin levels were significantly associated with aortic pulse wave velocity in the patients with peripheral arterial disease as well as in the healthy subjects. In addition, serum osteoprotegerin was related to oxidized low-density lipoprotein in the patient group. These findings would suggest that aortic stiffness is related to calcification in patients with atherosclerosis and in healthy subjects.
2. Plasma β_2-microglobulin was significantly higher among the patients with peripheral arterial disease. The β_2-microglobulin levels were independently associated with aortic pulse wave velocity and fetuin-A level in the patients with symptomatic peripheral arterial disease. These findings indicate that the inflammatory biomarker β_2-microglobulin is associated with aortic stiffness and with calcification in patients with atherosclerosis.
3. The patients with peripheral arterial disease had significantly higher aortic calcification score but lower vitamin D levels compared with the controls. The extent of aortic calcification was independently associated with increased aortic pulse wave velocity in the patients with peripheral arterial disease and in the clinically healthy subjects. Aortic calcification score showed positive correlation with vitamin D levels in the patient group and negative correlation among the control subjects. These findings suggest that calcification of the aorta is related to increased aortic stiffness in patients with atherosclerosis and in healthy subjects. The extent of aortic calcification is positively related to serum levels of vitamin D in patients with atherosclerosis and negatively related to vitamin D levels in healthy subjects.
4. Osteopontin levels were significantly higher in the patients with symptomatic peripheral arterial disease compared with the controls. Aortic pulse wave velocity was independently related to serum levels of osteopontin and oxidized low-density lipoprotein in the patients with atherosclerosis and in the clinically healthy subjects. These findings indicate that aortic stiffness is associated with calcification and oxidative stress in patients with atherosclerosis and in controls.
5. Aortic pulse wave velocity was independently associated with angiographic score in the patients with peripheral arterial disease, suggesting that aortic stiffness is related to the distribution and severity of atherosclerotic lesions in the lower extremity arteries in patients with the established diagnosis of peripheral arterial disease.

REFERENCES

Adji A, O'Rourke M, Namasivayam M. Arterial stiffness, its assessment, prognostic value, and implications for treatment. Am J Hypertens 2011; 24: 5–17.

Agabiti-Rosei E, Mancia G. O'Rourke M, Roman M, Safar M, Smulyan H, Wang J, Wilkinson I, Williams B, Vlachopoulos C. Central blood pressure measurements and antihypertensive therapy: a consensus document. Hypertension 2007a; 50: 154–160.

Agabiti-Rosei E, Muiesan M. Carotid atherosclerosis, arterial stiffness and stroke events. Adv Cardiol 2007b; 44: 173–186.

Agnholt J, Kelsen J, Schack L, Hvas C, Dahlerup J, Sorensen E. Osteopontin, a protein with cytokine-like properties, is associated with inflammation in Crohn's disease. Scand J Immunol 2007; 65: 453–460.

Ahimastos A, Dart A, Lawler A, Blombery P, Kingwell B. Reduced arterial stiffness may contribute to angiotensin-converting enzyme inhibitor induced improvements in walking time in peripheral arterial disease patients. J Hypertens 2008; 26: 1037–1042.

Ahlgren A, Hansen F, Sonesson B, Länne T. Stiffness and diameter of the common carotid artery and abdominal aorta in women. Ultrasound Med Biol 1997; 23: 983–988.

Ahmadi N, Tsimikas S, Hajsadeghi F, Saeed A, Nabavi V, Bevinal M, Kadakia J, Flores F, Ebrahimi R, Budoff M. Relation of oxidative biomarkers, vascular dysfunction, and progression of coronary artery calcium. Am J Cardiol 2010; 105: 459–466.

Alexopoulos N, Raggi P. Calcification in atherosclerosis. Nat Rev Cardiol 2009; 6: 681–688.

Al Mheid I, Patel R, Murrow J, Morris A, Rahman A, Fike L, Kavtaradze N, Uphoff I, Hooper C, Tangpricha V, Alexander R, Brigham K, Quyyumi A. Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. J Am Coll Cardiol 2011; 58: 186–192.

Amighi J, Hoke M, Mlekusch W, Schlager O, Exner M, Haumer M, Pernicka E, Koppensteiner R, Minar E, Rumpold H, Schillinger M, Wagner O. Beta 2 microglobulin and the risk for cardiovascular events in patients with asymptomatic carotid atherosclerosis. Stroke 2011; 42: 1826–1833.

Anand D, Lahiri A, Lim E, Hopkins D, Corder R. The relationship between plasma osteoprotegerin levels and coronary artery calcification in uncomplicated type-2 diabtetic subjects. J Am Coll Cardiol 2006; 47: 1850–1857.

Anand D, Lim E, Darko D, Bassett P, Hopkins D, Lipkin D, Corder R, Lahiri A. Determinants of progression of coronary artery calcification in type 2 diabetes: role of glycemic control and inflammatory/vascular calcification markers. J Am Coll Cardiol 2007; 50: 2218–2225.

Andersson J, Sundström J, Kurland L, Gustavsson T, Hulthe J, Elmgren A, Zilmer K, Zilmer M, Lind L. The carotid artery plaque size and echogenicity are related to different cardiovascular risk factors in the elderly: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study. Lipids 2009; 44: 397–403.

Anselmi M, Garbin U, Agostoni P, Fusaro M, Pasini A, Nava C, Keta D, Turri M, Zardini P, Vassanelli C, Lo Cascio V, Cominacini L. Plasma levels of oxidized-low-density lipoproteins are higher in patients with unstable angina and correlated with angiographic coronary artery complex plaques. Atherosclerosis 2006; 185: 114–120.

Asou Y, Rittling S, Yoshitake H, Tsuji K, Shinomiya K, Nifuji A, Denhardt D, Noda M. Osteopontin facilitates angiogenesis, accumulation os osteoblasts, and resorption in ectopic bone. Endocrinology 2001; 142: 1325–1332.

Astrand H, Stalhand J, Karlsson J, Karlsson M, Sonesson B, Länne T. In vivo estimation of the contribution of elastin and collagen to the mechanical properties in the human abdominal aorta: effect of age and sex. J Appl Physiol 2011; 110: 176–187.

Avolio A, Jones D, Tafazzoli-Shadpour M. Quantification of alterations in structure and function of elastin in the media. Hypertension 1998; 32: 170–175.

Aydin H, Deyneli O, Yavuz D, Yüksel M, Tarcin O, Yazici D, Tutepe H, Akalin S. Effect of oxidative stress on aorta and tibia osteoprotegerin gene expression in ovariectomized rats. Minerva Endocrinol 2011; 36: 107–115.

Badimon L, Storey R, Vilahur G. Update on lipids, inflammation and atherothrombosis. Thromb Haemost 2011; 105: 34–42.

Barreto D, Lenglet A, Liabeuf S, Kretschmer A, Barreto F, Nollet A, Slama M, Choukroun G, Brazier M, Massy Z. Prognostic implication of plasma osteopontin levels in patients with chronic kidney disease. Nephron Clin Pract 2011; 117: 363–372.

Bazzichi L, Ghiadoni L, Rossi A, Bernardini M, Lanza M, De Feo F, Giacomelli C, Mencaroni I, Raimo K, Rossi M, Mazzone A, Taddei S, Bombardieri S. Osteopontin is associated with increased arterial stiffness in rheumatoid arthritis. Mol Med 2009; 15: 402–406.

Beard J. Chronic lower limb ischemia. West J Med 2000; 173: 60–63.

Bellasi A, Raggi P. Techniques and technologies to assess vascular calcification. Semin Dial 2007; 20: 129–133.

Benetos A, Laurent S, Hoeks A, Boutouyrie P, Safar M. Arterial alterations with aging and high blood pressure. A noninvasive study of carotid and femoral arteries. Arterioscler Thromb 1993; 13: 90–97.

Bennett B, Scatena M, Kirk E, Rattazi M, Varon R, Averill M, Schwartz S, Giachelli C, Rosenfeld M. Osteoprotegerin inactivation accelerates advanced atherosclerotic lesion progression and calcification in older ApoE–/– mice. Arterioscler Thromb Vasc Biol 2006; 26: 2117–2124.

Bjarnegård N, Bengtsson C, Brodszki J, Sturfelt G, Nived O, Länne T. Increased aortic pulse wave velocity in middle aged women with systemic lupus erythematosus. Lupus 2006; 15: 644–650.

Blacher J, Guerin A, Pannier B, Marchais S, London G. Arterial calcifications, arterial stiffness, and cardiovascular risk in end-stage renal disease. Hypertension 2001; 38: 938–942.

Blacher J, Guerin A, Pannier B, Marchais S, Safar M, London G. Impact of aortic stiffness on survival in end-stage renal disease. Circulation 1999; 99: 2434–2439.

Blacher J, Protogerou A, Henry O, Czernichow S, Iaria P, Zhang Y, Agnoletti D, Safar M. Aortic stiffness, inflammation, denutrition and type 2 diabetes in the elderly. Diabetes Metab 2012; 38: 68–75.

Blankenberg S, Rupprecht H, Bickel C, Peetz D, Hafner G, Tiret L, Meyer J. Circulating cell adhesion molecules and death in patients with coronary artery disease. Circulation 2001; 104: 1336–1342.

Blazquez-Medela A, Garcia-Ortiz L, Gomez-Marcos M, Recio-Rodriguez J, Sanchez-Rodriguez A, Lopez-Novoa J, Martinez-Salgado C. Osteoprotegerin is associated with cardiovascular risk in hypertension and/or diabetes. Eur J Clin Invest 2012; 42: 548–556.

Bollinger A, Breddin K, Hess H, Heystraten F, Kollath J, Konttila A, Pouliadis G, Marshall M, Mey T, Mietaschk A, Roth F, Schoop W. Semiquantitative assessment of lower limb atherosclerosis from routine angiographic images. Atherosclerosis 1981; 38: 339–346.

Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer H, Lieben L, Mathieu C, Demay M. Vitamin D and human health: lessons from vitamin D receptor null mice. Endocr Rec 2008; 29: 726–776.

Boutouyrie P, Tropeano A, Asmar R, Gautier I, Benetos A, Lacolley P, Laurent S. Aortic stiffness is an independent predictor of primary coronary events in hypertensive patients: a longitudinal study. Hypertension 2002; 39: 10–15.

Boutouyrie P, Vermeersch S. Reference Values for Arterial Stiffness' Collaboration. Determinants of pulse wave velocity in healthy people and in the presence of cardiovascular risk factors: establishing normal and reference values. Eur Heart J 2010; 31: 2338–2350.

Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilatation to ankle-brachial pressure index. Circulation 2003; 108: 2093–2098.

Brewer L, Michos E, Reis J. Vitamin D in atherosclerosis, vascular disease, and endothelial function. Current Drug Targets 2011; 12: 54–60.

Brinkley T, Nicklas B, Kanaya A, Satterfield S, Lakatta E, Simonsick E, Sutton-Tyrrell K, Kritchevsky S. Plasma oxidized low-density lipoprotein levels and arterial stiffness in older adults: the health, aging, and body composition study. Hypertension 2009; 53: 846–852.

Brosius F, Hostetter T, Kelepouris E Mitsnefes M, Moe M, Moore M, Pennathur S, Smith G, Wilson P. Detection of chronic kidney disease in patients with or at increased risk of cardiovascular disease: a science advisory from the American Heart Association Kidney And Cardiovascular Disease Council; the Councils on High Blood Pressure Research, Cardiovascular Disease in the Young, and Epidemiology and Prevention; and the Quality of Care and Outcomes Research Interdisciplinary Working Group: developed in collaboration with the National Kidney Foundation. Circulation 2006; 114: 1083–1087.

Browner W, Lui L, Cummings S. Associations of serum osteorpotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. J Clin Endorinol Metab 2001; 86: 631–637.

Bucay N, Sarosi I, Dunstan C, Morony S, Tarpley J, Capparelli C, Scully S, Tan H, Xu W, Lacey D, Boyle W, Simonet W. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998; 12: 1260–1268.

Cai H, Harrison D. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000; 87: 840–844.

Cermak J, Key N, Bach R, Balla J, Jacob H, Vercellotti G. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood 1993; 82: 513–520.

Cheng K, Tiwari A, Baker C, Morris R, Hamilton G, Seifalian A. Impaired carotid and femoral viscoelastic properties and elevated intima-media thickness in peripheral vascular disease. Atherosclerosis 2002; 164: 113–120.

Chiba S, Okamoto H, Kon S, Kimura C, Murakami M, Inobe M, Matsui Y, Sugawara T, Shimizu T, Uede T, Kitabatake A. Development of atherosclerosis in osteopontin transgenic mice. Heart Vessels 2002; 16: 111–117.

Cigolini M, Iagulli M, Miconi V, Galiotto M, Lombardi S, Targher G. Serum 25-hydroxyvitamin D3 concentrations and prevalence of cardiovascular disease among type 2 diabetic patients. Diabetes Care 2006; 29: 722–724.

Cimmino G, D'Amico C, Vaccaro V, D'Anna M, Golino P. The missing link between atherosclerosis, inflammation and thrombosis: is it tissue factor? Expert Rev Cardiovasc Ther 2011; 9: 517–523.

Collins R, Burch J, Cranny G, Aguiar-Ibanez R, Craig D, Wright K, Berry E, Gough M, Kleijnen J, Westwood M. Duplex ultrasonography, magnetic resonance angiography, and computed tomography angiography for diagnosis and assessment of symptomatic, lower limb peripheral arterial disease: systematic review. BMJ 2007; 334: 1257–1265.

Criqui M, Denenberg J, Langer R, Fronek A. The epidemiology of peripheral arterial disease: importance of identifying the population at risk. Vasc Med 1997; 2: 221–226.

Criqui M. Peripheral arterial disease – epidemiological aspects. Vasc Med 2001; 6: 3–7.

Cruickshank K, Riste L, Anderson S, Wright J, Dunn G, Gosling R. Aortic pulse wave velocity and its relationship to mortality in diabetes and glucose intolerance: an integrated index of vascular function? Circulation 2002; 106: 2085–2090.

Danesh J, Wheeler J, Hirschfield G, Eda S, Eiriksdottir G, Rumley A, Lowe G, Pepys M, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med 2004; 350: 1387–1397.

Danielsson P, Truedsson L, Eriksson K, Norgren L. Inflammatory markers and IL-6 polymorphism in peripheral arterial disease with and without diabetes mellitus. Vasc Med 2005; 10: 191–198.

Dao H, Essalihi R, Bouvet C, Moreau P. Evolution and modulation of age-related medial elastocalcinosis: impact on large artery stiffness and isolated systolic hypertension. Cardiovasc Res 2005; 66: 307–317.

Davies M, Hruska K. Pathophysiological mechanisms of vascular calcification in end-stage renal disease. Kidney Int 2001; 60: 472–479.

de Kleijn D, Moll F, Hellings W, Ozsarlak-Sozer G, de Bruin P, Doevendans P, Vink A, Catanzariti L, Schoneveld A, Algra A, Daemen M, Biessen E, de Jager W, Zhang H, de Vries J, Falk E, Lim S, van der Spek P, Sze S, Pasterkamp G. Local atherosclerotic plaques are a source of prognostic biomarkers for adverse cardiovascular events. Arterioscler Thromb Vasc Biol 2010; 30: 612–619.

Delius W, Erikson U. Correlation between angiographic and hemodynamic findings in occlusions of arteries of the extremities. Vasc Surg 1969; 3: 201–210.

DeLoach S, Townsend R. Vascular stiffness: its measurement and significance for epidemiologic and outcome studies. Clin J Am Soc Nephrol 2008; 3: 184–192.

Demer L, Tintut Y. Vascular calcification: pathobiology of a multifaceted disease. Circulation 2008; 117: 2938–2948.

Denhardt D, Noda M, O'Regan A, Pavlin D, Berman J. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodelling, and cell survival. J Clin Invest 2001; 107: 1055–1061.

Derlin T, Toth Z, Papp L, Wisotzki C, Apostolova I, Habermann C, Mester J, Klutmann S. Correlation of inflammation assessed by 18F-FDG PET, active mineral deposition assessed by 18F-fluoride PET, and vascular calcification in atherosclerotic plaque: a dual-tracer PET/CT study. J Nucl Med 2011; 52: 1020–1027.

De Silva D, Woon F, Gan H, Cameron J, Kingwell B, Koh T, Chen C, Chang H, Wong M. Arterial stiffness, metabolic syndrome and inflammation amongst Asian ischaemic stroke patients. Eur J Neurol 2008; 15: 872–875.

Dimmeler S, Zeiher A. Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. Regul Pept 2000; 90: 19–25.

Donadio C, Lucchesi A, Ardini M, Giordani R. Cystatin C, beta 2-microglobulin, and retinol-binding protein as indicators of glomerular filtration rate: comparison with plasma creatinine. J Pharm Biomed Anal 2001; 24: 835–842.

Dong Y, Stallmann-Jorgensen I, Pollock N, Harris R, Keeton D, Huang Y, Li K, Bassali R, Guo D, Thomas J, Pierce G, White J, Holick M, Zhu H. A 16-week randomized clinical trial of 2000 international units daily vitamin D3 supplementation in black youth: 25-hydroxyvitamin D, adiposity, and arterial stiffness. J Clin Endocrinol Metab 2010; 95: 4584–4591.

Drummond G, Selemidis S, Griendling K, Sobey C. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. Nat Rev Drug Discov 2011; 10: 453–471.

Duprez D, Cohn J. Arterial stiffness as a risk factor for coronary atherosclerosis. Curr Atheroscler Rep 2007; 9: 139–144.

Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M, Komatsu R, Matsuo T, Itabe H, Takano T, Tsukamoto Y, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker A. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001; 103: 1955–1960.

Elahi M, Kong Y, Matata B. Oxidative stress as a mediator of cardiovascular disease. Oxid Med Cell Longev 2009; 2: 259–269.

El-Shehaby A, Zakaria A, El-Khatib M, Mostafa N. Association of fetuin-A and cardiac calcification and inflammation levels in hemodialysis patients. Scand J Clin Lab Invest 2010; 70: 575–582.

Faglia E, Favales F, Quarantiello A, Calia P, Clelia P, Brambilla G, Rampoldi A, Morabito A. Angiographic evaluation of peripheral arterial occlusive disease and its role as a prognostic determinant for major amputation in diabetic subjects with foot ulcers. Diab Care 1998; 21: 625–630.

Fahrleitner A, Dobnig H, Obernosterer A, Pilger E, Leb G, Weber K, Kudlacek S, Obermayer-Pietsch B. Vitamin D deficiency and secondary hyperparathyroidism are common complications in patients with peripheral arterial disease. J Gen Intern Med 2002; 17: 663–669.

Farrar D, Bond M, Riley W, Sawyer J. Anatomic correlates of aortic pulse wave velocity and carotid artery elasticity during atherosclerosis progression and regression in monkeys. Circulation 1991; 83: 1754–1763.

Figueiredo V, Yugar-Toledo J, Martins LC, Martins LB, de Faria A, de Haro Moraes C, Sierra C, Coca A, Moreno H. Vascular stiffness and endothelial dysfunction: correlations at different levels of blood pressure. Blood Press 2012; 21: 31–38.

Fitzgerald P, Ports T, Yock P. Contribution of localized calcium deposits to dissection after angioplasty. An observational study using intravascular ultrasound. Circulation 1992; 86: 64–70.

Fitzpatrick L, Severson A, Edwards W, Ingram R. Diffuse calcification in human coronary arteries. Association of osteopontin with atherosclerosis. J Clin Invest 1994; 94: 1597–1604.

Ford M, Smith E, Tomlinson L, Chatterjee P, Rajkumar C, Holt S. FGF-23 and osteoprotegerin are independently associated with myocardial damage in chronic

kidney disease stages 3 and 4. Another link between chronic kidney disease-mineral bone disorder and the heart. Nephrol Dial Transplant 2012; 27: 727–733.
Ford M, Tomlinson L, Smith E, Rajkumar C, Holt S. Fetuin-A is an independent determinant of change of aortic stiffness over 1 year in non-diabetic patients with CKD stages 3 and 4. Nephrol Dial Transplant 2010; 25: 1853–1858.
Forman J, Giovannucci E, Holmes M, Bischoff-Ferrari H, Tworoger S, Willett W, Curhan G. Plasma 25-hydroxyvitamin D levels and risk of incident hypertension. Hypertension 2007; 49: 1063–1069.
Fowkes F. The measurement of atherosclerotic peripheral arterial disease in epidemiological surveys. Int J Epidemiol 1988; 17: 248–254.
Fowkes F, Housley E, Cawood E, Macintyre C, Ruckley C, Prescott R. Edinburgh Artery Study: prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population. Int J Epidemiol 1991; 20: 384–392.
Fowkes F, Murray G, Butcher I, Heald C, Lee R, Chambless L, Folsom A, Hirsch A, Dramaix M, deBacker G, Wautrecht J, Kornitzer M, Newman A, Cushman M, Sutton-Tyrrell K, Fowkes F, Lee A, Price J, d'Agostino R, Murabito J, Norman P, Jamrozik K, Curb J, Masaki K, Rodríguez B, Dekker J, Bouter L, Heine R, Nijpels G, Stehouwer C, Ferrucci L, McDermott M, Stoffers H, Hooi J, Knottnerus J, Ogren M, Hedblad B, Witteman J, Breteler M, Hunink M, Hofman A, Cruqui M, Langer R, Fronek A, Hiatt W, Hamman R, Resnick H, Guralnik J, McDermott M, Ankle Brachial Index Collaboration. Ankle brachial index combined with Framingham Risk Score to predict cardiovascular events and mortality: a meta-analysis. JAMA 2008; 300: 197–208.
Frink R, Achor R, Brown A, Kincaid O, Brandenburg R. Significance of calcification of the coronary arteries. Am J Cardiol 1970; 26: 241–247.
Frost M, Grella R, Millasseau S, Jiang B, Hampson G, Fogelman I, Chowienczyk P. Relationship of calcification of atherosclerotic plaque and arterial stiffness to bone mineral density and osteoprotegerin in postmenopausal women referred for osteoporosis screening. Calcif Tissue Int 2008; 83: 112–120.
Fung E, Wilson A, Zhang F, Harris N, Edwards K, Olin J, Cooke J. A biomarker panel for peripheral arterial disease. Vasc Med 2008; 13: 217–224.
Fuseini M, Goodwin W, Ferris E, Mehta J. Does electron beam computer tomography provide added value in the diagnosis of coronary artery disease? Curr Opin Cardiol 2003; 8: 385–393.
Galis Z, Khatri J. Martix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 2002; 90: 251–262.
Garcia-Canton C, Bosch E, Ramirez A, Gonzalez Y, Auyanet I, Guerra R, Perez M, Fernández E, Toledo A, Lago M, Checa M. Vascular calcification and 25-hydroxyvitamin D levels in non-dialysis patients with chronic kidney disease stages 4 and 5. Nephrol Dial Transplant 2011; 26: 2250–2256.
Ge J, Chirillo F, Schwedtmann J, Gorge G, Haude M, Baumgart D, Shah V, von Birgelen C, Sack S, Boudoulas H, Erbel R. Screening of ruptured plaques in patients with coronary artery disease by intravascular ultrasound. Heart 1999; 81: 621–627.
Ghaisas N, Shani C, Foley B, Goggins M, Crean P, Kelly A, Kelleher D, Walsch M. Elevated levels of circulating soluble adhesion molecules in peripheral blood of patients with unstable angina. Am J Cardiol 1997; 80: 617–619.
Giachelli C, Steitz S. Osteopontin: a versatile regulator of inflammation and biomineralization. Matrix Biol 2000; 19: 615–622.

Giulietti A, van Etten E, Overbergh L, Stoffels K, Bouillon R, Mathieu C. Monocytes from type 2 diabetic patients have a pro-inflammatory profile. 1,25-dihydroxyvitamin D3 works as anti-inflammatory. Diabetes Res Clin Pract 2007; 77: 47–57.

Golledge J, Leicht A, Crowther R, Glanville S, Clancy P, Sangla KS, Spinks W, Quigley F. Determinants of endothelial function in a cohort of patients with peripheral artery disease. Cardiology 2008; 111: 51–56.

Greenwald S. Ageing of the conduit arteries. J Pathol 2007; 211: 157–172.

Gross M, Bielinski S, Suarez-Lopez J, Reiner A, Bailey K, Thyagarajan B, Carr J, Duprez D, Jacobs D Jr. Circulating soluble intercellular adhesion molecule 1 and subclinical atherosclerosis: the Coronary Artery Risk Development in Young Adults Study. Clin Chem 2012; 58: 411–420.

Guerin A, London G, Marchais S, Metivier F. Arterial stiffening and vascular calcifications in end-stage renal disease. Nephrol Dial Transplant 2000; 15: 1014–1021.

Guerin A, Pannier B, Marchais S, London G. Arterial structure and function in end-stage renal disease. Curr Hypertens Rep 2008; 10: 107–111.

Guo X, Li J, Pang W, Zhao M, Luo Y, Sun Y, Hu D. Sensitivity and specifity of ankle-brachial index for detecting angiographic stenosis of peripheral arteries. Circ J 2008; 72: 605–610.

Guzman R, Brinkley D, Schumacher P, Donahue R, Beavers H, Qin X. Tibial artery calcification as a marker of amputation risk in patients with peripheral arterial disease. J Am Coll Cardiol 2008; 51: 1967–1974.

Ha C, Kim J, Paik J, Kim O, Paik Y, Lee E, Lee J. The association of specific metabolites of lipid metabolism with markers of oxidative stress, inflammation and arterial stiffness in men with newly diagnosed type 2 diabetes. Clin Endocrinol 2012; 76: 674–682.

Halcox J, Schenke W, Zalos G, Mincemoyer R, Prasad A, Waclawiw M, Nour K, Quyyumi A. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106: 653–658.

Halliwell B. Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. Nutr Rev 1999; 57: 104–113.

Haslacher H, Perkmann T, Gruenewald J, Exner M, Endler G, Scheichenberger V, Wagner O, Schillinger M. Plasma myeloperoxidase level and peripheral arterial disease. Eur J Clin Invest 2012; 42: 463–469.

Hassoun P, Mouthon L, Barbera J, Eddahibi S, Flores S, Grimminger F, Jones P, Maitland M, Michelakis E, Morrell N, Newman J, Rabinovitch M, Schermuly R, Stenmark K, Voekel N, Yuan J, Humbert M. Inflammation, growth factors, and pulmonary vascular remodeling. J Am Coll Cardiol 2009; 54: 10–19.

Haydar A, Covic A, Colhoun H, Rubens M, Goldsmith D. Coronary artery calcification and aortic pulse wave velocity in chronic kidney disease patients. Kidney Int 2004; 65: 1790–1794.

Heald C, Fowkes F, Murray G, Price J, Ankle Brachial Index Collaboration. Risk of mortality and cardiovascular disease associated with the ankle-brachial index: systematic review. Atherosclerosis 2006; 189: 61–69.

Heilman K, Zilmer M, Zilmer K, Lintrop M, Kampus P, Kals J, Tillmann V. Arterial stiffness, carotid artery intima-media thickness and plasma myeloperoxidase level in children with type 1 diabetes. Diabetes Res Clin Pract 2009a; 84: 168–173.

Heilman K, Zilmer M, Zilmer K, Tillmann V. Lower bone mineral density in children with type 1 diabetes is associated with poor glycemic control and higher serum ICAM-1 and urinary isoprostane levels. J Bone Miner Metab 2009b; 27: 598–604.

Heitzer T, Schlinzig T, Krohn K, Meinertz T, Münzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001; 104: 2673–2678.

Henrion D, Chillon J, Godeau G, Muller F, Capdeville-Atkinson C, Hoffman M, Atkinson J. The consequences of aortic calcium overload following vitamin D3 plus nicotine treatment in young rats. J Hypertens 1991; 9: 919–926.

Heslop C, Frohlich J, Hill J. Myeloperoxidase and C-reactive protein have combined utility for long-term prediction of cardiovascular mortality after coronary angiography. J Am Coll Cardiol 2010; 55: 1102–1109.

Hirsch A, Haskal Z, Hertzer N, Bakal C, Creager M, Halperin J, Hiratzka L, Murphy W, Olin J, Puschett J, Rosenfield K, Sacks D, Stanley J, Taylor L Jr, White C, White J, White R, Antman E, Smith S Jr, Adams C, Anderson J, Faxon D, Fuster V, Gibbons R, Halperin J, Hiratzka L, Hunt S, Jacobs A, Nishimura R, Ornato J, Page R, Riegel B. ACC/AHA 2005 guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): executive summary a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease) endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. J Am Coll Cardiol 2006; 47: 1239–1312.

Hirsch A, Murphy T, Lovell M, Twillman G, Treat-Jacobson D, Harwood E, Mohler E, Creager M, Hobson R, Robertson R, Howard W, Schroeder P, Criqui M, Peripheral Arterial Disease Coalition. Gaps in public knowledge of peripheral arterial disease: the first national PAD public awareness survey. Circulation 2007; 116: 2086–2094.

Holick M. Vitamin D deficiency. N Engl J Med 2007; 357: 266–281.

Holvoet P, Collen D. Oxidized lipoproteins in atherosclerosis and thrombosis. FASEB J 1994; 8: 1279–1284.

Hopper K, Strollo D, Mauger D. Comparison of electron-beam and ungated helical CT in detecting coronary arterial calcification by using a working heart phantom and artificial coronary arteries. Radiology 2002; 222: 474–482.

Huang Y, Hu Y, Mai W, Cai X, Song Y, Wu Y, Dong Y, Huang H, He Z, Li W, Yang Y, Rao S. Plasma oxidized low-density lipoprotein is an independent risk factor in young patients with coronary artery disease. Dis Markers 2011; 31: 295–301.

Hulsmans M, Holvoet P. The vicious circle between oxidative stress and inflammation in atherosclerosis. J Cell Mol Med 2010; 14: 70–78.

Inoue N, Maeda R, Kawakami H, Shokawa T, Yamamoto H, Ito C, Sasaki H. Aortic pulse wave velocity predicts cardiovascular mortality in middle-aged and elderly Japanese men. Circ J 2009; 73: 549–553.

Iribarren C, Sidney S, Sternfeld B, Browner W. Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke, and peripheral vascular disease. JAMA 2000; 283: 2810–2815.

Isoda K, Kamezawa Y, Ayaori M, Kusuhara M, Tada N, Ohsuzu F. Osteopontin transgenic mice fed a high-cholesterol diet develop early fatty-streak lesions. Circulation 2003; 107: 679–681.

Isoda K, Nishikawa K, Kamezawa Y, Yoshida M, Kusuhara M, Moroi M, Tada N, Ohsuzu F. Osteopontin plays an important role in the development of medial thickening and neointimal formation. Circ Res 2002; 91: 77–82.

Ix J, Barrett-Connor E, Wassel C, Cummins K, Bergstrom J, Daniels L, Laughlin G. The associations of fetuin-A with subclinical cardiovascular disease in community-dwelling persons: the Rancho Bernardo Study. J Am Coll Cardiol 2011; 58: 2372–2379.

Jayalath R, Mangan S, Golledge J. Aortic calcification. Eur J Vasc Endovasc Surg 2005; 30: 476–488.

Johnson R, Leopold J, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res 2006; 99: 1044–1059.

Jones D, Go Y. Redox compartmentalization and cellular stress. Diabetes Obes Metab 2010; 12: 116–125.

Jono S, Otsuki S, Higashikuni Y, Shioi A, Mori K, Hara K, Hashimoto H, Ikari Y. Serum osteoprotegerin levels and long-term prognosis in subjects with stable coronary artery disease. J Thromb Haemost 2010; 8: 1170–1175.

Jovanovic D, Krstivojevic P, Obradovic I, Durdevic V, Dukanovic L. Serum cystatin C and beta2-microglobulin as markers of glomerular filtration rate. Ren Fail 2003; 25: 123–133.

Jung C, Lee W, Kim S, Jung J, Rhee E, Park C, Mok J, Oh K, Kim C, Park S, Kim S. The relationship between coronary artery calcification score, plasma osteoprotegerin level and arterial stiffness in asymptomatic type 2 DM. Acta Diabetol 2010; 47: 145–152.

Jung H, Baek H, Kim S. Fetuin-A, coronary artery calcification and outcome in maintenance hemodialysis patients. Clin Nephrol 2011; 75: 391–396.

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 2006a; 185: 368–374.

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. Am J Hypertens 2006b; 19: 902–908.

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 associated differently with endothelial function and arterial stiffness in healthy subjects and in patients with atherosclerosis. Scand J Clin Invest 2008; 68: 594–601.

Kampus P, Kals J, Ristimäe T, Fischer K, Zilmer M, Teesalu R. High-sensitivity C-reactive protein affects central haemodynamics and augmentation index in apparently healthy persons. J Hypertens 2004; 22: 1133–1139.

Kampus P, Kals J, Ristimäe T, Muda P, Ulst K, Zilmer K, Salonen R, Tuomainen T, Teesalu R, Zilmer M. Augmentation index and carotid intima-media thickness are differently related to age, C-reactive protein and oxidized low-density lipoprotein. J Hypertens 2007; 25: 819–825.

Kampus P, Muda P, Kals J, Ristimäe T, Fischer K, Teesalu R, Zilmer M. The relationship between inflammation and arterial stiffness in patients with essential hypertension. Int J Cardiol 2006; 112: 46–51.

Kampus P, Serg M, Kals J, Zagura M, Muda P, Karu K, Zilmer M, Eha J. Differential effects of nebivolol and metoprolol on central aortic pressure and left ventricular wall thickness. Hypertension 2011; 57: 1122–1128.
Kanda T, Takahashi T. Interleukin-6 and cardiovascular diseases. Jpn Heart J 2004; 45: 183–193.
Kato R, Momiyama Y, Ohmori R, Tanaka N, Taniguchi H, Arakawa K, Kusuhara M, Nakamura H, Ohsuzu F. High plasma levels of osteopontin in patients with restenosis after percutaneous coronary intervention. Arterioscler Thromb Vasc Biol 2006; 26: 1–2.
Kawamoto R, Kohara K, Tabara Y, Miki T, Ohtsuka N, Kusunoki T, Yorimitsu N. An association between decreased estimated glomerular filtration rate and arterial stiffness. Inter Med 2008; 47: 593–598.
Kelly R, Smith T. Cytokines and cardiac contracile function. Circulation 1997; 95: 778–781.
Kennedy M, Solomon C, Manolio T, Criqui M, Newman A, Polak J, Burke G, Enright P, Cushman M. Risk factors for declining ankle-brachial index in men and women 65 years or older: the Cardiovascular Health Study. Arch Intern Med 2005; 165: 1896–1902.
Ketteler M, Rothe H, Krüger T, Biggar P, Schlieper G. Mechanisms and treatment of extraosseous calcification in chronic kidney disease. Nat Rev Nephrol 2011; 7: 509–516.
Khandapour N, Armon M, Jennings B, Clark A, Meyer F. The association between ankle brachial pressure index and pulse wave velocity: clinical implication of pulse wave velocity. Angiology 2009; 60: 732–738.
Kiechl S, Schett G, Wenning G, Redlich K, Oberhollenzer M, Mayr A, Santer P, Smolen J, Poewe W, Willeit J. Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. Circulation 2004; 109: 2175–2180.
Kiechl S, Werner P, Knoflach M, Furtner M, Willeit J, Schett G. The osteoprotegerin/ RANK/RANKL system: a bone key to vascular disease. Expert Rev Cardiovasc Ther 2006; 4: 801–811.
Kiekara O, Riekkinen H, Soimakallio S, Länsimies E. Correlation of angiographically determined reduction of vascular lumen with lower-limb systolic pressures. Acta Chir Scand 1985; 151: 437–440.
Kim S, Lee J, Ryu O, Lee K, Kim H, Seo J, Kim S, Kim N, Baik S, Choi D, Choi K. Serum osteoprotegerin levels are associated with inflammation and pulse wave velocity. Clin Endocrinol 2005; 63: 594–598.
Kingwell B, Waddell T, Medley T, Cameron J, Dart A. Large artery stiffness predicts ischemic threshold in patients with coronary artery disease. J Am Coll Cardiol 2002; 40: 773–779.
Koos R, Brandenburg V, Mahnken A, Mühlenbruch G, Stanzel S, Günther R, Floege J, Jahnen-Dechent W, Kelm M, Kühl H. Association of fetuin-A levels with the progression of aortic valve calcification in non-dialyzed patients. Eur Heart J 2009; 30: 2054–2061.
Koshikawa M, Aizawa K, Kasai H, Izawa A, Tomita T, Kumazaki S, Tsutsui H, Koyama J, Shimodaira S, Takahashi M, Ikeda U. Elevated osteopontin levels in patients with peripheral arterial disease. Angiology 2009; 60: 42–45.
Kurata M, Okura T, Watanabe S, Fukuoka T, Higaki J. Osteopontin and carotid atherosclerosis in patients with essential hypertension. Clin Sci 2006; 111: 319–324.

Lacey D, Timms E, Tan H, Kelley M, Dunstan C, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian Y, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle W. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998; 93: 165–176.

Lamb D, Wilkins G, Leake D. The oxidative modification of low density lipoprotein by human lymphocytes. Atherosclerosis 1992; 92: 187–192.

Landry C, Ruppe M, Grubbs E. Vitamin D receptors and parathyroid glands. Endocr Pract 2011; 17: 63–68.

Laurent S, Boutouyrie P, Asmar R, Gautier I, Laloux B, Guize L, Ducimetiere P, Benetos A. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. Hypertension 2001; 37: 1236–1241.

Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, Pannier B, Vlachopoulos C, Wilkinson I, Struijker-Boudier H, European Network for Non-invasive Investigation of Large Arteries. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J 2006; 27: 2588–2605.

Laurent S, Katsahian S, Fassot C, Tropeano A, Gautier I, Laloux B, Boutouyrie P. Aortic stiffness is an independent predictor of fatal stroke in essential hypertension. Stroke 2003; 34: 1203–1206.

Lee J, Oh S, Ha W, Kwon H, Sohn T, Son H, Cha B. Serum 25-hydroxyvitamin D concentration and arterial stiffness among type 2 diabetes. Diabetes Res Clin Pract 2012; 95: 42–47.

Lehto S, Niskanen L, Suhonen M, Ronnemaa T, Laakso M. Medial artery calcification. A neglected harbinger of cardiovascular complications in non-insulin-dependent diabetes mellitus. Arterioscler Thromb Vasc Biol 1996; 16: 978–983.

Lemogoum D, Flores G, Van den Abeele W, Ciarka A, Leeman M, Degaute J, Van de Borne P, Van Bortel L. Validity of pulse pressure and augmentation index as surrogate measures of arterial stiffness during beta-adrenergic stimulation. J Hypertens 2004; 22: 511–517.

Leng G, Fowkes F, Lee A, Dunbar J, Housley E, Ruckley C. Use of ankle brachial pressure index to predict cardiovascular events and death: a cohort study. BMJ 1996; 313: 1440–1444.

Li Y, Kong J, Wei M, Chen Z, Liu S, Cao L. 1,25-Dihydroxyvitamin D_3 is a negative endocrine regulator of the renin-angiotensin system. J Clin Invest 2002; 110: 229–238.

Libby P, Ridker P, Maseri A. Inflammation and atherosclerosis. Circulation 2002; 105: 1135–1143.

Lind L. Arterial stiffness, but not endothelium-dependent vasodilation, is related to a low ankle-brachial index. Clin Physiol Funct Imaging 2011; 31: 182–187.

Liu L, Liu Z, Chen H, Zhang G, Kong Y, Kang X. Oxidized low-density lipoprotein and β-glycerophosphate synergistically induce endothelial progenitor cell ossification. Acta Pharmacol Sin 2011; 32: 1491–1497.

Llaurado G, Ceperuelo-Mallafre V, Vilardell C, Simo R, Freixenet N, Vendrell J, Gonzalez-Clemente J. Arterial stiffness is increased in patients with type 1 diabetes without cardiovascular disease: a potential role of low-grade inflammation. Diabetes Care 2012; 35: 1083–1089.

Lobbes M, Lutgens E, Heeneman S, Cleutjens K, Kooi M, van Engelshoven J, Daemen M, Nelemans P. Is there more than C-reactive protein and fibrinogen? The

prognostic value of soluble CD40 ligand, interleukin-6 and oxidized low-density lipoprotein with respect to coronary and cerebral vascular disease. Atherosclerosis 2006; 187: 18–25.
Loffredo L, Marcoccia A, Pignatelli P, Andreozzi P, Borgia M, Cangemi R, Chiarotti F, Violi F. Oxidative-stress-mediated arterial dysfunction in patients with peripheral arterial disease. Eur Heart J 2007; 28: 608–612.
Lomashvili K, Cobbs S, Hennigar R, Hardcastle K, O'Neill W. Phosphate-induced vascular calcification: role of pyrophosphate and osteopontin. J Am Soc Nephrol 2004; 15: 1392–1401.
London G, Blacher J, Pannier B, Guerin A, Marchais S, Safar M. Arterial wave reflections and survival in end-stage renal failure. Hypertension 2001; 38: 434–438.
London G, Guerin A, Marchais S, Metivier F, Pannier B, Adda H. Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. Nephrol Dial Transplant 2003; 18: 1731–1740.
London G, Pannier B. Arterial functions: how to interpret the complex physiology. Nephrol Dial Transplant 2010; 25: 3815–3823.
Mackenzie I, Wilkinson I, Cockcroft J. Assessment of arterial stiffness in clinical practice. QJM 2002; 95: 67–74.
Mackey R, Venkitachalam L, Sutton-Tyrrell K. Calcifications, arterial stiffness and atherosclerosis. Adv Cardiol 2007; 44: 234–244.
Madamanchi N, Vendrov A, Runge M. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 2005; 25: 29–38.
Mahmud A, Feely J. Effect of smoking on arterial stiffness and pulse pressure amplification. Hypertension 2003; 41: 183–187.
Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, Grassi G, Heagerty A, Kjeldsen S, Laurent S, Narkiewicz K, Ruilope L, Rynkiewicz A, Schmieder R, Struijker-Boudier H, Zanchetti A, Vahanian A, Camm J, De Caterina R, Dean V, Dickstein K, Filippatos G, Funck-Brentano C, Hellemans I, Kristensen S, McGregor K, Sechtem U, Silber S, Tendera M, Widimsky P, Zamorano J, Kjeldsen S, Erdine S, Narkiewicz K, Kiowski W, Agabiti-Rosei E, Ambrosioni E, Cifkova R, Dominiczak A, Fagard R, Heagerty A, Laurent S, Lindholm L, Mancia G, Manolis A, Nilsson P, Redon J, Schmieder R, Struijker-Boudier H, Viigimaa M, Filippatos G, Adamopoulos S, Agabiti-Rosei E, Ambrosioni E, Bertomeu V, Clement D, Erdine S, Farsang C, Gaita D, Kiowski W, Lip G, Mallion J, Manolis A, Nilsson P, O'Brien E, Ponikowski P, Redon J, Ruschitzka F, Tamargo J, van Zwieten P, Viigimaa M, Waeber B, Williams B, Zamorano J. 2007 Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur Heart J 2007; 28: 1462–1536.
Martins D, Wolf M, Pan D, Zagshir A, Tareen N, Thadhani R, Felsenfeld A, Levine B, Mehrotra R, Norris K. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: data from the Third National Health and Nutrition Examination Survey. Arch Intern Med 2007; 167: 1159–1165.
Matsubara K, Stenvinkel P, Qureshi A, Carrero J, Axelsson J, Heimbürger O, Barany P, Alvestrand A, Lindholm B, Suliman M. Inflammation modifies the association of osteportegerin with mortality in chronic kidney disease. J Nephrol 2009; 22: 774–782.

Matsuura E, Kobayashi K, Koike T, Shoenfeld Y, Khamashta M, Hughes G. Oxidized low-density lipoprotein as a risk factor of thrombosis in antiphospholipid syndrome. Lupus 2003; 12: 550–554.

Mattace-Raso F, van der Cammen T, Hofman A, van Popele N, Bos M, Schalekamp M, Asmar R, Reneman R, Hoeks A, Breteler M, Witteman J. Arterial stiffness and risk of coronary heart disease and stroke: the Rotterdam Study. Circulation 2006; 113: 657–663.

Mayer O, Filipovsky J, Seidlerova J, Vanek J, Dolejšova M, Vrzalova J, Cifkova R. The association between low 25-hydroxyvitamin D and increased aortic stiffness. J Hum Hypertens 2012 (in press).

Mazière C, Gomila C, Mazière J. Oxidized low-density lipoprotein increases osteopontin expression by generation of oxidative stress. Free Radic Biol Med 2010; 48: 1382–1387.

McDermott M, Kerwin D, Liu K, Martin G, O'Brien E, Kaplan H, Greenland P. Prevalence and significance of unrecognized lower extremity peripheral arterial disease in general medicine practice. J Gen Intern Med 2001; 16: 384–390.

McDermott M, Liu K, Greenland P, Guralnik J, Criqui M, Chan C, Pearce W, Schneider J, Ferrucci L, Celic L, Taylor L, Vonesh E, Martin G, Clark E. Functional decline in peripheral arterial disease: associations with ankle brachial index and leg symptoms. JAMA 2004; 292: 453–461.

McEniery C, McDonnell B, So A, Aitken S, Bolton C, Munnery M, Hickson S, Yasmin, Maki-Petaja K, Cockcroft J, Dixon A, Wilkinson I, Anglo-Cardiff Collaboration Trial Investigators. Aortic calcification is associated with aortic stiffness and isolated systolic hypertension in healthy individuals. Hypertension 2009; 53: 524–531.

McEniery C, Qasem A, Schmitt M, Avolio A, Cockcroft J, Wilkinson I. Endothelin-1 regulates arterial pulse wave velocity in vivo. J Am Coll Cardiol 2003; 42: 1975–1981.

McEniery C, Yasmin, Hall I, Qasem A, Wilkinson I, Cockcroft J, Anglo-Cardiff Collaboration Trial Investigators. Normal vascular aging: differential effects on wave reflection and aortic pulse wave velocity: the Anglo-Cardiff Collaborative Trial (ACCT). J Am Coll Cardiol 2005; 46: 1753–1760.

McEniery C, Yasmin, Maki-Petaja K, McDonnell B, Munnery M, Hickson S, Franklin S, Cockcroft J, Wilkinson I, Anglo-Cardiff Collaboration Trial Investigators. The impact of cardiovascular risk factors on aortic stiffness and wave reflections depends on age: the Anglo-Cardiff Collaborative Trial (ACCT III). Hypertension 2010; 56: 591–597.

McEniery C, Yasmin, McDonnell B, Munnery M, Wallace S, Rowe C, Cockcroft J, Wilkinson I, Anglo-Cardiff Collaboration Trial Investigators. Central pressure: variability and impact of cardiovascular risk factors: the Anglo-Cardiff Collaborative Trial II. Hypertension 2008; 51: 1476–1482.

Melamed M, Michos E, Post W, Astor B. 25-Hydroxyvitamin D levels and the risk of mortality in the general population. Arch Intern Med 2008a; 168: 1629–1637.

Melamed M, Muntner P, Michos E, Uribarri J, Weber C, Sharma J, Raggi P. Serum 25-hydroxyvitamin D levels and the prevalence of peripheral arterial disease: results from NHANES 2001 to 2004. Arterioscler Thromb Vasc Biol 2008b; 28: 1179–1185.

Milan A, Tosello F, Fabbri A, Vairo A, Leone D, Chiarlo M, Covella M, Veglio F. Arterial stiffness: from physiology to clinical implications. High Blood Press Cardiovasc Prev 2011; 18: 1–12.

Minoretti P, Falcone C, Calcagnino M, Emanuele E, Buzzi M, Coen E, Geroldi D. Prognostic significance of plasma osteopontin levels in patients with chronic stable angina. Eur Heart J 2006; 27: 802–807.

Mitchell G, Parise H, Benjamin E, Larson M, Keyes M, Vita J, Vasan R, Levy D. Changes in arterial stiffness and wave reflection with advancing age in healthy men and women: the Framingham Heart Study. Hypertension 2004; 43: 1239–1245.

Mody N, Parhami F, Sarafian T, Demer L. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. Free Radic Biol Med 2001; 31: 509–519.

Momiyama Y, Ohmori R, Fayad Z, Kihara T, Tanaka N, Kato R, Taniguchi H, Nagata M, Nakamura H, Ohsuzu F. Associations between plasma osteopontin levels and the severities of coronary and aortic atherosclerosis. Atherosclerosis 2010; 210: 668–670.

Moneta G, Yeager R, Antonovic R, Hall L, Caster J, Cummings C, Porter J. Accuracy of lower extremity arterial duplex mapping. J Vasc Surg 1992; 15: 275–283.

Montero I, Orbe J, Varo N, Beloqui O, Monreal J, Rodriguez J, Diez J, Libby P, Paramo J. C-reactive protein induces matrix metalloproteinase-1 and -10 in human endothelial cells: implications for clinical and subclinical atherosclerosis. J Am Coll Cardiol 2006; 47: 1369–1378.

Moore K, Freeman M. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol 2006; 26: 1702–1711.

Morony S, Tintut Y, Zhang Z, Cattley R, Van G, Dwyer D, Stolina M, Kostenuik P, Demer L. Osteoprotegerin inhibits vascular calcification without affecting atherosclerosis in ldlr(–/–) mice. Circulation 2008; 117: 411–420.

Motobe K, Tomiyama H, Koji Y, Yambe M, Gulinisa Z, Arai T, Ichihashi H, Nagae T, Ishimaru S, Yamashina A. Cut-off value of the ankle-brachial pressure index at which the accuracy of brachial-ankle pulse wave velocity measurement is diminished. Circ J 2005; 69: 55–60.

Mottram P, Haluska B, Leano R, Carlier S, Case C, Marwick T. Relation of arterial stiffness to diastolic dysfunction in hypertensive heart disease. Heart 2005; 91: 1551–1556.

Mourad J, Pannier B, Blacher J, Rudnichi A, Benetos A, London G, Safar M. Creatinine clearance, pulse wave velocity, carotid compliance and essential hypertension. Kidney Int 2001; 59: 1834–1841.

Mueller T, Dieplinger B, Gebenhuber A, Haidinger D, Schmid N, Roth N, Ebner F, Landl M, Poelz W, Haltmayer M. Serum 8-iso-prostaglandin F2alpha: a new and independent predictor of peripheral arterial disease. J Vasc Surg 2004; 40: 768–773.

Muller-Buhl U, Wiesemann A, Oser B, Kirchberger I, Strecker E. Correlation of hemodynamic and functional variables with the angiographic extent of peripheral arterial occlusive disease. Vasc Med 1999; 4: 247–251.

Nakashima A, Carrero J, Qureshi A, Hirai T, Takasugi N, Ueno T, Taniguchi Y, Lindholm B, Yorioka N. Plasma osteoprotegerin, arterial stiffness, and mortality in normoalbuminemic Japanese hemodialysis patients. Osteoporos Int 2011; 22: 1695–1701.

Newman A, Shemanski L, Manolio T, Cushman M, Mittelmark M, Polak J, Powe N, Siscovick D. Ankle-arm index as a predictor of cardiovascular disease and mortality in the Cardiovascular Health Study. Arterioscler Thromb Vasc Biol 1999; 19: 538–545.

Newman A, Siscovick D, Manolio T, Polak J, Fried L, Borhani N, Wolfson S. Ankle-arm index as a marker of atherosclerosis in the Cardiovascular Heath Study. Circulation 1993; 88: 837–845.
Nichols W. Clinical measurement of arterial stiffness obtained from noninvasive pressure waveforms. Am J Hypertens 2005; 18: 3–10.
Nichols W, O'Rourke M. McDonald's blood flow in arteries. Theoretical, Experimental and Clinical Principles. 4th edition. Oxford University Press. 1998.
Niederhoffer N, Bobryshev Y, Lartaud-Idjouadiene I, Giummelly P, Atkinson J. Aortic calcification produced by vitamin D3 plus nicotine. J Vasc Res 1997; 34: 386–398.
Niskanen L, Siitonen O, Suhonen M, Uusitupa M. Medial artery calcification predicts cardiovascular mortality in patients with NIDDM. Diabetes Care 1994; 17: 1252–1256.
Niskanen L, Suhonen M, Siitonen O, Lehtinen J, Uusitupa M. Aortic and lower limb artery calcification in type 2 (non-insulin-dependent) diabetic patients and non-diabetic control subjects. A five-year follow-up study. Atherosclerosis 1990; 84: 61–71.
Nishi K, Itabe H, Uno M, Kitazato K, Horiguchi H, Shinno K, Nagahiro S. Oxidized LDL in carotid plaques and plasma associates with plaque instability. Arterioscler Thromb Vasc Biol 2002; 22: 1649–1654.
Norgren L, Hiatt W, Dormandy J, Nehler M, Harris K, Fowkes F; TASC II Working Group, Bell K, Caporusso J, Durand-Zaleski I, Komori K, Lammer J, Liapis C, Novo S, Razavi M, Robbs J, Schaper N, Shigematsu H, Sapoval M, White C, White J, Clement D, Creager M, Jaff M, Mohler E, Rutherford R, Sheehan P, Sillesen H, Rosenfield K. Inter-society consensus for the management of peripheral arterial disease (TASC II). Eur J Vasc Endovasc Surg 2007; 33: 1–75.
Norman P, Powell J. Vitamin D, shedding light on the development of disease in peripheral arteries. Arterioscler Thromb Vasc Biol 2005; 25: 39–46.
Nylaende M, Kroese A, Stranden E, Morken B, Sandbaek G, Lindahl A, Arnesen H, Seljeflot I. Markers of vascular inflammation are associated with the extent of atherosclerosis assessed as angiographic score and treadmill walking distances in patients with peripheral arterial occlusive disease. Vasc Med 2006; 11: 21–28.
Ohmori R, Momiyama Y, Taniguchi H, Takahashi R, Kusuhara M, Nakamura H, Ohsuzu F. Plasma osteopontin levels are associated with the presence and extent of coronary artery disease. Atherosclerosis 2003; 170: 333–337.
Okuno S, Ishimura E, Kohno K, Fujino-Katoh Y, Maeno Y, Yamakawa T, Inaba M, Nishizawa Y. Serum beta2-microglobulin level is a significant predictor of mortality in maintenance haemodialysis patients. Nephrol Dial Transplant 2009; 24: 571–577.
Olson J, Edmundowicz D, Becker D, Kuller L, Orchard T. Coronary calcium in adults with type 1 diabetes: a stronger correlate of clinical coronary artery disease in men than in women. Diabetes 2000; 49: 1571–1578.
O'Rourke M. How stiffening of the aorta and elastic arteries leads to compromised coronary flow. Heart 2008; 94: 690–691.
O'Rourke M, Hashimoto J. Mechanical factors in arterial aging: a clinical perspective. J Am Coll Cardiol 2007; 50: 1–13.
Pannier B, Guerin A, Marchais S, Safar M, London G. Stiffness of capacitive and conduit arteries: prognostic significance for end-stage renal disease patients. Hypertension 2005; 45: 592–596.

Parthasarathy S, Printz D, Boyd D, Joy L, Steinberg D. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. Arteriosclerosis 1986; 6: 505–510.
Parthasarathy S, Raghavamenon A, Garelnabi M, Santanam N. Oxidized low-density lipoprotein. Methods Mol Biol 2010; 610; 403–417.
Patel R, Al Mheid I, Morris A, Ahmed Y, Kavtaradze N, Ali S, Dabhadkar K, Brigham K, Hooper W, Alexander R, Jones D, Quyyumi A. Oxidative stress is associated with impaired arterial elasticity. Atherosclerosis 2011; 218: 90–95.
Patange A, Valentini R, Du W, Pettersen M. Vitamin D and arterial wall stiffness in children with chronic kidney disease. Pediatr Cardiol 2012; 33: 122–128.
Peeters W, Moll F, Vink A, van der Spek P, de Kleijn D, de Vries J, Verheijen J, Newby A, Pasterkamp G. Collagenase matrix metalloproteinase-8 expressed in atherosclerotic carotid plaques is associated with systemic cardiovascular outcome. Eur Heart J 2011; 32: 2314–2325.
Pilz S, Tomaschitz A, Friedl C, Amrein K, Drechsler C, Ritz E, Boehm B, Grammer T, März W. Vitamin D status and mortality in chronic kidney disease. Nephrol Dial Transplant 2011; 26: 3603–3609.
Pini R, Cavallini M, Palmieri V, Marchionni N, Di Bari M, Devereux R, Masotti G, Roman M. Central but not brachial blood pressure predicts cardiovascular events in an unselected geriatric population: the ICARe Dicomano Study. J Am Coll Cardiol 2008; 51: 2432–2439.
Poole K, Loveridge N, Barker P, Halsall D, Rose C, Reeve J, Warburton E. Reduced vitamin D in acute stroke. Stroke 2006; 37: 243–245.
Pradhan A, Rifai N, Ridker P. Soluble intercellular adhesion molecule-1, soluble vascular adhesion molecule-1, and the development of symptomatic peripheral arterial disease in men. Circulation 2002; 106: 820–825.
Provan S, Angel K, Semb A, Mowinckel P, Agewall S, Atar D, Kvien T. Early prediction of increased arterial stiffness in patients with chronic inflammation: a 15-year follow-up study of 108 patients with rheumatoid arthritis. J Rheumatol 2011; 38: 606–612.
Rabkin S, Chan S, Sweeney C. Ankle-brachial index as an indicator of arterial stiffness in patients without peripheral arterial disease. Angiology 2012; 63: 150–154.
Raggi P, Bellasi A, Ferramosca E, Islam T, Muntner P, Block G. Association of pulse wave velocity with vascular and valvular calcification in hemodialysis patients. Kidney Int 2007; 71: 802–807.
Rajagopalan S, Meng X, Ramasamy S, Harrison D, Galis Z. Reactive oxygen species produced by macrophage-derived foam cells regulate the acitivity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. J Clin Invest 1996; 98: 2572–2579.
Ramaiah S, Rittling S. Pathophysiological role of osteopontin in hepatic inflammation, toxicity, and cancer. Toxicol Sci 2008; 103: 4–13.
Rasmussen L, Tarnow L, Hansen T, Parving H, Flyvbjerg A. Plasma osteoprotegerin levels are associated with glycaemic status, systolic blood pressure, kidney function and cardiovascular morbidity in type 1 diabetic patients. Eur J Endocrinol 2006; 154: 75–81.
Roy C. The elastic properties of the arterial wall. J Physiol 1881; 3: 125–159.
Recio-Mayoral A, Banerjee D, Streather C, Kaski J. Endothelial dysfunction, inflammation and atherosclerosis in chronic kidney disease – a cross-sectional study of

predialysis, dialysis and kidney-transplantation patients. Atherosclerosis 2011; 216: 446–451.
Redheuil A, Yu W, Wu C, Mousseaux E, de Cesare A, Yan R, Kachenoura N, Bluemke D, Lima J. Reduced ascending aortic strain and distensibility: earliest manifestations of vascular aging in humans. Hypertension 2010; 55: 319–326.
Reis J, Michos E, von Mühlen D, Miller E. Differences in vitamin D status as a possible contributor to the racial disparity in peripheral arterial disease. Am J Clin Nutr 2008; 88: 1469–1477.
Rennenberg R, Kessels A, Schurgers L, van Engelshoven J, de Leeuw P, Kroon A. Vascular calcifications as a marker of increased cardiovascular risk: a meta-analysis. Vasc Health Risk Manag 2009; 5: 185–197.
Resnick H, Lindsay R, McDermott M, Devereux R, Jones K, Fabsitz R, Howard B. Relationship of high and low ankle brachial index to all-cause and cardiovascular disease mortality: the Strong Heart Study. Circulation 2004; 109: 733–739.
Resnick L, Militianu D, Cunnings A, Pipe J, Evelhoch J, Soulen R. Direct magnetic resonance determination of aortic distensibility in essential hypertension: relation to age, abdominal visceral fat, and in situ intracellular free magnesium. Hypertension 1997; 30: 654–659.
Reynolds J, Skepper J, McNair R, Kasama T, Gupta K, Weissberg P, Jahnen-Dechent W, Shanahan C. Multifunctional roles for serum protein fetuin-A in inhibition of human vascular smooth muscle cell calcification. J Am Soc Nephrol 2005; 16: 2920–2930.
Ridker P, Cannon C, Morrow D, Rifai N, Rose L, McCabe C, Pfeffer M, Braunwald E, Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 (PROVE IT – TIMI 22) Investigators. C-reactive protein levels and outcomes after statin therapy. N Engl J Med 2005; 352: 20–28.
Ridker P, Cushman M, Stampfer M, Tracy R, Hennekens C. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997; 336: 973–979.
Ridker P, Danielson E, Fonseca F, Genest J, Gotto A, Kastelein J, Koenig W, Libby P, Lorenzatti A, Macfadyen J, Nordestgaard B, Shepherd J, Willerson J, Glynn R, JUPITER Trial Study Group. Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial. Lancet 2009; 373: 1175–1182.
Ridker P, Hennekens C, Roitman-Johnson B, Stampfer M, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998; 351–388.
Ridker P, Rifai N, Stampfer M, Hennekens C. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 2000; 101: 1767–1772.
Rohde L, Hennekens C, Ridker P. Cross-sectional study of soluble intercellular adhesion molecule-1 and cardiovascular risk factors in apparently healthy men. Arterioscler Thromb Vasc Biol 1999; 19: 1595–1599.
Rojas A, Romay S, Gonzalez D, Herrera B, Delgado R, Otero K. Regulation of endothelial nitric oxide synthase expression by albumin-derived advanced glycosylation end products. Circ Res 2000; 86: 50–54.
Roos M, Richart T, Kouznetsova T, von Eynatten M, Lutz J, Heemann U, Baumann M, Staessen J. Fetuin-A and arterial stiffness in patients with normal kidney function. Regul Pept 2009; 154: 39–43.

Rosoky R, Wolosker N, Nasser M, Zerati A, Gidlund M, Puech-Leao P. Oxidized low-density lipoprotein and ankle-brachial pressure index in patients with clinically evident periphral arterial disease. Clinics 2010; 65: 383–387.

Ross R. Atherosclerosis – an inflammatory disease. N Engl J Med 1999; 340: 115–26.

Rumberger J, Simons D, Fitzpatrick L, Sheedy P, Schwartz R. Coronary artery calcium area by electron-beam computed tomography and coronary atheroslerotic plaque area. A histopathologic correlative study. Circulation 1995; 92: 2157–2162.

Safar M, Blacher J, Pannier B, Guerin A, Marchais S, Guyonvarch P, London G. Central pulse pressure and mortality in end-stage renal disease. Hypertension 2002; 39: 735–738.

Safar M, Levy B, Struijker-Boudier H. Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. Circulation 2003; 107: 2864–2869.

Saijo Y, Utsugi M, Yoshioka E, Horikawa N, Sato T, Gong Y, Kishi R. Relationship of beta2-microglobulin to arterial stiffness in Japanese subjects. Hypertens Res 2005; 28: 505–511.

Sakuragi S, Abhayaratna W. Arterial stiffness: methods of measurement, physiologic determinants and prediction of cardiovascular outcomes. Int J Cardiol 2010; 138: 112–118.

Salum E, Zilmer M, Kampus P, Kals J, Unt E, Serg M, Zagura M, Blöndal M, Zilmer K, Eha J. Effects of a long-term military mission on arterial stiffness, inflammation markers, and vitamin D level. Int J Cardiol 2011; 151: 106–107.

Scatena M, Liaw L, Giachelli C. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. Arterioscler Thromb Vasc Biol 2007; 27: 2302–2309.

Schäfer C, Heiss A, Schwartz A, Westenfeld R, Ketteler M, Floege J, Müller-Esterl W, Schinke T, Jahnen-Dechent W. The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systematically acting inhibitor of ectopic calcification. J Clin Invest 2003; 112: 357–366.

Schierbeck L, Jensen T, Bang U, Jensen G, Kober L, Jensen J. Parathyroid hormone and vitamin D – markers for cardiovascular and all cause mortality in heart failure. Eur J Heart Fail 2011; 13: 626–632.

Schinke T, Amendt C, Trindl A, Pöschke O, Müller-Esterl W, Jahnen-Dechent W. The serum protein alpha2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. J Biol Chem 1996; 271: 20789–20796.

Schnabel R, Larson M, Dupuis J, Lunetta K, Lipinska I, Meigs J, Yin X, Rong J, Vita J, Newton-Cheh C, Levy D, Keaney J, Vasan R, Mitchell G, Benjamin E. Relations of inflammatory biomarkers and common genetic variants with arterial stiffness and wave reflection. Hypertension 2008; 51: 1651–1657.

Schoppet M, Preissner K, Hofbauer L. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler Tromb Vasc Biol 2002; 22: 549–553.

Schulz E, Gori T, Münzel T. Oxidative stress and endothelial dysfunction in hypertension. Hypertens Res 2011; 34: 665–673.

Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brümmer J, Gutzki F, Berger J, Frölich J, Böger R. Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study. Circulation 2004; 109: 843–848.

Scialla J, Leonard M, Townsend R, Appel L, Wolf M, Budoff M, Chen J, Lustigova E, Gadegbeku C, Glenn M, Hanish A, Raj D, Rosas S, Seliger S, Weir M, Parekh R, CRIC Study Group. Correlates of osteoprotegerin and association with aortic pulse wave velocity in patients with chronic kidney disease. Clin J Am Soc Nephrol 2011; 6: 2612–2619.

Scragg R, Jackson R, Holdaway I, Lim T, Beaglehole R. Myocardial infarction is inversely associated with plasma 25-hydroxyvitamin D3 levels: a community-based study. Int J Epidemiol 1990; 19: 559–563.

Selvin E, Erlinger T. Prevalence of and risk factors for peripheral arterial disease in the United States: results form the National Health and Nutrition Examination Survey 1999–2000. Circulation 2004; 110: 738–743.

Shan J, Resnick L, Lewanczuk R, Karpinski E, Li B, Pang P. 1,25-Dihydroxyvitamin D as a cardiovascular hormone. Effects on calcium current and cytosolic free calcium in vascular smooth muscle cells. Am J Hypertens 1993; 6: 983–988.

Shao J, Cai J, Towler D. Molecular mechanisms of vascular calcification: lessons learned from the aorta. Arterioscler Thromb Vasc Biol 2006; 26: 1423–1430.

Sharman J, Lim R, Qasem A, Coombes J, Burgess M, Franco J, Garrahy P, Wilkinson I, Marwick T. Validation of a generalized transfer function to noninvasively derive central blood pressure during exercise. Hypertension 2006; 47: 1203–1208.

Shi C, Zhu Y, Su Y, Chung L, Cheng T. Beta2-microglobulin: emerging as a promising cancer therapeutic target. Drug Discov Today 2009; 14: 25–30.

Shinkai S, Chaves P, Fujiwara Y, Watanabe S, Shibata H, Yoshida H, Suzuki T. Beta2-microglobulin for risk stratification of total mortality in the elderly population: comparison with cystatin C and C-reactive protein. Arch Intern Med 2008; 168: 200–206.

Shokawa T, Imazu M, Yamamoto H, Toyofuku M, Tasaki N, Okimoto T, Yamane K, Kohno N. Pulse wave velocity predicts cardiovascular mortality: findings from the Hawaii-Los Angeles-Hiroshima study. Circ J 2005; 69: 259–264.

Shirwany N, Zou M. Arterial stiffness: a brief review. Acta Pharmacol Sin 2010; 31: 1267–1276.

Shroff R, Egerton M, Bridel M, Shah V, Donald A, Cole T, Hiorns M, Deanfield J, Rees L. A bimodal association of vitamin D levels and vascular disease in children on dialysis. J Am Soc Nephrol 2008a; 19: 1239–1246.

Shroff R, McNair R, Figg N, Skepper J, Schurgers L, Gupta A, Hiorns M, Donald A, Deanfield J, Rees L, Shanahan C. Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis. Circulation 2008b; 118: 1748–1757.

Sies H. Oxidative stress: from basic research to clinical application. Am J Med 1991; 91: 31–38.

Sigrist M, Taal M, Bungay P, McIntyre C. Progressive vascular calcification over 2 years is associated with arterial stiffening and increased mortality in patients with stages 4 and 5 chronic kidney disease. Clin J Am Soc Nephrol 2007; 2: 1241–1248.

Silvestro A, Scopacasa F, Ruocco A, Oliva G, Schiano V, Zincarelli C, Brevetti G. Inflammatory status and endothelial function in asymptomatic and symptomatic peripheral arterial disease. Vasc Med 2003; 8: 225–232.

Simionescu M. Implications of early structural-functional changes in the endothelium for vascular disease. Arterioscler Thromb Vasc Biol 2007; 27: 266–274.

Soejima H, Irie A, Fukunaga T, Sugamura K, Kojima S, Sakamoto T, Yoshimura M, Kishikawa H, Nishimura Y, Ogawa H. Elevated plasma osteopontin levels were

associated with osteopontin expression of CD4+ T cells in patients with unstable angina. Circ J 2006; 70: 851–856.
Sonesson B, Hansen F, Stale H, Länne T. Compliance and diameter in the human abdominal aorta – the influence of age and sex. Eur J Vasc Surg 1993; 7: 690–697.
Speer G, Fekete B, El Hadj Othmane T, Szabo T, Egresits J, Fodor E, Kiss I, Logan A, Nemcsik J, Szabo A, Nemeth Z, Szathmari M, Tisler A. Serum osteoprotegerin level, carotid-femoral pulse wave velocity and cardiovascular survival in haemodialysis patients. Nephrol Dial Transplant 2008; 23: 3256–3262.
Speer M, McKee M, Guldberg R, Liaw L, Yang H, Tung E, Karsenty G. Giachelli C. Inactivation of the osteopontin gene enhances vascular calcification of matrix Gla protein-deficient mice: evidence for osteopontin as an inducible inhibitor of vascular calcification in vivo. J Exp Med 2002; 196: 1047–1055.
Steitz S, Speer M, McKee M, Liaw L, Almeida M, Yang H, Giachelli C. Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. Am J Pathol 2002; 161: 2035–2046.
Stocker R, Keaney J. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004; 84: 1381–1478.
Sugden J, Davies J, Witham M, Morris A, Struthers A. Vitamin D improves endothelial function in patients with type 2 diabetes mellitus and low vitamin D levels. Diabet Med 2008; 25: 320–325.
Sutliff R, Walp E, El-Ali A, Elkhatib S, Lomashvili K, O'Neill W. Effect of medial calcification on vascular function in uremia. Am J Physiol Renal Physiol 2011; 301: 78–83.
Szmitko P, Wang C, Weisel R, de Almeida J, Anderson T, Verma S. New markers of inflammation and endothelial cell activation: Part I. Circulation 2003; 108: 1917–1923.
Takayama F, Miyazaki S, Morita T, Hirasawa Y, Niwa T. Dialysis-related amyloidosis of the heart in long-term hemodialysis patients. Kidney Int Suppl 2001; 78: 172–176.
Talib A, Nakagawa N, Saito E, Matsuki M, Kobayashi M, Akasaka K, Hirayama T, Ishida H, Sato N, Hasebe N. The balance of fetuin-A and osteoprotegerin is independently associated with disatolic dysfunction in hemodialysis patients. Hypertens Res 2012; 35: 426–433.
Tanaka H, DeSouza C, Seals D. Absence of age-related increase in central arterial stiffness in physically active women. Arterioscler Thromb Vasc Biol 1998; 18: 127–132.
Tang F, Chen S, Wu X, Wang T, Chen J, Li J, Bao L, Huang H, Liu P. Hypercholesterolemia accelerates vascular calcification induced by excessive vitamin D via oxidative stress. Calcif Tissue Int 2006; 79: 326–339.
Tang F, Wu X, Wang T, Wang P, Li R, Zhang H, Gao J, Chen S, Bao L, Huang H, Liu P. Tashinone II A attenuates atherosclerotic calcification in rat model by inhibition of oxidative stress. Vascul Pharmacol 2007; 46: 427–438.
Teng M, Wolf M, Ofsthun M, Lazarus J, Hernan M, Camargo C, Thadhani R. Activated injectable vitamin D and hemodialysis survival: a historical cohort study. J Am Soc Nephrol 2005; 16: 1115–1125.
Toprak A, Reddy J, Chen W, Srinivasan S, Berenson G. Relation of pulse pressure and arterial stiffness to concentric left ventricular hypertrophy in young men (from the Bogalusa Heart Study). Am J Cardiol 2009; 103: 978–984.

Tsimikas S, Bergmark C, Beyer R, Patel R, Pattison J, Miller E, Juliano J, Witztum J. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. J Am Coll Cardiol 2003; 41: 360–370.

Tsimikas S, Kiechl S, Willeit J, Mayr M, Miller E, Kronenberg F, Xu Q, Bergmark C, Weger S, Oberhollenzer F, Witztum J. Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. J Am Coll Cardiol 2006; 47: 2219–2228.

Tsuchikura S, Shoji T, Kimoto E, Shinohara K, Hatsuda S, Koyama H, Emoto M, Nishizawa Y. Central versus peripheral arterial stiffness in association with coronary, cerebral and peripheral artery disease. Atherosclerosis 2010; 211: 480–485.

Tsuchiya M, Suzuki E, Egawa K, Nishio Y, Maegawa H, Morikawa S, Inubushi T, Kashiwagi A. Abnormal peripheral circulation in type 2 diabetic patients with normal ankle-brachial index associates with coronary atherosclerosis, large artery stiffness, and peripheral vascular resistance. Diabetes Res Clin Pract 2005; 70: 253–262.

Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and heart failure. Am J Physiol Heart Circ Physiol 2011; 301: 2181–2190.

Turesson C, Jacobsson L, Ryden Ahlgren A, Sturfelt G, Wollmer P, Länne T. Increased stiffness of the abdominal aorta in women with rheumatoid arthritis. Rheumatology 2005; 44: 896–901.

Tuttolomondo A, Di Raimondo D, Pecoraro R, Serio A, D'Aguanno G, Pinto A, Licata G. Immune-inflammatory markers and arterial stiffness indexes with acute ischemic stroke. Atherosclerosis 2010; 213: 311–318.

Tzoulaki I, Murray G, Lee A, Rumley A, Lowe G, Fowkes F. C-reactive protein, interleukin-6, and soluble adhesion molecules as predictors of progressive peripheral atherosclerosis in the general population: Edinburgh Artery Study. Circulation 2005; 112: 976–983.

Urbonaviciene G, Frystyk J, Flyvbjerg A, Urbonavicius S, Henneberg E, Lindholt J. Markers of inflammation in relation to long-term cardiovascular mortality in patients with lower-extremity peripheral arterial disease. Int J Cardiol 2012 (in press).

Van Bortel L, Laurent S, Boutouyrie P, Chowienczyk P, Cruickshank J, De Backer T, Filipovsky J, Huybrechts S, Mattace-Raso F, Protogerou A, Schillaci G, Segers P, Vermeersch S, Weber T, Artery Society, European Society of Hypertension Working Group on Vascular Structure and Function, European Network for Noninvasive Investigation of Large Arteries. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. J Hypertens 2012; 30: 445–448.

Van Campenhout A, Golledge J. Osteoprotegerin, vascular calcification and atherosclerosis. Atherosclerosis 2009; 204: 321–329.

Van der Feen C, Neijens F, Kanters S, Mali W, Stolk R, Banga J. Angiographic distribution of lower extremity atherosclerosis in patients with and without diabetes. Diabet Med 2002; 19: 366–370.

Van Der Meer I, De Maat M, Hak A, Kiliaan A, Del Sol A, Van Der Kuip D, Nijhuis R, Hofman A, Witteman J. C-reactive protein predicts progression of atherosclerosis measured at various sites in the arterial tree: the Rotterdam Study. Stroke 2002; 33: 2750–2755.

Van Popele N, Grobbee D, Bots M, Asmar R, Topouchian J, Reneman R, Hoeks A, van der Kuip D, Hofman A, Witteman J. Association between arterial stiffness and atherosclerosis: the Rotterdam Study. Stroke 2001; 32: 454–460.

Vattikuti R, Towler D. Osteogenic regulation of vascular calcification: an early perspective. Am J Physiol Endocrinol Metab 2004; 286: 686–696.

Venugopal S, Devaraj S, Yuhanna I, Shaul P, Jialal I. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. Circulation 2002; 106: 1439–1441.

Verbeke F, Van Biesen W, Honkanen E, Wikström B, Jensen P, Krzesinski J, Rasmussen M, Vanholder R, Rensma P, CORD Study Investigators. Prognostic value of aortic stiffness and calcification for cardiovascular events and mortality in dialysis patients: outcome of the calcification outcome in renal disease (CORD) study. Clin J Am Soc Nephrol 2011; 6: 153–159.

Verbeke F, Vanholder R, Rensma P, Wikström B, Jensen P, Krzesinski J, Rasmussen M, Van Biesen W, Honkanen E. Role of aortic calcification, stiffness and wave reflections in cardiovascular risk in dialysis patients: baseline data from the CORD study. Artery Res 2010; 4: 81–90.

Vidula H, Tian L, Liu K, Criqui M, Ferrucci L, Pearce W, Greenland P, Green D, Tan J, Garside D, Guralnik J, Ridker P, Rifai N, McDermott M. Biomarkers of inflammation and thrombosis as predictors of near-term mortality in patients with peripheral arterial disease: a cohort study. Ann Intern Med 2008; 148: 85–93.

Virmani R, Burke A, Farb A. Plaque morphology in sudden coronary death. Cardiologia 1998; 43: 267–271.

Vita J, Hamburg N. Does endothelial dysfunction contribute to the clinical status of patients with peripheral arterial disease? Can J Cardiol 2010; 26: 45–50.

Vitovski S, Phillips J, Sayers J, Croucher P. Investigating the interaction between osteoprotegerin and receptor activator of NF-kappa B or tumor necrosis factor-related apoptosis inducing ligand: evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways. J Biol Chem 2007; 282: 31601–31609.

Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. J Am Coll Cardiol 2010; 55: 1318–1327.

Wada T, McKee M, Steitz S, Giachelli C. Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. Circ Res 1999; 84; 166–178.

Waller A, Sanchez-Ross M, Kaluski E, Klapholz M. Osteopontin in cardiovascular disease: a potential therapeutic target. Cardiol Rev 2010; 18: 125–131.

Wang A, Woo J, Lam C, Wang M, Chan I, Gao P, Lui S, Li P, Sanderson J. Associations of serum fetuin-A with malnutrition, inflammation, atherosclerosis and valvular calcification syndrome and outcome in peritoneal dialysis patients. Nephrol Dial Transplant 2005; 20: 1676–1685.

Wang H, Zhang M, Soda K, Sama A, Tracey K. Fetuin protects the fetus from TNF. Lancet 1997; 350: 861–862.

Wang W, Hein T, Zhang C, Zaweija D, Liao J, Kuo L. Oxidized low-density lipoprotein inhibits coronary arterial dilatation by up-regulating endothelial arginase I. Microcirculation 2011; 18: 36–45.

Wang Y, Halks-Miller M, Vergona R, Sullivan M, Fitch R, Mallari C, Martin-McNulty B, da Cunha V, Freay A, Rubanyi G, Kauser K. Increased aortic stiffness assessed by pulse wave velocity in apolipoprotein E-deficient mice. Am J Physiol Heart Circ Physiol 2000; 278: 428–434.

Watson N, Sutton-Tyrrell K, Youk A, Boudreau R, Mackey R, Simonsick E, Rosano C, Hardy S, Windham B, Harris T, Najjar S, Lakatta E, Atkinson H, Johnson K, Bauer D, Newman A, Health ABC study. Arterial stiffness and gait in older adults with and without peripheral arterial disease. Am J Hypertens 2011; 24: 90–95.

Weber T, Auer J, O'Rourke M, Kvas E, Lassnig E, Lamm G, Stark N, Rammer M, Eber B. Increased arterial wave reflections predict severe cardiovascular events in patients undergoing percutaneous coronary interventions. Eur Heart J 2005; 26: 2657–2663.

Wendt T, Harja E, Bucciarelli L, Qu W, Lu Y, Rong L, Jenkins D, Stein G, Schmidt A, Yan S. RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. Atherosclerosis 2006; 185: 70–77.

Wilkinson I, Franklin S, Hall I, Tyrrell S, Cockcroft J. Pressure amplification explains why pulse pressure is unrelated to risk in young subjects. Hypertension 2001; 38: 1461–1466.

Wilkinson I, Fuchs S, Jansen I, Spratt J, Murray G, Cockcroft J, Webb D. Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. J Hypertens 1998; 16: 2079–2084.

Wilkinson I, Prasad K, Hall I, Thomas A, MacCallum H, Webb D, Frenneaux M, Cockcroft J. Increased central pulse pressure and augmentation index in subjects with hypercholesterolemia. J Am Coll Cardiol 2002; 39: 1005–1011.

Willum-Hansen T, Staessen J, Torp-Pedersen C, Rasmussen S, Thijs L, Ibsen H, Jeppesen J. Prognostic value of aortic pulse wave velocity as index of arterial stiffness in the general population. Circulation 2006; 113: 664–670.

Wilson P, Kauppila L, O'Donnell C, Kiel D, Hannan M, Polak J, Cupples L. Abdominal aortic calcific deposits are an important predictor of vascular morbidity and mortality. Circulation 2001; 103: 1529–1534.

Wilson A, Kimura E, Harada R, Nair N, Narasimhan B, Meng X, Zhang F, Beck K, Olin J, Fung E, Cooke J. Beta2-microglobulin as a biomarker in peripheral arterial disease: proteomic profiling and clinical studies. Circulation 2007; 116: 1396–1403.

Witkowska A, Borawska M. Soluble intercellular adhesion molecule-1 (sICAM-1): an overview. Eur Cytokine Netw 2004; 15: 91–98.

Wong N, Lopez V, Allison M, Detrano R, Blumenthal R, Folsom A, Folsom A, Ouyang P, Criqui M. Abdominal aortic calcium and multi-site atherosclerosis: the multi-ethnic study of atherosclerosis. Atherosclerosis 2011; 214: 436–41.

Xie J, Yi Q. Beta2-microglobulin as a potential initiator of inflammatory responses. Trends Immunol 2003; 24: 228–229.

Yasmin, McEniery C, Wallace S, Dakham Z, Pulsalkar P, Maki-Petaja K, Ashby M, Cockcroft J, Wilkinson I. Matrix metalloproteinase-9 (MMP-9), MMP-2, and serum elastase activity are associated with systolic hypertension and arterial stiffness. Arterioscler Thromb Vasc Biol 2005; 25: 372–378.

Yasmin, McEniery C, Wallace S, Mackenzie I, Cockcroft J, Wilkinson I. C-reactive protein is associated with arterial stiffness in apparently healthy individuals. Arterioscler Thromb Vasc Biol 2004; 24: 967–974.

Yudkin J, Kumari M, Humphries S, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 2000; 148: 209–214.

Zagura M, Serg M, Kampus P, Zilmer M, Eha J, Unt E, Lieberg J, Cockcroft J, Kals J. Aortic stiffness and vitamin D are independent markers of aortic calcification in patients with peripheral arterial disease and in healthy subjects. Eur J Vasc Endovasc Surg 2011; 42: 689–695.

Zhang J, Fu M, Myles D, Zhu X, Du J, Cao X, Chen Y. PDGF induces osteoprotegerin expression in vascular smooth muscle cells by multiple signal pathways. FEBS Lett 2002; 521: 180–184.

Zhou R, Vendrov A, Tchivilev I, Niu X, Molnar K, Rojas M, Carter J, Tong H, Stouffer G, Madamanchi N, Runge M. Mitochondrial oxidative stress in aortic stiffening with age: the role of smooth muscle cell function. Arterioscler Thromb Vasc Biol 2012; 32: 745–755.

Ziegler S, Kudlacek S, Luger A, Minar E. Osteoprotegerin plasma concentrations correlate with severity of peripheral artery disease. Atherosclerosis 2005; 182: 175–180.

Zilmer M, Karelson E, Vihalemm T, Rehema A, Zilmer K. Inimorganismi biomolekulid ja nende meditsiiniliselt olulised ülesanded. Inimorganismi metabolism, selle häired ja haigused. Avita. 2010.

Zittermann A, Schleithoff S, Koerfer R. Putting cardiovascular disease and vitamin D insufficiency into perspective. Br J Nutr 2005; 94: 483–492.

Zittermann A, Schleithoff S, Tenderich G, Berthold H, Körfer R, Stehle P. Low vitamin D status: a contributing factor in the pathogenesis of congestive heart failure? J Am Coll Cardiol 2003; 41: 105–112.

Zumrutdal A, Sezer S, Demircan S, Seydaoglu G, Ozdemir F, Haberal M. Cardiac troponin I and beta 2 microglobulin as risk factors for early-onset atherosclerosis in patients on haemodialysis. Nephrology 2005; 10: 453–458.

SUMMARY IN ESTONIAN

Arterikahjustuste biokeemiline, funktsionaalne ja strukturaalne profileerimine ateroskleroosi korral

Arterite jäikus iseloomustab arterite laienemisvõimet vererõhu tõusu mõjul. Jäigad arterid tõstavad vasaku vatsakese järelkoormust, mis omakorda põhjustab vasaku vatsakese hüpertroofiat ja suurendab müokardi hapnikuvajadust. Lisaks langetab arterite jäigenemine diastoolset vererõhku ja vähendab veelgi koronaarperfusiooni, mis viib *circulus vitiosus*'e tekkeni. Pulsilaine leviku kiiruse registreerimine aordis on tänapäeval arteriaalse jäikuse hindamise kuldstandard. Aordi suurenenud jäikus ennustab iseseisvalt üld- ja kardiovaskulaarset suremust kõrge riskiga patsientidel ja üldrahvastikus. Tuginedes mitmete rahvastikupõhiste uuringute andmetele, on kindlaks tehtud pulsilaine kiiruse referentsväärtused. Euroopa Hüpertensiooni Ühingu ja Euroopa Kardioloogide Seltsi hüpertensiooni ravijuhendis peetakse pulsilaine kiiruse suurenemist üle 12 m/s subkliinilise organkahjustuse markeriks.

Arterite kaltsifikatsioonil on oluline roll ateroskleroosi patogeneesis. Mitmed uuringud on näidanud, et arterite kaltsifikatsioon ja jäigenemine on omavahel seotud, kuid nende muutuste täpne vahekord aterogeneesis on veel lõplikult teadmata. Arterite kaltsifikatsioon haarab arterite sise- ja/või keskkesta. Sisekesta kaltsifikatsioon esineb eeskätt ateroskleroosi korral. Seevastu meedia kaltsifikatsioon (arteriioskleroos) on seotud diabeedi, kroonilise neerupuudulikkuse, hüpertensiooni ja vananemisega. Nii eksperimentaalsete kui kliiniliste uuringute tulemused viitavad sellele, et kaltsiumi ladestumine aordi seinas on reguleeritud mitmete kaltsifikatsiooni aktivaatorite ja inhibiitoritega.

Osteoprotegeriin on kaltsifikatsiooni inhibiitor, mis kuulub tuumornekroosifaktori retseptorite perekonda. Osteoprotegeriini toodetakse luudes, endoteelis ja veresoonte silelihasrakkudes. Hiirtel on osteoprotegeriini puudulikkus seotud raskekujulise osteoporoosi ja kaltsiumi ladestumisega aordi ja neeruarterite seinte keskkestas. Seega loomkatsete tulemused viitavad sellele, et osteoprotegeriinil on protektiivne roll ateroskleroosi korral. Seevastu inimuuringud on näidanud, et osteoprotegeriini taseme suurenemine on seotud südame isheemiatõve, arterite kaltsifikatsiooni, diabeetilise mikroangiopaatia ja alajäsemete arterite ateroskleroosiga.

Osteopontiin kuulub samuti kaltsifikatsiooni inhibiitorite hulka. Loommudelites on näidatud, et osteopontiini defitsiit on seotud arterite väljendunud kaltsifikatsiooniga. Tänapäeval on kindlaks tehtud, et osteopontiin osaleb põletiku poolt vahendatud aterosklerootilises protsessis. Osteopontiini toodetakse aterosklerootilistes naastudes, mis soodustab makrofaagide tungimist subendoteliaalsesse kihti ning põletikuliste tsütokiinide produktsiooni. Seerumi osteopontiini tase on seotud südame isheemiatõve raskusastmega ja ennustab iseseisvalt kardiovaskulaarsete tüsistuste teket.

Vitamiin D on oluline lüli luu ainevahetuse ning südame- ja veresoonkonnahaiguste vahel. Vitamiin D roll arterite kaltsifikatsiooni tekkimises ja arenemises on vastuoluline. Uuringute tulemused viitavad sellele, et nii vitamiin D defitsiit kui liigsus on seotud arterite väljendunud kaltsifikatsiooniga. Lisaks kaltsiumi ainevahetuse regulatsioonile vähendab vitamiin D reniin-angiotensiin-süsteemi aktiivsust ja pärsib põletikuliste tsütokiinide sekretsiooni.

Tänapäeval on kindlaks tehtud, et põletikul on keskne roll ateroskleroosi patogeneesis. Suured prospektiivsed uuringud on näidanud, et C-reaktiivse valgu taseme suurenemine ennustab sõltumatult teistest riskifaktorites kardiovaskulaarset suremust nii üldrahvastikus kui suure riskiga patsientidel. β_2-mikroglobuliin osaleb samuti põletikulise protsessi regulatsioonis. β_2-mikroglobuliini sisaldus seerumis on suurenenud infektsioonide, autoimmuunhaiguste ja ateroskleroosi korral ning on üldsuremuse riski sõltumatuks teguriks.

Tugev kestev oksüdatiivne stress on tähtis aterosklerootilise protsessi vallandaja ja edasiviija. Reaktiivsete osakeste liigteke kahjustab endoteeli funktsiooni, suurendab arterite jäikust, soodustab vahtrakkude teket ja veresoonte silelihasrakkude proliferatsiooni. Oksüdeeritud madala tihedusega lipoproteiin on oksüdatiivse stressi marker, mis omab tugevat proaterogeenset toimet ja ennustab kardiovaskulaarseid tüsistusi ja suremust.

Alajäsemete arterite ateroskleroos on levinud haigus, mis avaldub vahelduva lonkamise ja rahuolekuvaludena, kuid võib raskematel juhtudel tüsistuda gangreeniga. Samuti on nendel haigetel oluliselt suurenenud ka müokardi- ja ajuinfarkti tekke risk. Varasemate uuringute tulemused viitavad sellele, et süsteemne põletik, tugev oksüdatiivne stress ja arterite kaltsifikatsioon ning jäigenemine võivad mõjutada alajäseme arterite ateroskleroosi kliinilist kulgu. Ometi on vähe andmeid nende tegurite vaheliste seoste kohta alajäseme arterite ateroskleroosi korral.

Uurimuse eesmärgid

Käesoleva töö eesmärgiks oli uurida aordi jäikust (funktsionaalne profileerimine), aordi kaltsifikatsiooni ja angiograafilist skoori (strukturaalne profileerimine); samuti hinnata kaltsifikatsiooni, põletiku ja oksüdatiivse stressi biomarkerite taset (biokeemiline profileerimine) alajäsemete arterite ateroskleroosiga patsientidel ja kliiniliselt tervetel uuritavatel. Lisaks uurida arterikahjustuste funktsionaalse, biokeemilise ja strukturaalse profiili vahelist seost ja hinnata nende potentsiaalset rolli ateroskleroosi korral.

Uurimuse täpsed eesmärgid olid järgmised:

1. Võrrelda pulsilaine kiirust aordis (funktsionaalne profileerimine) ja osteoprotegeriini ning oksüdeeritud madala tihedusega lipoproteiini seerumi taset (biokeemiline profileerimine) ja hinnata nendevahelist seost alajäsemete arterite ateroskleroosiga patsientidel ja kliiniliselt tervetel uuritavatel.

2. Määrata pulsilaine kiirust aordis (funktsionaalne profileerimine), β_2-mikroglobuliini ja fetuiin-A plasma taset (biokeemiline profileerimine) alajäsemete arterite ateroskleroosiga patsientidel ja kliiniliselt tervetel uuritavatel ja testida, kas β_2-mikroglobuliini suurenenud tase on seotud aordi jäigenemise ja fetuiin-A taseme suurenemisega.
3. Võrrelda aordi kaltsifikatsiooni skoori (strukturaalne profileerimine), pulsilaine kiirust aordis, Doppler-indeksit (funktsionaalne profileerimine) ja vitamiin D seerumi taset (biokeemiline profileerimine) ning hinnata nende parameetrite vahelist seost alajäsemete arterite ateroskleroosiga patsientidel ja kliiniliselt tervetel uuritavatel.
4. Uurida pulsilaine kiirust aordis (funktsionaalne profileerimine), osteopontiini ning oksüdeeritud madala tihedusega lipoproteiini seerumi taset (biokeemiline profileerimine) ja hinnata funktsionaalse ja biokeemilise profiili vahelist seost alajäsemete arterite ateroskleroosiga patsientidel ja kliiniliselt tervetel uuritavatel.
5. Hinnata pulsilaine kiiruse aordis (funktsionaalne profileerimine) ja angiograafilise skoori (strukturaalne profileerimine) vahelist seost alajäsemete arterite ateroskleroosiga patsientidel.

Uuringute meetodid

Uuriti 79 alajäsemete arterite ateroskleroosiga meespatsienti (II – IV staadium Fontaine järgi) ja 84 kliiniliselt tervet meest. Aordi jäikust hinnati pulsilaine kiiruse registreerimise kaudu Tartu Ülikooli Kardioloogiakliiniku Endoteeli Keskuses. Kaltsifikatsiooni, põletiku ja oksüdatiivse stressi biomarkerite tase määrati Tartu Ülikooli Biokeemia instituudis ja Sihtasutus Tartu Ülikooli Kliinikumi Ühendlaboris. Aordi kaltsifikatsiooni skoor ja angiograafiline skoor hinnati SA TÜK Radioloogiakliinikus.

Tulemused ja järeldused

1. Pulsilaine kiirus aordis ja osteoprotegeriini ning oksüdeeritud madala tihedusega lipoproteiini seerumi tase olid suurenenud alajäsemete arterite ateroskleroosiga patsientidel. Osteoprotegeriini seerumi tase oli seotud pulsilaine kiirusega aordis nii alajäsemete arterite ateroskleroosiga patsientidel kui ka kliiniliselt tervetel uuritavatel. Lisaks sellele esines statistiliselt oluline seos osteoprotegeriini ja oksüdeeritud madala tihedusega lipoproteiini sisalduse vahel ateroskleroosiga patsientide rühmas. Saadud informatsioon näitab, et aordi jäikus on seotud kaltsifikatsiooniga nii ateroskleroosiga patsientidel kui ka tervetel inimestel.
2. β_2-mikroglobuliini plasma tase oli suurenenud alajäsemete arterite ateroskleroosiga patsientidel. β_2-mikroglobuliini tase oli sõltumatult seotud pulsilaine kiirusega aordis ja fetuiin-A tasemega ateroskleroosiga patsientidel. Antud tulemused viitavad võimalusele, et põletiku marker β_2-mikroglobuliin

on seotud nii aordi jäikusega kui ka kaltsifikatsiooniga ateroskleroosiga haigetel.

3. Alajäsemete arterite ateroskleroosiga patsientidel oli suurenenud aordi kaltsifikatsiooni skoor, kuid vähenenud vitamiin D sisaldus seerumis. Aordi kaltsifikatsiooni skoor oli sõltumatult seotud aordi pulsilaine kiirusega nii ateroskleroosiga haigetel kui ka kontrollrühma uuritavatel. Lisaks oli aordi kaltsifikatsiooni skoor seotud vitamiin D taseme suurenemisega ateroskleroosiga haigetel, kuid vitamiin D taseme vähenemisega tervetel inimestel. Uuringu tulemustest võib järeldada, et aordi kaltsifikatsioon on seotud nii aordi jäikuse kui vitamiin D tasemega nii ateroskleroosiga haigetel kui ka tervetel inimestel.
4. Osteopontiini tase oli suurenenud alajäsemete arterite ateroskleroosiga haigetel. Pulsilaine kiirus aordis oli sõltumatult seotud osteopontiini ja oksüdeeritud madala tihedusega lipoproteiini seerumi tasemega ateroskleroosiga haigetel ja kontrollrühma uuritavatel. Saadud informatsioon näitab, et aordi jäikus on seotud kaltsifikatsiooni ja oksüdatiivse stressiga nii ateroskleroosiga patsientidel kui ka tervetel inimestel.
5. Pulsilaine kiirus aordis on sõltumatult seotud angiograafilise skooriga alajäsemete arterite ateroskleroosiga patsientidel. Antud tulemus näitab, et aordi jäikus on seotud ateroskleerootilise kahjustuse ulatuse ja raskusastmega alajäsemete arterite ateroskleroosiga haigetel.

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PUBLICATIONS

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