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Beneficial effects of vitamin D and angiotensin II receptor blocker on arterial damage

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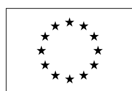

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To my family

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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 Salum E, Kampus P, Zilmer M, Eha J, Butlin M, Avolio AP, Põdramägi T, Arend A, Aunapuu M, Kals J. Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. Cardiovascular Diabetology 2012; 11: 58.

II Salum E, Butlin M, Kals J, Zilmer M, Eha J, Avolio AP, Arend A, Aunapuu M, Kampus P. Angiotensin II receptor blocker telmisartan attenuates aortic stiffening and remodelling in STZ-diabetic rats. (Submitted for publication).

III Salum E, Kals J, Kampus P, Salum T, Zilmer K, Aunapuu M, Arend A, Eha J, Zilmer M. Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. Diabetes Research and Clinical Practice 2013; 100: 243–249.

IV 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. International Journal of Cardiology 2011; 151: 106–107.

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Papers I–III: Design of experiments, collection of experimental data, data analysis, main writer of the manuscript.

Paper IV: Participation in clinical data collection, data analysis, main writer of the manuscript.

ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D
ABTS	2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate)
ADMA	asymmetric N^G,N^G-dimethyl-L-arginine
AGE	advanced glycation end-product
AIx	augmentation index
AIx@75	augmentation index corrected for a heart rate 75 beats/minute
ANCOVA	analysis of covariance
Ang II	angiotensin II
ANOVA	analysis of variance
ARB	angiotensin II type 1 receptor blocker
ATP	adenosine triphosphate
BP	blood pressure
CML	*N*ε-(carboxymethyl)lysine
CRP	C-reactive protein
CV	cardiovascular
CVD	cardiovascular disease
DBP	diastolic blood pressure
DCCT	Diabetes Control and Complications Trial
DHA	dehydroascorbic acid
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dP/dt	maximal change in blood pressure
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
ESH	European Society of Hypertension
GSH	glutathione
HbA_{1c}	glycated haemoglobin
hsCRP	high-sensitivity C-reactive protein
IU	international unit
MAP	mean arterial pressure
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMMA	N^G-monomethyl-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
NTG	nitroglycerin
OSI	oxidative stress index
OxS	oxidative stress
PE	phenylephrine
PP	pulse pressure
PPAR-γ	peroxisome proliferator-activated receptor-gamma

PTH	parathyroid hormone
PWA	pulse wave analysis
PWV	pulse wave velocity
RAGE	receptor for advanced glycation end-products
RAS	renin-angiotensin system
ROS	reactive oxygen species
SBP	systolic blood pressure
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAC	total antioxidant capacity
TMB	tetra-methyl-benzidine
TPX	total peroxides
UVB	ultraviolet B
VDR	vitamin D receptor
VSM	vascular smooth muscle

1. INTRODUCTION

The physiological function of vitamin D in the maintenance of calcium homeostasis and bone metabolism is well-recognised (Holick *et al.* 1972; Boyle *et al.* 1972). Concurrent studies on vitamin D metabolism have identified the vitamin D receptor (VDR) expression in a variety of tissues and cells, including, but not limited to, the pancreatic β-cells, immune cells, vascular endothelial cells, cardiomyocytes, and neurons (Walters 1992). These findings support the view that vitamin D has beneficial effects on other important functions in organs and tissues not inherently associated with calcium metabolism. Clinical studies have reported an inverse relationship between vitamin D status and incidence of cardiovascular disease (CVD) (Scragg *et al.* 1990; Zittermann *et al.* 2003), while replenishing vitamin D stores has been shown to reduce blood pressure (BP) and left ventricular hypertrophy (Pfeifer *et al.* 2001; Park *et al.* 1999). The relevance of maintaining adequate vitamin D status is highlighted by epidemiological studies demonstrating high prevalence of vitamin D insufficiency in Northern Europe (Barger-Lux and Heaney 2002; Kull *et al.* 2009).

Arterial stiffening is a manifest of adverse structural and functional modifications within the arterial wall that develop with advancing age and in the presence of cardiovascular (CV) risk factors (Laurent *et al.* 2006). Large artery stiffness is a powerful predictor of CVD progression and is now considered an independent CV risk factor and a target for therapy (Blacher *et al.* 1999; Laurent *et al.* 2001a; Mattace-Raso *et al.* 2006). Detection of changes within the arterial wall using different diagnostic methods enables to identify patients at risk of CV complications and allows early prevention and intervention in the progress of CVD. A variety of methods are available for assessment of arterial stiffness. Pulse wave velocity (PWV) is an index of arterial stiffness that can be measured with high accuracy and reproducibility in experimental animals and human subjects. The acknowledgment of using PWV as a marker of asymptomatic target organ damage and an integral tool for diagnosis and risk stratification is evidenced by its inclusion in the 2007 and 2013 European guidelines for management of arterial hypertension (Mancia *et al.* 2007; Mancia *et al.* 2013).

Morbidity and mortality of patients with diabetes mellitus (DM) are mainly determined by the development of vascular complications as a result of threefold increased CV risk compared to general population (Stamler *et al.* 1993). The deleterious impact of diabetic vascular complications warrants early detection of abnormalities in the vascular structure and function and early interventional strategies. Prevention and treatment of diabetic vascular damage by correcting underlying metabolic abnormalities is the mainstay in the treatment of DM. However, pharmacological interventions may be of limited value in the modulation of complications once they have developed. For this purpose, studying the pathogenesis of diabetic vascular complications in experimental animal studies provides valuable means for understanding of the course of the disease and finding new targets for intervention.

Long-term hyperglycaemia, one of the hallmarks of DM, induces non-enzymatic modifications of proteins and other biomolecules, producing advanced glycation end-products (AGEs) and their deposition in tissues, including the arterial wall (Brownlee *et al.* 1984). Modification of extracellular matrix proteins, including collagen and elastin, impairs the biomechanical integrity of the arterial wall and subsequently decreases arterial elasticity (Mizutani *et al.* 1999). The formation of AGEs is exacerbated by concurrent high-grade oxidative stress (OxS), which overwhelms the capacity of antioxidant defence to counterbalance elevated OxS (Mullarkey *et al.* 1990). Collectively, the OxS-AGE axis is considered central in the development of DM-induced vascular complications (Brownlee 2005), and therapeutic strategies targeted at different steps of AGE formation may significantly improve DM-induced vascular damage.

The activated renin-angiotensin system (RAS) is an important contributor to adverse vascular outcomes in DM (Miller 1999). The interplay between angiotensin II-induced vasoconstriction and arterial wall remodelling via non-haemodynamic mechanisms leads to arterial stiffening (Dzau *et al.* 1991; Griffin *et al.* 1991). The RAS blockers (e.g., angiotensin converting enzyme inhibitors [ACEIs] and angiotensin II type 1 receptor blockers [ARBs]) are well-established agents for treatment of hypertension, but they also have equally important effects beyond BP reduction (UK Prospective Diabetes Study Group 1998; Brenner *et al.* 2001). Among ARBs, telmisartan may be distinguished as a favourable agent for use in patients with DM owing to its profile of cardiometabolic protection (Yamagishi and Takeuchi 2005; Derosa *et al.* 2004). Collective evidence suggests that telmisartan may provide unique possibilities for the prevention and treatment of DM-associated vascular complications.

The main purpose of the current thesis was to investigate the role of vitamin D in the arterial structure and function in experimental DM and in well-trained human subjects, as well as to study the effect of an ARB telmisartan on arterial damage associated with experimental DM.

2. REVIEW OF THE LITERATURE

2.1. Vitamin D

Vitamin D belongs to a group of lipid-soluble sterol-like compounds, with the two most important forms being vitamin D_2 (ergocalciferol), which is obtained from plant products, and vitamin D_3 (cholecalciferol), which is produced by the skin as a result of ultraviolet B (UVB) radiation or obtained from animal sources. Vitamin D is biologically inactive in humans and requires conversion to the pro-hormone 25-hydroxyvitamin D (25[OH]D) in the liver (Henry 1992). This pro-hormone is then further converted into the active hormone 1,25-dihydroxyvitamin D or calcitriol by the enzyme 1α-hydroxylase which takes place mainly in the renal tubular epithelium (Henry 1992). Vitamin D and its metabolites are transported in the blood bound to the vitamin D binding protein to its effector sites. The biological actions of calcitriol are mediated by the nuclear VDR that is present in various tissues, including the heart, brain, skin, and pancreas (Baker *et al.* 1988; Walters 1992).

2.1.1. The role of vitamin D in health and disease

2.1.1.1. Calcium metabolism

The main function of vitamin D is the regulation of calcium homeostasis in the organism. Calcium is one of the most tightly regulated substances in the plasma because its concentration as ionised calcium is maintained constantly within a narrow range (Jones *et al.* 1998). Calcium is essential for a variety of metabolic processes, including muscle contraction, neurotransmission, signal transduction, coagulation, enzyme activation, and maintenance of osmotic pressure in the blood (Holick 2003). In response to even a slight decrease in plasma calcium level the parathyroid glands react to secrete the parathyroid hormone (PTH) that initiates a sequence of events resulting in mobilisation of calcium to restore its level in the plasma. In the kidneys, PTH activates the conversion of 25(OH)D to the active hormone calcitriol (Garabedian *et al.* 1972). Calcitriol is transported to the small intestine where it increases the expression of calcium channels and calcium binding protein, thereby enhancing the absorption of calcium from the food (Boyle *et al.* 1972). Calcitriol also stimulates osteoclasts which results in increased bone resorption and increased mobilisation of calcium from the bone tissue to plasma (Holick *et al.* 1972). In the distal renal tubule, calcitriol, in concert with PTH, increases the reabsorption of calcium into the plasma (Yamamoto *et al.* 1984). Negative feedbacks from increasing plasma calcium and calcitriol concentrations inhibit the secretion of PTH.

2.1.1.2. Cardiovascular diseases

The importance of vitamin D in calcium homeostasis and bone metabolism is well established. With increasing number of studies investigating the influence of vitamin D on non-classical target tissues a wide range of non-calciotropic actions of vitamin D has been discovered. The biological plausibility of these activities is supported by the findings of VDR, a transcription factor, expression in a variety of cells, including pancreatic β-cells, immune cells, myocytes, cardiomyocytes, endothelial cells, and neurons (Walters 1992).

There is substantial evidence to support the implication of vitamin D in the pathogenesis of CVD (Watson *et al.* 1997; Zittermann *et al.* 2005; Lee *et al.* 2008). Calcitriol is a negative regulator of renin expression and thereby inhibits the RAS, which is an important contributor to the development of arterial hypertension (Li *et al.* 2002). Vascular effects of calcitriol include the modulation of smooth muscle proliferation (Mitsuhashi *et al.* 1991) and inhibition of vascular calcification (Watson *et al.* 1997). Calcitriol is also known to regulate the cellular production of pro-inflammatory (e.g., interleukin-1 and tumour necrosis factor-alpha) and anti-inflammatory cytokines (e.g., interleukin-10), a mechanism that may be important in the prevention of CVD (Müller *et al.* 1992; Canning *et al.* 2001).

Pharmacological, but not physiological, doses of vitamin D have been reported to cause hypertension, arterial stiffening, and atherosclerosis in rodent models; however, the importance of these findings for humans is uncertain (Norman and Powell 2005). Vitamin D toxicity and the resultant hypercalcaemia, which can lead to arterial stiffening and hypertension, have been observed when circulating 25(OH)D levels are higher than 374 nmol/L (Holick 2007). Data from clinical trials have not shown vitamin D toxicity with daily doses of up to 10,000 international units (IU) vitamin D which approximates the level of vitamin D production that can be achieved by endogenous vitamin D synthesis in the skin (Vieth 1999; Heaney *et al.* 2003).

Cross-sectional studies investigating the association between vitamin D status and CVD risk have demonstrated low levels of vitamin D in individuals with acute myocardial infarction (Scragg *et al.* 1990), stroke (Poole *et al.* 2006), heart failure (Zittermann *et al.* 2003), hypertension (Scragg *et al.* 2007), and peripheral artery disease (van de Luijtgaarden *et al.* 2012). In clinical trials, vitamin D supplementation has been shown to decrease BP (Lind *et al.* 1989; Pfeifer *et al.* 2001) and left ventricular hypertrophy (Park *et al.* 1999). However, there is relatively little information regarding the role of vitamin D in arterial stiffness. Vitamin D deficiency has been correlated with increased aortic stiffness in patients with end-stage renal disease (London *et al.* 2007) and carotid artery calcification (Freedman *et al.* 2010). Inverse relationships between serum 25(OH)D levels and aortic stiffness have been shown in healthy subjects (Al Mheid *et al.* 2011) and in general population (Mayer *et al.* 2012). Interventional studies have produced conflicting results, reporting either improvement of arterial stiffness (Dong *et al.* 2010) or no effect of vitamin D supplementation on arterial stiffness (Larsen *et al.* 2012; Stricker *et al.* 2012). In this thesis, we

have investigated the association between vitamin D status and arterial stiffness in experimental DM (Paper I) and in healthy individuals (Paper IV).

2.1.2. Environmental determinants of vitamin D status

Vitamin D insufficiency is an increasingly recognised worldwide health problem (Holick 2005). Vitamin D status in the organism is assessed by the concentration of its circulating metabolite 25(OH)D since its level is mainly regulated by substrate availability (Zittermann 2006). Serum concentration of 25(OH)D is dependent on several modifiable and non-modifiable factors such as diet, latitude, season, time spent outdoors, skin pigmentation, clothing, and tanning habits (Sherman *et al.* 1990).

Ultraviolet B radiation initiates vitamin D production in the skin. The 7-dehydrocholesterol, a cutaneous membrane lipid, absorbs UVB radiation and is converted into pre-vitamin D, which will isomerise into cholecalciferol (Holick and DeLuca 1974). Vitamin D intoxication due to prolonged exposure to UVB is not possible because any excess vitamin D is converted to inert isomers in the skin (Webb *et al.* 1989). Increasing latitude decreases the availability and intensity of UVB radiation. At latitudes above 40° (the latitude of Estonia is 59° N), synthesis of vitamin D is absent during winter and is blunted even in the summer months (Barger-Lux and Heaney 2002). In a recent cross-sectional study in Estonia, the prevalence of vitamin D insufficiency and deficiency were 73% and 8%, respectively (Kull *et al.* 2009). This indicates that Estonian population is at high risk for vitamin D deficiency.

While most of vitamin D in the organism is synthesised by the skin, food is also a considerable source of vitamin D. Fish and fish products are among the best sources of vitamin D_3, small amounts are also found in cheese and egg yolks (Lamberg-Allardt 2006). Food fortification (mostly dairy products) and vitamin D supplements are also increasingly common in many countries.

The cut-off value for normal circulating 25(OH)D levels has been a subject of much dispute. Vitamin D insufficiency is most commonly defined by serum 25(OH)D level below 50 nmol/L (Holick 2007), but there are also studies suggesting that 25(OH)D level less than 37.5 nmol/L should be considered inadequate (Jacques *et al.* 1997; Thomas *et al.* 1998). However, a number of recent studies suggest that 25(OH)D level above 75 nmol/L should be considered ideal in regard of bone metabolism and non-calcaemic functions of vitamin D (Chapuy *et al.* 1997; Bischoff-Ferrari *et al.* 2006; Bischoff-Ferrari 2007). In these studies, this level of 25(OH)D was found to be efficient in maintaining normal PTH secretion, considering that high levels of PTH may have adverse effects on the CV system by promoting ventricular hypertrophy (Schlüter and Piper 1992) and vascular remodelling (Amann *et al.* 1995; Perkovic *et al.* 2003).

2.2. Aortic stiffness

Large arteries, in particular the aorta and its main branches, are regarded as a complex organ with distinct functions, regulating blood flow and BP as well as buffering the pulsations generated by the heart (Nichols and O'Rourke 2005). The buffering function is the essential ability of large arteries to accommodate the blood volume ejected by the heart and absorb the energy generated by the pulsation, thereby reducing the load on the left ventricle and providing a steady blood flow to the peripheral organs. With increasing age, athero- and arterio-sclerosis, and hypertension, functional and structural modifications occur within the arterial wall, resulting in increased arterial stiffness (Laurent 1995; O'Rourke 1995). These changes predominantly appear in the central elastic arteries than in the distal muscular arteries (Benetos *et al.* 1993). Stiffening of the aortic wall impairs the inherent ability of the aorta to expand and convert pulsatile blood flow ejected from the heart into a continuous blood flow to the peripheral organs. Increased aortic stiffness also increases the left ventricular afterload leading to impaired contractility and heart failure (O'Rourke 1982; Roman *et al.* 2000; Nitta *et al.* 2004). Aortic stiffness is predictive of CV mortality and all-cause mortality, in a range of high-risk patients (Laurent *et al.* 2001a; van Popele *et al.* 2001) and in general population (Mattace-Raso *et al.* 2006). The utility of assessment of aortic stiffness in clinical practice is facilitated by a range of methods developed to measure parameters indicative of aortic stiffness.

2.2.1. Aortic wall morphology

The capacitance and resistance of the aorta are determined by the geometry, proportion, and action of the components of the aortic wall. The aortic wall consists of three layers: *tunica intima*, *tunica media*, and *tunica adventitia.* The adventitia is the outmost layer consisting of collagen fibres and extracellular matrix components. The media contains vascular smooth muscle (VSM) cells embedded in the network of collagen and elastin fibres. The intima is composed of a monolayer of endothelial cells lining the lumen of the vessel, anchored to the internal elastic lamina by the subendothelial layer of elastin and collagen fibres.

Each of the wall layers has a distinctive role in the regulation of arterial function. The adventitia, abundant with longitudinally arranged elastin and collagen bundles, provides mechanical support and serves as a connection to surrounding tissues. The medial layer contributes to circumferential stiffness of the aorta. The spatial arrangement and quantity of collagen and elastin fibres in the media are important in determining the overall mechanical properties of the aortic wall. At low distending pressure, the majority of the load is transferred to elastic fibres. The elastic fibres extend proportionally to the distending pressure (Wolinsky and Glagov 1967). As the pressure increases, stiffer collagen fibres are being gradually recruited, and their stiffness limits the aortic distension

(Clark and Glagov 1985; Armentano *et al.* 1991). The resultant classic non-linear relationship between the stress applied to the aortic wall and change of aortic diameter (the increase in diameter is reduced with increasing pressure) can be assigned to the different elastic properties of both elastin and collagen fibres in the aortic media (Figure 1). Collagen fibres in the adventitia are not well organised and normally not stretched at physiological BP (Wolinsky and Glagov 1967).

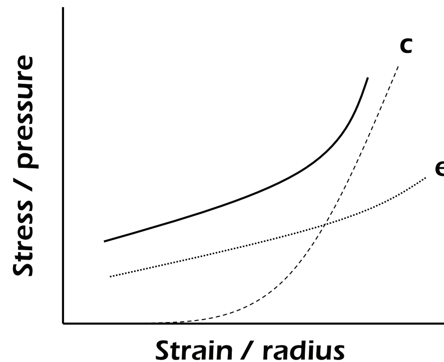

Figure 1. Schematic representation of a typical stress-strain relationship of an elastic artery, reproduced from Åstrand 2008. The contribution of collagen (c – dashed line) and elastin (e – dotted line) fibres constitute the biphasic global response of the arterial wall to the applied stress (solid line).

The distribution of load bearing components, elastin, collagen, and VSM, varies along the arterial tree. Elastin is the major component in the proximal part of the aorta, while collagen dominates in the distal aorta and remains relatively constant in the peripheral arteries (Harkness *et al.* 1957; Apter *et al.* 1966). Vascular smooth muscle content is lower in the proximal arteries than those more distal to the heart. In the aorta and other large arteries, VSM is helically arranged, while the alignment in the distal arteries is more circumferential (Milnor 1982). Due to proportion and structure of the load bearing components, large arteries more proximal to the heart are inherently more elastic than the distal arteries (Latham *et al.* 1985). These properties are essential in the arterial haemodynamics, as the aorta has a capacitive function, buffering the stroke volume and continuously supplying blood to the peripheral organs, while limiting the load on the left ventricle. Changes in the aortic wall geometry, both quantitatively and qualitatively, lead to increased stiffness of the vessel wall, as seen in the ageing aorta (Benetos *et al.* 1993; Boutouyrie *et al.* 1992; Länne *et al.* 1992). This aortic remodelling occurs in response to chronic applied stress such as pulse pressures imposed on the vessel wall (Laurent *et al.* 2001b), leading to degeneration of the wall structure. Increased aortic stiffness is characterised by fracture and degradation of elastic fibres and increased loading on collagen fibres (O'Rourke and Hashimoto 2007). The other important causes

of aortic remodelling include hypertension (Benetos *et al.* 1993; Avolio *et al.* 1998), atherosclerosis (van Popele *et al.* 2001; Zagura *et al.* 2011), and end-stage renal disease (Blacher *et al.* 1998).

2.2.2. Functional properties of the aorta

In addition to passive load bearing structural components, elastin and collagen, aortic wall also contains functional, dynamic component, smooth muscle. Dynamic alteration in aortic stiffness is mediated by changes in VSM tone induced by exogenous or endogenous chemical signalling pathways. There are two distinct phenotypes of VSM cells: contractile and synthetic (Shanahan and Weissberg 1998). Contractile VSM cells contain contractile filaments, while synthetic VSM cells have a high number of organelles required for protein synthesis (Hao *et al.* 2003). Contractile VSM cells may transform into synthetic phenotype which is regarded as a precondition for development of vascular disease (Shanahan and Weissberg 1998).

The vascular endothelium has an important role in the regulation of arterial function by producing vasoactive substances, including nitric oxide (NO), an important vasodilator that mediates VSM tone (Lekakis *et al.* 2011). The nitric oxide, synthesised from L-arginine by endothelial NO synthase (NOS), is the main vasodilating and major antiatherogenic biomolecule in the arterial wall (Davignon and Ganz 2004). Endothelial NO also inhibits platelet aggregation, cell adhesion, and the proliferation of VSM cells, thereby preventing vascular remodelling (Moncada *et al.* 1991). Under basal conditions, NO synthesis is continuously stimulated by shear stress on endothelial cells, and the release of NO is responsible for maintenance of VSM relaxation (Haynes *et al.* 1993; Kinlay *et al.* 2001). Exogenous NO donors such as nitroglycerin (NTG) release NO directly and act on VSM similarly to endogenously produced NO (Ignarro 2002). Overall, the continuous generation of NO by the endothelium is essential for the maintenance of aortic elasticity, while inhibition of NO production has been shown to increase the stiffness of the aortic wall (Wilkinson *et al.* 2002).

2.2.3. Pulse wave velocity

The contraction of the left ventricle generates a pressure pulse wave that travels along the arterial wall throughout the body. The velocity at which the pulse wave propagates along the artery is dependent on the physical properties and geometry of the arterial wall. Hence, the velocity of pulse wave propagation provides an index of arterial distensibility and stiffness so that higher velocity corresponds to higher arterial stiffness. Pulse wave velocity can be simply calculated by dividing the distance between the two sites at which the pressure pulses are being recorded with time needed by the wave front to travel between those two sites.

Assessment of PWV is very useful in the evaluation of changes in the vascular system because increasing age (Avolio *et al.* 1983) and various diseases are known to affect the stiffness of the arteries. Several large studies have demonstrated that PWV is an independent predictor of all-cause and CV mortality in patients with hypertension (O'Rourke 1982), DM (Mattace-Raso *et al.* 2006), and end-stage renal disease (Blacher *et al.* 1999). The importance of PWV is not limited to specific disease groups. Increased PWV has also been shown to predict CV mortality in general population (Willum-Hansen *et al.* 2006). Accordingly, PWV can be regarded as a useful diagnostic tool in different clinical settings. The importance of aortic PWV in the assessment of aortic stiffness is illustrated by inclusion of PWV measurement in the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) guidelines for assessment of asymptomatic target organ damage in arterial hypertension (Mancia *et al.* 2007; Mancia *et al.* 2013). Reference values for aortic PWV have been published recently to help identify people with higher CV risk (Boutouyrie and Vermeersch 2010).

Pulse wave velocity assessment is dependent on several critical factors, including accurate measurement of distance between the recording sites, consistent determination of the pressure wave front, and the dependency of PWV on distending pressure. The distance between the two pressure sensors in non-invasive PWV measurements in human subjects can be estimated by direct superficial measurement (over the skin) with acceptable accuracy owing to relatively long distances between the two sites. In experimental studies using small animals, inaccurate measurement of short distances may introduce a significant error in PWV calculations. These errors can be eliminated by simultaneous recording of the pulse waves using a high-fidelity dual pressure sensor catheter with two pressure sensors fixed an exact distance apart.

Accurate determination of the wave front is important because of continuous changes in the pulse contour as it travels due to viscoelastic properties of the arterial wall and wave reflection. The forward propagating wave is reflected from sites of impedance mismatch in the arterial tree as present at branchings and segments with tapering or changes in vessel wall stiffness. The peak of the pulse wave is not practical for calculating PWV because it is strongly affected by the wave reflection (Nichols and O'Rourke 2005). The foot of the pressure wave is regarded to be relatively free of reflections because of the time delay between the forward and backward propagating waves. The pressure wave foot-to-foot measurement of PWV (Figure 2) is used throughout this thesis due to its simplicity, accuracy, and repeatability.

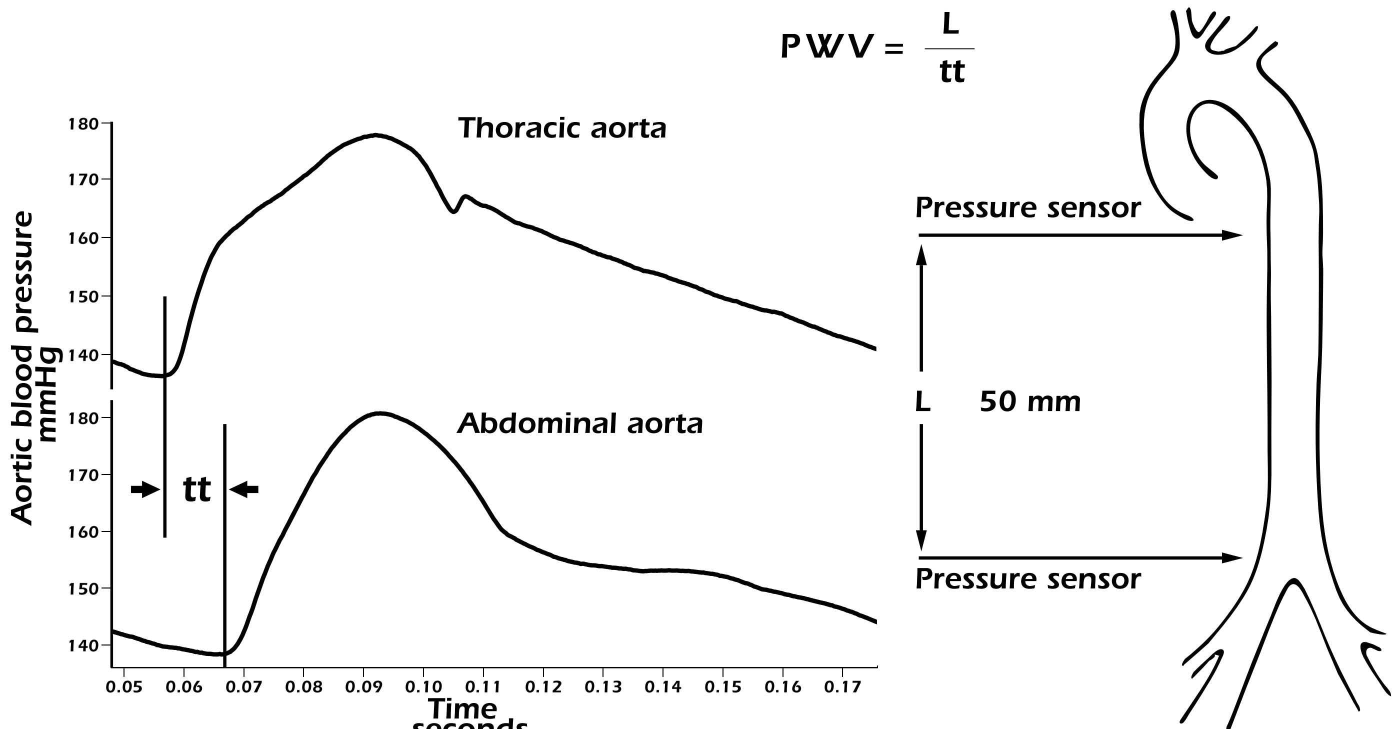

Figure 2. Measurement of pulse wave velocity by the foot-to-foot method. Pulse wave velocity (PWV) is calculated by dividing the known distance (L) between the two sites of pressure sensors with the transit time (tt) between the recorded pressure waveforms.

It is well established that PWV is a complex function of arterial wall structure, vasomotor tone, and transmural pressure (Shadwick 1999). Therefore, PWV varies as a function of arterial pressure at which it is measured. This means that comparison of PWV between individuals should be made in isobaric conditions. In animal experiments, manipulation of arterial pressure is feasible through infusion of pharmacological agents, e.g., phenylephrine (PE) and NTG, for increasing and decreasing arterial pressure, respectively. Due to nonlinear elastic properties of the arterial wall, measuring PWV at an arbitrarily chosen pressure may provide limited information compared to the assessment of PWV over a wide range of arterial pressures. In recent years, pressure-dependent PWV measurement has gained increasing use as a standard for the assessment of aortic stiffness in a range of experimental settings (Ng *et al.* 2011; Tan *et al.* 2012; Jung *et al.* 2013). In this thesis, we have investigated aortic stiffness assessed by PWV under pressure-independent conditions in a rat model of type 1 DM (T1DM) (Papers I and II).

2.2.4. Arterial stiffness and inflammation

Systemic inflammation has been established as a major contributor for atherosclerotic processes (Libby *et al.* 2002) and is considered a risk factor for CVD (Ridker *et al.* 1997; Willerson and Ridker 2004). Inflammation is associated with endothelial dysfunction since activated inflammatory system is paralleled by decreased NO availability and normalisation of inflammatory activity has been shown to improve endothelium-dependent vasodilation (Yki-Järvinen *et al.* 2003; Fichtlscherer *et al.* 2000). Circulating markers of inflammation, including C-reactive protein (CRP), have been shown to be associated with arterial stiffness indices such as augmentation index (AIx) (Kampus *et al.* 2004; Kampus *et al.* 2006; Kampus *et al.* 2007) and aortic and brachial PWV (Mattace-Raso *et al.* 2004; Yasmin *et al.* 2004) in various sub-populations. Similar correlations have been demonstrated between circulating pro-inflammatory cytokine interleukin-6 levels and aortic PWV in patients with arterial hypertension (Mahmud and Feely 2005) and in general population (Schnabel *et al.* 2008). Paper IV addresses the relationship between inflammation and arterial stiffness in well-trained young individuals under long-term heavy physical and environmental stress.

2.3. Investigation of vascular complications in diabetes mellitus

Diabetes mellitus represents one of the most common public health problems affecting more than 350 million people worldwide at an increasing rate (Danaei *et al.* 2011). Diabetes mellitus comprises a group of metabolic diseases mainly characterised by elevated blood glucose either because of insufficient production of insulin (T1DM) and/or decreased response of cells to insulin

(T2DM). Regardless of the classification, the resulting metabolic abnormalities of DM are associated with 2–4 fold higher CV risk than in general population even after adjustment to other known CV risk factors (Stamler *et al.* 1993). Vascular complications of DM represent the principal cause of morbidity and mortality. Due to the natural course of DM, it takes years for the complications to develop which makes it difficult to assess the effects of the interventions to modulate the development of complications. This concern may be addressed by studying DM in animal models which also has the advantage to eliminate such factors as ethnicity, geographic variables, diet, age and gender differences, and drug interactions that inevitably set limits to clinical studies. Studying DM in experimental settings is fundamental for the advancement of knowledge of its pathogenesis and finding new therapies for DM and its complications.

2.3.1. Streptozotocin-induced model of type 1 diabetes mellitus

Streptozotocin (STZ) is a metabolite from *Streptomyces Achromogenes* with antibiotic, antitumour, and carcinogenic properties. STZ is composed of the cytotoxic moiety, 1-methyl-1-nitrosourea, attached to the C-2 position of D-glucose. The glucose moiety is an important factor that specifically directs STZ toxicity to the β-cells, sparing α-cells and δ-cells (Agarwal 1980), by mediating the uptake of STZ via the glucose transporter GLUT2 (Schnedl *et al.* 1994).

The diabetogenic action of STZ was first reported on 1963 (Rakieten *et al.* 1963). The finding of its selective toxicity to β-cells in the pancreatic islets suggested that the drug can be used for inducing DM in animal models (Mansford and Opie 1968). Since then, it has been used in numerous studies, probably making it the second most used animal model of human disease, after the spontaneously hypertensive rat.

Acting as an alkylating agent, STZ has been shown to interfere with glucose transport (Wang and Gleichmann 1998) and glucokinase function (Zahner and Malaisse 1990) and to cause multiple deoxyribonucleic acid (DNA) strand breaks (Bolzan and Bianchi 2002). A single large dose of STZ can be used to produce hyperglycaemia in experimental animals, evidently due to direct toxic effects on β-cells (Junod *et al.* 1967). Alternatively, multiple low doses of STZ can be used which leads to insulitis and death of β-cells (Like and Rossini 1976). The metabolic abnormalities after administration of STZ doses of 50–65 mg/kg body weight are characteristic to those seen in patients with T1DM. Hyperglycaemia (20–30 mmol/L) is observed within 48 hours concomitantly with a decrease in blood insulin levels, but severe ketosis does not develop, even if insulin is not administered (Junod *et al.* 1969). Higher doses of STZ (>75 mg/kg body weight) result in fatal ketosis within days if insulin is not given (Junod *et al.* 1967).

2.3.2. Endothelial dysfunction

Disturbed endothelial function is generally defined as the imbalance between vasoconstricting and vasodilating substances released by the endothelial cells (Deanfield *et al.* 2005). Endothelial dysfunction is a common characteristic in patients with atherosclerosis (Kals *et al.* 2006a) and DM (Dogra *et al.* 2001) and a critical factor for the development of CVD (Gokce *et al.* 2003) and diabetic vascular complications (Johnstone *et al.* 1993).

The synthesis of NO, a major endothelial-derived vasodilative substance, is dependent on its substrate, L-arginine. Two types of endogenous competitive inhibitors of NOS are present in the circulation, N^G-monomethyl-L-arginine (L-NMMA) and asymmetric N^G,N^G-dimethyl-L-arginine (ADMA) (Vallance *et al.* 1992). Higher concentrations of ADMA in plasma suggest that the latter is the major endogenous NOS inhibitor, compared to L-NMMA. Reduced NO synthesis leads to impaired endothelium-dependent vasodilation (Giugliano *et al.* 1996), thus contributing to increased CV morbidity.

The utility of the measurement of ADMA as a surrogate marker of endothelial dysfunction has been demonstrated in studies using venous occlusion plethysmography (Juonala *et al.* 2007) and flow-mediated dilation (Perticone *et al.* 2005) that are both well-established methods for assessment of endothelial function. Endothelial vasodilatory dysfunction and accumulation of ADMA may be important mechanisms underlying reduced arterial elasticity in healthy subjects (Kals *et al.* 2007). Elevated levels of ADMA have been reported in a wide range of clinical conditions associated with increased CV risk, including hypertension (Goonasekera *et al.* 1997), peripheral artery disease (Böger *et al.* 1997), pulmonary hypertension (Gorenflo *et al.* 2001), chronic heart failure (Kielstein *et al.* 2003), and cerebral small vessel disease (Khan *et al.* 2007). Asymmetric dimethylarginine has been shown to predict all-cause mortality in healthy subjects (Böger *et al.* 2009) and CV events in patients with coronary artery disease (Schnabel *et al.* 2005) and peripheral artery disease (Mittermayer *et al.* 2006).

2.3.3. Structural changes in the aorta

Short-term reversible functional changes in the aortic distensibility are mediated by alterations in the endothelium-dependent smooth muscle tension. During contraction, smooth muscle cells transfer stress to collagen, while elastin lamellae are the load-bearing elements during relaxation (O'Rourke and Avolio 1985). Over longer periods of time, the aortic wall undergoes changes in the spatial arrangement and quantities of smooth muscle, elastin, and collagen, which leads to mechanical adaptation to chronic applied forces such as pulse pressures altering aortic wall stiffness, wall thickness, and luminal diameter (Mulvany 1993). These changes, if observed morphologically, are considered essential for defining vascular remodelling (Lee *et al.* 1997).

Vascular remodelling is a fundamental basis of normal vessel growth and adaptation. However, the mechanical properties of the aorta and resistance

arteries gradually decline with increasing age in parallel with increasing aortic stiffness (Mitchell *et al.* 2007). Abnormalities in the vascular structure associated with DM have been described as accelerated aging (Lee and Oh 2010). Diabetes mellitus is known to induce modifications of extracellular matrix proteins such as collagen and elastin (Reddy 2004) that may compromise the biomechanical integrity of the vessel wall and consequently alter vascular elasticity (Mizutani *et al.* 1999). This thesis investigates two specific aspects of aortic wall remodelling, namely the quantitative and qualitative changes in the elastin and collagen fibres (Papers I and II).

2.3.4. Advanced glycation end-products in diabetes mellitus

Long-term high levels of glucose in blood and tissues induce non-enzymatic modifications of proteins, lipids, and nucleic acids, forming AGEs through a series of reactions (Vlassara *et al.* 1984; Brownlee *et al.* 1984). Protein glycation is initiated by a reaction between a free amino group and the carbonyl group of glucose to form a reversible Schiff base (within hours). Over a period of days, the latter rearranges into a more stable ketoamine or Amadori product (Brownlee *et al.* 1984). The Amadori product can be fragmented by oxidation to produce a number of highly reactive compounds such as Nε-(carboxymethyl)lysine (CML) or pentosidine which react again with other free amino groups thus forming different intermediate and advanced glycation end-products (McCance *et al.* 1993; Reddy *et al.* 1995). While the process of early protein glycation is dependent on ambient glucose levels, the contribution of oxidative stress becomes more important in the development of AGEs (Baynes 1991). Advanced glycoxidation occurs over a period of weeks or even months, thereby affecting proteins with a slow turnover. Structural components of the connective tissue matrix such as collagen and elastin are the prime targets of advanced glycation (Brownlee *et al.* 1984).

A common consequence of irreversible AGE formation is the accelerated development of covalent cross-links between structural proteins (Fu *et al.* 1994). The pathological formation of cross-links induced by AGEs increases the stiffness of the tissue. Several studies have indicated that accumulation of AGEs in the vascular wall, rather than quantitative changes in the structural proteins, contribute to the mechanical properties of the vascular wall. The amount of AGEs in the aortic wall has been positively correlated with wall stiffness in experimental (Brüel and Oxlund 1996) and human studies (Sims *et al.* 1996) suggesting that AGEs may be one of the causative factors of reduced aortic elasticity in DM. Advanced glycation end-products have also been implicated in other diabetic complications, including retinopathy (Stitt 2003), nephropathy (Sugiyama *et al.* 1996), and neuropathy (Sugimoto *et al.* 1997). Besides inducing the formation of protein cross-links, AGEs may also bind to cell membrane receptors and elicit intracellular damage. Interaction of AGEs with their receptors (RAGE) on such cells as macrophages, mesangial, or endothelial cells initiates many of the downstream effects, including superoxide radical

generation and apoptosis (Nitti *et al.* 2005; Vincent *et al.* 2007) which implies that AGEs directly modulate and exacerbate oxidative stress in DM.

2.4. Oxidative stress, arterial stiffness, and vascular protection

Reactive oxygen species (ROS), including oxygen free radicals and its derivatives are generated as by-products of normal aerobic metabolism (Pourova *et al.* 2010). Tightly controlled low levels of ROS are an integral part of physiological processes such as signal transduction, inflammatory response, phagocytosis, cellular growth and differentiation, apoptosis, and aging (Thannickal and Fanburg 2000). Due to their unpaired electron, they are highly reactive and may damage lipids, proteins, and DNA (Freeman and Crapo 1982). A network of intra- and extracellular antioxidants mediates the protection against potential damaging effects by ROS. The imbalance between the production of ROS and antioxidant defence may be defined as OxS. Prolonged excessive production of ROS and other reactive species, resulting in high-grade OxS, has been implicated in the pathogenesis of several CV diseases, including atherosclerosis, hypertension, and heart failure (Cai and Harrison 2000; Kals *et al.* 2006b). In the vasculature, high-grade OxS plays an important role in the deterioration of endothelial function, most importantly by disturbing the endothelium-dependent vasorelaxation and vascular remodelling (Drexler and Hornig 1999; Fortuno *et al.* 2005) which contribute to arterial stiffening.

2.4.1. Oxidative stress, vitamin D, and diabetes mellitus

Hyperglycaemia-induced high-grade OxS has been suggested as the unifying mechanism for the pathogenesis of diabetic complications (Brownlee 2005). Long-term hyperglycaemia may elevate OxS in several ways. Overproduction of ROS by the mitochondrial electron transport chain (Nishikawa *et al.* 2000), glucose autooxidation (Wolff and Dean 1987), and the polyol pathway (Lee *et al.* 1995) have all been described as potential sources of hyperglycaemia-driven OxS. Excessively produced free radicals also accelerate the formation of AGEs, which progressively produce more free radicals (Mullarkey *et al.* 1990), thus perpetuating the vicious cycle and leading to high-grade OxS. Although AGE formation is facilitated in hyperglycaemia, it has been shown that AGE levels were only weakly correlated with glycaemic control in the Diabetes Control and Complications Trial (DCCT) (Monnier *et al.* 1999). Furthermore, within the DCCT study, the AGE levels in skin were a better predictor of the development of complications compared to the glycated haemoglobin (HbA_{1c}) levels (Monnier *et al.* 1999). This is consistent with the hypothesis that other factors such as OxS may significantly contribute to the production and accumulation of AGEs regardless of good glycaemic control (Baynes 1991).

Achieving euglycaemia in patients with DM is a difficult task despite examples set by large-scale studies such as the DCCT or the United Kingdom Prospective Diabetes Study (Stratton *et al.* 2000). Therefore, therapies specifically targeted at preventing vascular complications in the presence of suboptimal control of hyperglycaemia are needed. Because of their central role in the development of diabetic complications, interventions aimed at reducing the accumulation of AGEs may provide significant improvement of DM-induced vascular disease. These strategies may be situated in different steps of AGE formation and AGE-mediated damage. From a practical point of view, the drugs can be distinguished as: 1) AGE inhibitors or AGE cross-link breakers; 2) RAGE signalling blockers; 3) other compounds possessing AGE inhibitor activity, including antioxidants and anti-inflammatory drugs. Due to heterogeneity of AGEs and their diverse distribution in tissues, there is no consensus on which therapeutic strategies would be most relevant regarding AGE inhibition in addition to the maintenance of adequate glycaemic control. Beneficial effects on arterial function and structure have been reported with a number of agents that are known to reduce AGE accumulation, including aminoguanidine (Brownlee *et al.* 1986), alagebrium (Vaitkevicius *et al.* 2001), RAS inhibitors (Monacelli *et al.* 2006; Saisho *et al.* 2006), vitamin B compounds (Stirban *et al.* 2006), and peroxisome proliferator-activated receptor (PPAR) activators (Wang *et al.* 2006).

Most of the AGE inhibitors listed above have also exhibited favourable effects on excess ROS generation within tissues. Furthermore, antioxidants and free radical trapping agents, including carnosine (Hipkiss and Chana 1998), benfotiamine (Babaei-Jadidi *et al.* 2003), pyridoxamine (Jain and Lim 2001), and curcumin (Sajithlal *et al.* 1998) have been reported to inhibit the formation of AGE-crosslinks or decrease tissue AGE levels. Increasing body of evidence suggests a role for vitamin D signalling pathways in redox homeostasis. Vitamin D has been reported to act as a membrane antioxidant by inhibiting lipid peroxidation *in vitro* (Wiseman 1993) and maintain a steady level of glutathione (GSH), a potent intracellular antioxidant, in rat hepatocytes *in vivo* (Sardar *et al.* 1996). In haemodialysis patients, vitamin D deficiency has been associated with elevated levels of malondialdehyde, nitrites, and carbonyl groups, that were improved by treatment with paricalcitol, a vitamin D analogue (Izquierdo *et al.* 2012). Vitamin D deficiency in asymptomatic subjects has been associated with high levels of thiobarbituric acid reactive substances, indicating lipid peroxidation, that were significantly decreased after vitamin D replacement (Tarcin *et al.* 2009). Experimentally induced vitamin D deficiency in healthy rats has been shown to increase BP and promote superoxide generation in the aortic wall (Argacha *et al.* 2011). However, the role of vitamin D as a potential antioxidant in different pathological conditions has not been fully investigated. In view of the beneficial effects of vitamin D on CV health, we have examined the potential impact of vitamin D supplementation on OxS and antioxidant defence in a rat model of STZ-induced DM (Paper III).

2.3.2. Renin-angiotensin system and vascular protection in diabetes mellitus

The renin-angiotensin system plays an important role in the regulation of arterial structure and function. Angiotensin II (Ang II) has powerful vasoconstrictive properties that are important for the regulation of BP (Dzau *et al.* 1991). However, it also contributes to vascular wall remodelling via non-haemodynamic mechanisms such as stimulation of VSM proliferation and re-arrangement of elastin and collagen fibres (Griffin *et al.* 1991). Hyperglycaemia enhances the production of Ang II in the vascular tissue (Miller 1999) promoting vascular hypertrophy and wall stiffness. Blockade of RAS with either ACEIs or ARBs has been reported to improve CV outcomes and reduce the risk of micro- and macrovascular complications of DM (UK Prospective Diabetes Study Group 1998; Heart Outcomes Prevention Evaluation Study Investigators 2000; Brenner *et al.* 2001). Furthermore, clinical studies have demonstrated that while RAS blockers are equally effective in BP reduction they may be superior to other antihypertensive agents in reducing the risk of CV events in patients with DM (Lindholm *et al.* 2002). It is suggested that the favourable influence of RAS blockers on the vasculature are, at least in part, due to reduction in the adaptive remodelling and stiffening of the arterial wall independently on their BP lowering effects.

Among ARBs, telmisartan has exhibited beneficial metabolic effects by increasing insulin sensitivity (Pershadsingh and Kurtz 2004) and improving plasma lipid profile (Derosa *et al.* 2004). Telmisartan has been reported to act as a partial agonist of PPAR-γ (Benson *et al.* 2004), which is involved in the regulation of carbohydrate and lipid metabolism (Takano *et al.* 2004). Furthermore, there is increasing evidence that PPAR-γ agonists exert anti-inflammatory, anti-oxidative, and antiproliferative effects on the vascular wall (Takano *et al.* 2004; Marx *et al.* 2004). Collectively, these properties indicate that telmisartan may provide unique possibilities for the prevention and treatment of DM and its vascular complications. We aimed to assess the antiproliferative effect of telmisartan within the diabetic aortic wall and hypothesised that this would be associated with reduction in aortic stiffness independently on BP (Paper II).

3. AIMS OF THE STUDY

The general aim of the study was to investigate the impact of vitamin D on the structural, functional, and biochemical parameters of the aortic wall, and on oxidative stress at a systemic and target organ level, as well as to study the effect of an angiotensin II receptor blocker telmisartan on arterial damage associated with experimental diabetes mellitus.

The specific aims of the study were as follows:

1. To assess aortic stiffness, as measured by aortic pulse wave velocity, in a rat model of streptozotocin-induced type 1 diabetes mellitus (STZ-T1DM).
2. To investigate the morphometric parameters in the histological preparations of the aorta and advanced glycation end-products in the aortic wall in STZ-T1DM.
3. To measure the serum levels of asymmetric dimethylarginine and assess oxidative stress level and total antioxidant capacity in the serum and liver in rats with STZ-T1DM.
4. To investigate the effects of treatment with vitamin D on aortic stiffness and aortic remodelling, oxidative stress level, and advanced glycation end-products in rats with STZ-T1DM.
5. To investigate the effects of treatment with telmisartan, an angiotensin II receptor blocker, on aortic stiffness and aortic remodelling in rats with STZ-T1DM.
6. To assess the relationship between aortic pulse wave velocity and vitamin D, and markers of inflammation, in young well-trained men exposed to long-term physical and environmental stress.

4. MATERIALS AND METHODS

4.1. Experimental animals

Male Wistar rats (RccHan:WIST, age 4 months, n=30 [Paper I], age 3 weeks, n=40 [Paper II]) were purchased from Harlan Laboratories (Harlan Laboratories Inc., The Netherlands). The animals were kept in a room with controlled temperature (21 ± 2 °C) and lighting (12:12-h light-dark cycle) with free access to food pellets and tap water. The animals were allowed to acclimatise for at least one week in the animal colony before entering experiments. All experimental procedures were approved by the Estonian National Board of Animal Experiments and were conducted in accordance with the European Communities Directive (86/609/EEC).

4.2. Study subjects

A total of 65 well-trained male soldiers (age 26 ± 4 years) from the Estonian ESTCOY-8 infantry company were examined before and after a 6-month military mission in Afghanistan. Pre-deployment measurements were performed two weeks before the company's deployment. Post-deployment measurements took place within one week after the return from Afghanistan. None of the participants showed any signs or symptoms of CVD (based upon history, electrocardiography, and exercise test), infection, or inflammatory disorders. None of the participants was currently taking any prescription medication.

4.3. Methods

4.3.1. Experimental protocol

4.3.1.1. Vitamin D experiment

Rats were randomly assigned to three groups of equal size: control group, diabetic group, and cholecalciferol-treated diabetic group (Papers I and III). Diabetes mellitus was induced by a single intraperitoneal injection of STZ 50 mg/kg (Sigma-Aldrich, St. Louis, MO, USA) freshly dissolved in 0.9% NaCl solution. Blood samples were taken 48 h later from the tail vein and glucose levels were measured with a glucometer (Glucocard X-meter, Arkray Inc., Japan). Diabetes was determined by blood glucose levels >15 mmol/L. Immediately after confirmation of diabetes, one diabetic group of animals was submitted to supplementation with cholecalciferol (Sigma-Aldrich, St. Louis, MO, USA) 12.5 µg (500 IU) kg^{-1} body weight, dissolved in 0.3 mL olive oil administered orally. Cholecalciferol was administered every other day by gavage for a period of 10 weeks. Weekly, body weights were monitored and glycosuria was assessed with reagent strips (Combur Test, Roche, Germany) to exclude ketosis. Haemodynamic measurements were performed 10 weeks after the DM induction.

4.3.1.2. Telmisartan experiment

Rats were randomly assigned to three groups: control (n=10), untreated diabetic (n=15), and telmisartan-treated diabetic (n=15) group (Paper II). Intraperitoneal injection of STZ 65 mg/kg (Sigma-Aldrich, St. Louis, MO, USA), freshly dissolved in 0.9% saline, was used to induce DM. After 48 hours, blood glucose was measured in samples taken from the tail vein, using a glucometer (Glucocard X-meter, Arkray Inc., Japan). Rats were considered diabetic if the blood glucose was >15 mmol/L. Treatment with telmisartan (Boehringer Ingelheim International GmbH, Germany) was started immediately after confirmation of DM. Telmisartan (10 mg/kg per day) was administered by gavage, dissolved in drinking water, for 10 weeks. Telmisartan was stopped one day prior to measurement of haemodynamic parameters. Weekly, body weights were recorded for all groups and glycosuria was assessed with reagent strips (Combur Test, Roche, Germany) to exclude ketosis in rats with DM.

4.3.2. Study protocol

The soldiers completed a questionnaire concerning their personal and familial medical history, lifestyle, physical activity/training habits, and basic demographic data. The participants were studied and blood samples were obtained following an overnight fast and abstinence from any medication, tobacco, alcohol, tea, and coffee. After 10 minutes of rest in the supine position BP was measured, pulse wave analysis (PWA) was performed, and aortic PWV was measured in all subjects. Venous blood samples were collected from the antecubital fossa. Height and weight of the soldiers were recorded and body mass index was calculated.

Informed written consent was obtained from each soldier in accordance with principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee, University of Tartu, Estonia.

4.3.3. *In vivo* haemodynamic measurements

4.3.3.1. Induction of anaesthesia

The animals were anaesthetised with a mixture of fentanyl (0.07 mg/kg, Gedeon-Richter Plc., Hungary), midazolam (5 mg/kg, Roche Pharma AG, Germany), and ketamine (75 mg/kg Vetoquinol Biowet Sp. z.o.o., Poland) administered subcutaneously. The optimal concentrations of the anaesthetic substances had been determined in pilot experiments. The depth of anaesthesia was monitored regularly by assessing the reflex response to noxious stimuli (hindpaw pinch) or tactile stimuli (corneal stroking). After induction of anaesthesia, animals were placed on a heating pad and body temperature was maintained at 37 °C.

4.3.3.2. Measurement of pulse wave velocity

A 2.5 F high-fidelity dual pressure sensor catheter with 50 mm separation between sensors (SPC-721, Millar Instruments Inc., TX, USA) was soaked in a water bath for 30 minutes to allow stability of the baseline before balancing and calibration against a mercury sphygmomanometer. The catheter was then introduced via the femoral artery into the descending aortic trunk so that the distal sensor was positioned at the beginning of the descending aorta and the resulting position of the proximal sensor was just proximal to the aortic bifurcation. The positioning of the distal sensor in the thoracic aorta was confirmed by the appearance of the incisura on the pressure waveform, indicating the aortic valve closure.

The rats were allowed to stabilise before resting blood pressures were recorded. Pulse pressure waves were recorded simultaneously at the two aortic sites and PWV was calculated offline by dividing the propagation distance by propagation time using an automated foot-to-foot method (Nichols and O'Rourke 2005; Mitchell *et al.* 1997). Data were acquired at a sampling rate of 2 kHz (PowerLab, ADInstruments, Australia) and feature extraction and calculations made with custom scripts in Spike2 v.6. software (Cambridge Electronic Design, United Kingdom).

4.3.3.3. Administration of vasoactive substances

Mean arterial pressure (MAP) was increased and decreased by infusion of PE (50 μg/min) and NTG (30 μg/min), respectively, via a catheter inserted into the femoral vein. Each drug was infused until the BP plateaued. Between the infusions, the rats were allowed a stabilisation period of at least 5 minutes and subsequent infusion of the opposing substance was given after the BP had returned to baseline. For PWV calculations, the range of pressure pulses during the decreasing MAP segment was selected after PE infusion, while the range of pressure pulses during the increasing MAP segment was selected after NTG infusion. These pressure ranges were assumed to be unaffected by the direct action of the vasoactive substances on the arterial function.

After the haemodynamic measurements were completed, blood samples were taken from the tail vein for assessment of glucose levels with a glucometer (Glucocard X-meter, Arkray Inc., Japan) [Paper I]. The rats were euthanised by drawing blood by cardiac puncture, followed by cervical dislocation. Samples of liver and aortic tissue were collected, soaked in ice-cold 0.9% NaCl, snap-frozen in liquid nitrogen, and stored at –60 ºC until use [Paper III]. Urine samples were obtained, drawn directly from the urinary bladder, and stored at –60 ºC until use [Paper II].

4.3.3.4. Haemodynamic data extraction

Simultaneous recording of multiple haemodynamic parameters enabled to analyse the relationship of BP and PWV on a pulse-by-pulse basis (Figure 3). The proximal arterial pressure waveform was used to construct a QRS complex in the electrocardiogram (ECG) waveform for calculation of heart rate (HR). The peak of the first derivative of the pressure waveform was used for calculations. The period of the waveform defined the R to R interval and the inverse of the period was output as a measure of HR.

Throughout the experiment, systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and MAP were determined from measurements made by the proximal pressure sensor in the abdominal aorta.

An algorithm designed and rigorously tested in previous studies (Butlin 2007) was employed to calculate PWV across a range of different pressure waveform shapes (Figure 4). Briefly, this algorithm of pressure foot location relies solely on the early segment of the systolic upstroke of the pressure waveform. The early systolic upstroke is not subject to the variability of late diastole, and is assumed to be free from augmentation by the reflected pressure wave (Nichols and O'Rourke 2005; Nitta *et al.* 2004). Using this algorithm, the foot of the pressure wave was defined by the peak of the second time derivative of pressure during each pulse (Chiu *et al.* 1991; Hermeling *et al.* 2007).

Pulse wave velocity was then plotted against MAP to construct phase plots to be used to characterise the PWV over a range of MAP from 50 to 200 mmHg in the aorta. Isobaric PWV indicates the comparison of PWV measured during the experiment at similar levels of BP when and compared among groups. This method for *in vivo* measurement of aortic BP and pulse-by-pulse pressure-dependent PWV has been employed in recent studies (Ng *et al.* 2011; Ng *et al.* 2012; Tan *et al.* 2012; Jung *et al.* 2013).

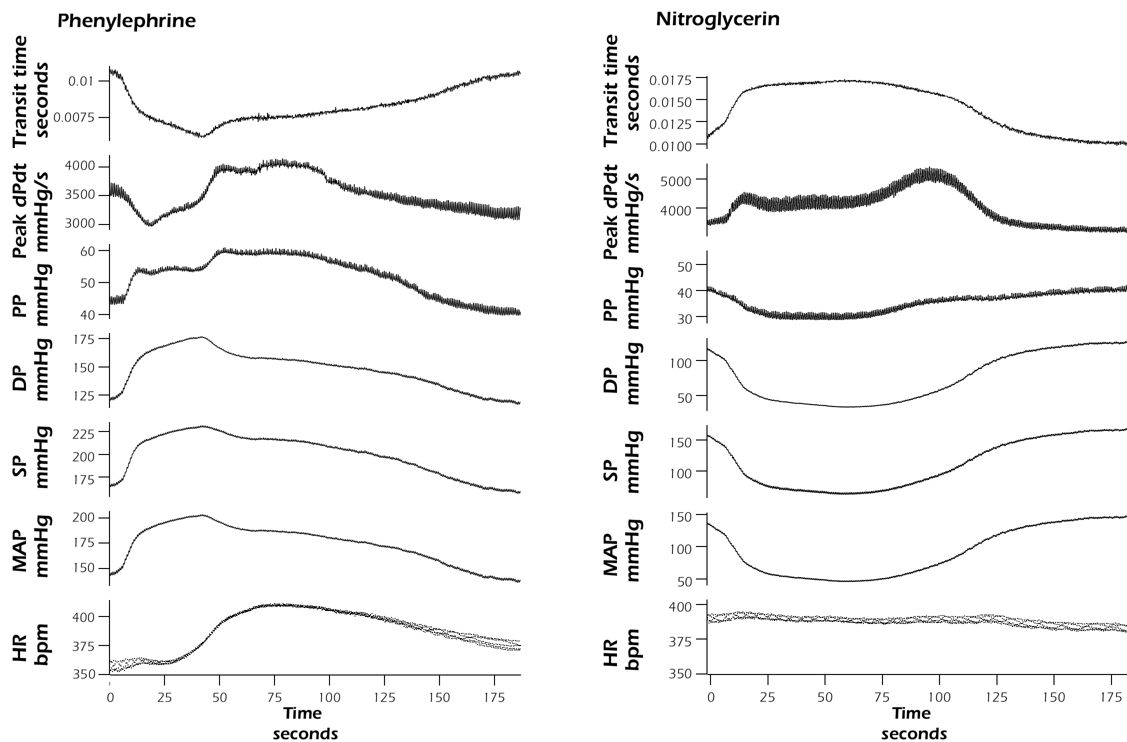

Figure 3. An example of the pulse-by-pulse analysis during intravenous infusion of phenylephrine (left) and nitroglycerin (right). Haemodynamic parameters measured are the heart rate (HR), mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), the maximum rate of pressure change (dP/dt), and transit time. Pulse wave velocity is calculated offline by dividing the known distance between the pressure sensors (50 mm) by the transit time (s). Each dot on the HR channel represents a single pulse.

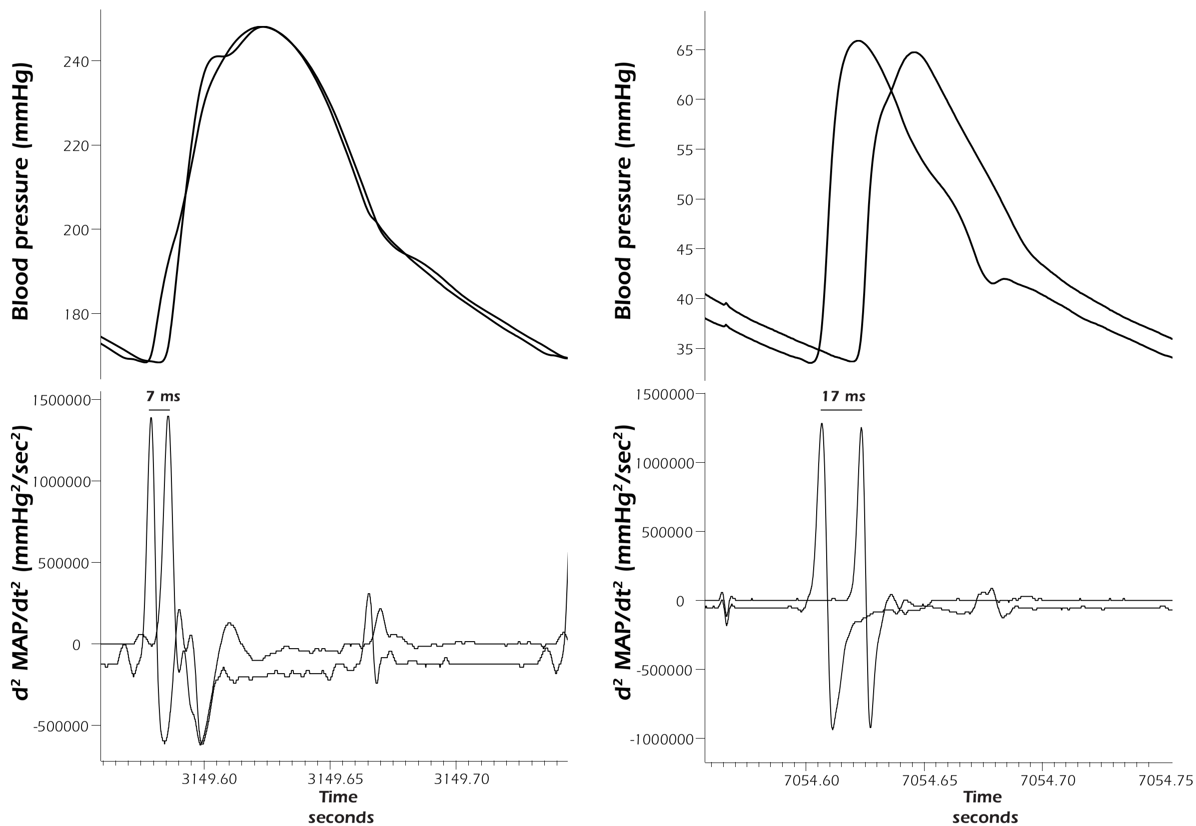

Figure 4. The pressure waveform foot is detected as the peak of the second derivative of the pressure wave which corresponds to the maximum rate of change in blood pressure during the early systole. The early systolic upstroke is generally considered to be not affected by the wave reflection. The transit time is the time difference between the peaks. Transit time decreases with increasing pressure, and the 7 ms transit time corresponds to pulse wave velocity of 7.14 m/s. When BP is decreased, the transit time increases, and the 17 ms transit time corresponds to PWV of 2.94 m/s.

4.3.4. Haemodynamic measurements in human subjects

4.3.4.1. Measurement of blood pressure

Peripheral BP was measured in the dominant arm using a validated oscillometric technique (OMRON M4-I, Omron Healthcare Europe, The Netherlands). Mean arterial pressure was obtained by integration of the radial pressure waveform using the Sphygmocor device with accordant software (SCOR Px, version 7.0; AtCor Medical, Australia). Peripheral PP was calculated as the difference between peripheral SBP and peripheral DBP. All measurements of BP were made in duplicate and mean values were used in subsequent analysis.

4.3.4.2. Aortic pulse wave velocity

Pulse wave velocity was measured by the foot-to-foot method using a Sphygmocor device. Pulse wave velocity was determined by sequentially recording ECG-gated carotid and femoral artery waveforms using a Millar tonometer (SPT-301B, Millar Instruments, TX, USA). 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. The difference between the carotid and the femoral path lengths was estimated from the distance of the sternal notch to the femoral pulse measured in a direct line. All measurements were made in duplicate and mean values were used in subsequent analysis.

4.3.4.3. Pulse wave analysis

Radial artery pressure waveforms were recorded with a high-fidelity applanation tonometer (SPT-301B, Millar Instruments, TX, USA) on the wrist of the left hand. After 20 sequential waveforms had been acquired, the integral software (SCOR Px, version 7.0; AtCor Medical, Australia) was used to generate a corresponding central (ascending aortic) waveform by a generalized transfer function, which has been prospectively validated for assessment of central BP (Sharman *et al.* 2006). Augmentation index, central SBP, central DBP, and central PP were derived from PWA. Augmentation index was calculated as the difference between the second and the first systolic peaks and expressed as the percentage of central PP (Wilkinson *et al.* 1998). Augmentation index was adjusted to a heart rate 75 beats/min using the Sphygmocor built-in algorithm.

4.3.5. Laboratory analyses

Leukocyte and platelet counts were assessed in whole blood using a QBC Autoread Plus autoanalyser (QBC Diagnostics, Inc., USA) (Paper IV). For other tests the blood samples were centrifuged within 15 minutes after collection at 3000 rpm for 15 minutes to obtain serum that was frozen at –60 °C until further analysis (Papers I–IV). Plasma glucose, total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglycerides were determined by standard laboratory methods, using certified assays, in a local clinical laboratory (Paper IV).

4.3.5.1. Biomarkers of inflammation

The plasma level of high-sensitivity CRP was measured by a validated latex particle-enhanced high-sensitivity immunoturbidimetric assay (CRP [Latex] HS, Roche Diagnostics Gmbh®, Mannheim, Germany), and analysed by the Hitachi 912 analyser (Roche Diagnostics®, Basel, Switzerland) (Paper IV).

Interleukin-1α, interleukin-2, interleukin-4, interleukin-6, interleukin-10, tumour necrosis factor alpha, interferon gamma, and monocyte chemoattractant protein-1were analysed with the Cytokine and Growth Factors High-sensitivity Array of the automated biochip immunoassay system, Evidence Investigator™ (Randox Laboratories Ltd., United Kingdom) (Paper IV).

4.3.5.2. Serum level of 25-hydroxyvitamin D

Serum 25(OH)D level was measured using a radioimmune assay (25-Hydroxyvitamin D, 125I Ria Kit, DiaSorin Corporation, MN, USA) (Papers I and IV). This assay has shown good correlation (r=0.74 to 0.96) with the liquid chromatography-tandem mass spectrometry that is currently regarded as the gold standard for the assessment of 25(OH)D (Zerwekh 2008). 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 radio-immune assay using 125I-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-assay and inter-assay coefficients of variation were 8.1% and 10.2%, respectively.

4.3.5.3. Serum levels of calcium and albumin

Calcium concentration in the serum was determined by a colorimetric test (Calcium liquicolor, HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Germany) (Papers I and III). This assay is based on the reaction between calcium ions and o-cresolphthalein-complexone in an alkaline medium,

resulting in a purple coloured complex. The absorbance of this complex was measured spectrophotometrically at 570 nm using a photometer (Tecan Sunrise, Tecan GmbH, Austria).

Serum albumin levels were measured using a colorimetric test (Albumin liquicolor, HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Germany) (Paper II). In this assay, bromocresol green forms with albumin in citrate buffer a coloured complex. The absorbance of this complex was measured spectrophotometrically at 578 nm. Serum calcium levels were corrected for albumin concentrations using the following formula: corrected calcium = serum calcium + 0.02 × (40 – serum albumin) (Gardner *et al.* 1981).

4.3.5.4. Glucose level in urine

Glucose concentration in the urine samples was measured by a colorimetric hexokinase glucose-6-phosphate dehydrogenase method (Glucose-HK kit, Spinreact, Spain) (Paper II). Hexokinase catalyses the adenosine triphosphate (ATP)-dependent phosphorylation of glucose to glucose-6-phosphate. The formed glucose-6-phosphate is reduced to 6-phosphogluconate in the presence of glucose-6-phosphate dehydrogenase with the subsequent reduction of nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH). The increase in the concentration of NADH, measured spectrophotometrically at 340 nm, is proportional to the glucose concentration in the sample.

4.3.5.5. Serum level of asymmetric dimethylarginine

Asymmetric dimethylarginine was determined from serum samples by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (DLD Diagnostika, Germany) (Paper I). ADMA is bound to the solid phase of the microtitre plate. In samples ADMA is acylated and competes with the solid phase-bound ADMA for a fixed number of rabbit anti-ADMA anti-serum binding sites. At equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase ADMA is detected by anti-rabbit/peroxidase. The substrate tetra-methyl-benzidine (TMB)/peroxidase reaction is analysed spectrophotometrically at 450 nm. The intra-assay and inter-assay coefficients of variation were 6.1% and 9.5%, respectively.

4.3.5.6. Measurement of total antioxidant capacity in the serum and liver

Liver samples were homogenised in ice-cold 0.9% NaCl solution and centrifuged at 15,000 x *g* for 10 minutes at 4ºC (Paper III). The insoluble pellets were discarded and the supernatants were used for analysis. An automated

colorimetric measurement method was employed (Erel 2004) using a colorimetric assay (Randox Laboratories Ltd., United Kingdom). By this method, a colourless molecule, reduced 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS), is oxidised to a blue-green ABTS*+, using hydrogen peroxide in acidic medium (the acetate buffer 30 mL/L pH 3.6). When the coloured ABTS*+ is mixed with any substance that can be oxidized, it is reduced to its original colourless ABTS form again. The 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate)*+ is decolourised by antioxidants according to their concentrations and antioxidant capacities. This change of colour is measured as a change in absorbance at 660 nm.

Briefly, 200 µL of reagent 1 (R1: acetate buffer 0.4 mol/L, pH 5.8) was carefully mixed with 5 µL of test sample in cuvette and incubated for 4 min at 37 °C in the spectrophotometer. The first absorbance was taken before the mixing with 20 µL R2 (R2: the ABTS*+ in acetate buffer 30 mmol/L, pH 3.6 [as sample blank]). The last absorbance was taken at the end of incubation (5 min). The bleaching rate is inversely related with the total antioxidant capacity (TAC) of sample. The reaction rate was calibrated with Trolox, which is used as a traditional standard for TAC measurement assays. The results are expressed in mmol Trolox equivalent/L. The data for the liver samples were expressed as mmol Trolox equivalent/mg protein with protein concentration measured spectrophotometrically using the Lowry method (Lowry *et al.* 1951).

Within- and between-batch precision data obtained by TAC method were 2.5 and 2.9% respectively.

4.3.5.7. Measurement of total peroxide concentrations and oxidative stress index in the liver

Liver samples were homogenised in ice-cold 0.9% NaCl solution and centrifuged at 15,000 x *g* for 10 minutes at 4ºC (Paper III). The insoluble pellets were discarded and the supernatants were used for analysis. Total peroxide concentrations (TPX) were measured with an OxyStat colorimetric assay kit (Biomedica Gruppe, Austria). Total peroxide concentration is determined by reaction of the biological peroxides with the enzyme peroxidase and a subsequent colour-reaction using TMB as substrate. After adding the stop solution the developed colour is measured spectrophotometrically at 450 nm. A calibrator is measured in parallel and used to calculate the concentration of circulating biological peroxides in the sample in a one-point calibration protocol. The results show a direct correlation between free radicals and circulating biological peroxides, and thus allow the characterisation of the oxidative status in biological samples. The detection limit of the assay is 7 µmol/L. The intra-assay and inter-assay coefficients of variation were 3.1% and 5.1%, respectively. The data were expressed as nmol/mg protein, with protein concentration measured spectrophotometrically using the Lowry method (Lowry *et al.* 1951).

Oxidative stress index (OSI) was expressed as per cent ratio of TPX level to TAC level (Harma *et al.* 2003). For calculation purposes, the resulting unit of TAC, mmol/L, was changed to μmol/L, and the OSI value was calculated according to the following formula: OSI = TPX (μmol/L) / TAC (μmol/L) × 100.

4.3.5.8. Measurement of Nε-(carboxymethyl)lysine levels in the aortic wall

Aortic strips (10 mm) were homogenised in ice-cold 0.9% NaCl solution, using a blade type homogeniser (Tekmar Tissumizer, Cincinnati, OH, USA) and centrifuged at 10,000 x *g* for 10 minutes at 4ºC (Paper III). The insoluble pellets were discarded and the supernatants were used for analysis. The concentration of CML was assessed using a commercial ELISA test OxiSelect™ N-epsilon-(Carboxymethyl) Lysine ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA). Ten μg/ml of total protein extracts were adsorbed onto a 96-well plate at 4ºC overnight, with protein concentration measured spectrophotometrically using the Lowry method (Lowry *et al.* 1951). The absorbance of each well was measured spectrophotometrically at 450 nm. The concentration of CML was calculated by comparison to a standard curve consisting of known concentrations of CML-BSA (bovine serum albumin). Results are presented as nanogram of CML per milliliter of solution.

4.3.6. Histological analysis of the aorta

4.3.6.1. Assessment of wall structure and morphometric parameters

The aortic samples for histological analysis were fixed in 10% formalin for 12 hours and embedded in paraffin with vacuum infiltration processor (Tissue-Tek® VIP™ 5 Jr, Sakura, USA) (Papers I and II). Specimens were cut with microtome Ergostar HM 200 (Microm, Germany) at four-μm thickness sections and stained using the haematoxylin-eosin, resorcin-fuchsin, and van Gieson methods for examination by light microscopy (Olympus BX50, Japan).

Estimation of the internal diameter of the aorta was performed by measuring two inner diameters at right angle for each cross-section of the thoracic aorta. At least eight different cross-sections of the aorta were analysed for each rat.

Thickness of the medial layer of the aorta was determined in the thoracic aorta cross-sections by ten consecutive measurements in a systematic manner to evaluate all segments of the circumference of the aorta. At least six different cross-sections of aorta were analysed for each rat. The staining intensities of the elastic fibres in the media and collagen fibres in the media and adventitia were evaluated on a subjective scale ranging from 0 to 3 (0 – no staining of fibres, 1 – poor staining of fibres, 2 – moderate staining of fibres, 3 – intensive staining of fibres). The evaluations were performed by two independent observers in a blinded fashion; the scores were summed and used for statistical analysis.

4.3.6.2. Immunohistochemical assessment of Nε-(carboxymethyl)lysine in the aortic wall

Three-μm thick paraffin sections mounted on poly-L-lysine coated SuperFrost slides (Menzel Gläser, Germany) were deparaffinised and rehydrated (Paper III). Peroxidase activity was blocked by 0.6% H_2O_2 (Merck, Germany) in methanol (Merck, Germany). Then the sections were washed in tap water and in phosphate buffered saline (pH = 7.4; Gibco, Invitrogen, CA, USA) for 10 minutes, treated with normal 1.5% goat serum (Gibco, Invitrogen Corporation, CA, USA) for 20 minutes at room temperature and incubated with the first antibody: anti-CML (mouse monoclonal antibody [CML26], abcam, United Kingdom) diluted 1:50 overnight at 4°C in the humidity chamber. On the next day the sections were incubated with the biotinylated horse anti-mouse secondary antibody for 30 min at room temperature. After a wash step the sections were incubated with the avidin-biotin peroxidase complex ELITE system (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA, USA) for 30 minutes. Peroxidatic activity was detected with 3,3'-diaminobenzidine (Vector Laboratories Inc., USA) and the sections were counterstained with hemalaun, dehydrated, and mounted with DPX (Fluka, Switzerland). The labelling was expressed on a semi-quantitative scale ranging from 0 to 4 (0 – no staining, 1 – weak staining, 2 – moderate staining, 3 – strong staining, 4 – very strong staining). Two independent observers in a blinded fashion performed the evaluation. Immunohistochemistry negative controls were performed by omitting the primary antibody (mouse IgG was used in place of the primary antibody).

4.3.7. Statistical analysis

Statistical comparisons were performed with the Statistica software (version 8; StatSoft, USA) (Papers I-IV) and with the software R (version 2.15.3 for Windows; The R Foundation for Statistical Computing, Austria) (Paper II). Continuous variables are shown as mean ± standard deviation or medians with interquartile ranges. All data were tested for normality using the Kolmogorov-Smirnov test (Papers I–IV).

In Paper II, all haemodynamic parameters were averaged into 5 mmHg MAP bins. Analysis was restricted to the MAP range of 60 to 185 mmHg. Rat S11 and S12 were merged to create one sample, due to lack of data in each rat. Rat S14 was removed due to lack of data. Second order polynomials were fitted using least squares regression to each individual rat data set, a second order polynomial being confirmed as a better fit than either linear or third order polynomial by Akaike Information Criterion (Table 1) (Akaike 1974). The intercepts (a) and coefficients (b, c) were extracted from the polynomial model (*Equation 1*), and comparison between groups made by analysis of variance (ANOVA), using a post-hoc, Bonferroni corrected t-tests to ascertain differences.

For analysis of the data as a whole, one of two methods was employed. *(1)* The data was transformed into a linear form (*Equation 2*) and analysis of covariance (ANCOVA) was employed if the assumption of equal slopes was maintained. *(2)* If the assumption of equal slopes was not maintained, robust analysis ANCOVA (Wilcox 2005) was employed with comparison of groups at 60, 90, 120, 150, and 180 mmHg MAP and a 20% trimmed mean. The robust analysis ANCOVA accommodates non-linearity, heteroscedasticity, and unequal slopes. The assumption of equal slopes was tested by ANCOVA as in method (1) with an interaction term between PWV and MAP, and PWV and MAP2. If either interaction was significant, showing that slopes were not equal, method (2) was employed.

Equation 1 $$PWV = a + b \times MAP + c \times MAP2$$

Equation 2 $$MAP2 = MAP^2$$

Differences in resting haemodynamic variables and basic and laboratory parameters between the groups were evaluated using the one-way ANOVA followed by Tukey's *post-hoc* analysis for multiple comparisons of group means. (Papers I–III). Semi-quantitative data were compared by the Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U test (Papers I–III).

Skewed variables were log-transformed before the analysis to obtain a normal distribution (Paper IV). Comparisons of two different groups were performed using the unpaired *t*-test (Paper IV). The Pearson correlation analysis was used to examine the associations between the parameters (Paper IV). Statistical significance was defined as P <0.05 (Papers I–IV).

Table 1. Model 1: linear; Model 2: second order polynomial; Model 3: third order polynomial fit. A second order polynomial provides the best fit to the grouped data.

	Df	AIC
Model 1	5.00	970.37
Model 2	6.00	786.11
Model 3	7.00	787.55

Df, degrees of freedom; AIC, Akaike information criterion.

5. RESULTS

5.1. Aortic stiffening and remodelling in experimental diabetes (Papers I and II)

5.1.1. Basic and biochemical characteristics

5.1.1.1. Vitamin D experiment

The results are presented in Table 2. The initial body weights were similar in control and diabetic groups. The final body weights in the diabetic groups were significantly lower than in the control group. The levels of blood glucose and HbA_{1c} were found to be significantly increased in the diabetic rats. Serum 25(OH)D levels were significantly decreased in the untreated diabetic group, compared to the control group. Serum levels of ADMA were significantly lower in the control group compared with untreated diabetic group.

5.1.1.2. Telmisartan experiment

The initial body weights were similar in all groups (Table 3). In the course of the experiment, rats in both treated and untreated diabetic groups presented with abnormalities associated with persistent hyperglycaemia: hyperphagia, polydipsia, polyuria, and wasting of stored fat as evidenced by retarded weight gain. The final body weights were significantly lower in diabetic groups compared to the control group, while no difference was found between the treated and untreated diabetic groups. The ratio of heart weight to body weight as a surrogate index of cardiac hypertrophy was significantly increased in both diabetic groups, compared to the control group. Glucose concentration in the urine samples was significantly higher in both diabetic groups, compared to the control group. Treatment with telmisartan resulted in slight but statistically significant reduction in urinary glucose excretion.

Table 2. Basic and laboratory parameters. Body weight was assessed at the beginning and at the end of the experiment.

Group	Body weight (g)		Blood glucose (mmol/L)	HbA_{1c} (%)	25(OH)D (nmol/L)	ADMA (µmol/L)	Calcium (mmol/L)
	Before	After					
Control	405 ± 26	450 ± 30	6.3 ± 1.6	4.0 ± 0.1	$140 \pm 21^{\#}$	0.68 ± 0.18	2.7 ± 0.3
Diabetes	406 ± 56	$370 \pm 50^{*}$	$28.3 \pm 3.9^{*}$	$10.3 \pm 0.7^{*}$	$108 \pm 38^{\#¶}$	$0.87 \pm 0.14^{¶}$	2.6 ± 0.3
Diabetes + vitamin D	406 ± 51	$352 \pm 43^{*}$	$28.5 \pm 5.9^{*}$	$9.5 \pm 1.3^{*}$	494 ± 125	$0.85 \pm 0.16^{¶}$	2.7 ± 0.2

HbA_{1c}, glycated haemoglobin; ADMA, asymmetric dimethylarginine; 25(OH)D, 25-hydroxyvitamin D.
¶ P <0.05 vs Control; * P <0.001 vs Control; $^{\#}$ P <0.001 vs Diabetes + vitamin D

Table 3. Basic and laboratory parameters. Body weight was assessed at the beginning and at the end of the experiment.

Group	Body weight (g)		Heart weight (g)	Cardiac index (%)	Urine glucose (mmol/L)
	Before	After			
Control	130 ± 10	408 ± 24	0.95 ± 0.09	2.32 ± 0.22	1.6 ± 0.9
Diabetes	135 ± 12	$216 \pm 59^{*}$	$0.62 \pm 0.13^{*}$	$2.79 \pm 0.25^{¶}$	$511.6 \pm 97.2^{*\#}$
Diabetes + telmisartan	135 ± 14	$254 \pm 62^{*}$	$0.67 \pm 0.12^{*}$	$2.67 \pm 0.35^{¶}$	$424.9 \pm 63.5^{*}$

¶ P <0.05 vs Control; * P <0.001 vs Control; $^{\#}$ P <0.05 vs Diabetes + telmisartan
Cardiac index = heart weight (mg) / body weight (g).

5.1.2. Haemodynamic parameters

5.1.2.1. Vitamin D experiment

Before administration of vasoactive substances, resting SBP, DBP, PP, MAP, and HR were not statistically different between all groups (Table 4). Intravenous infusion of PE increased MAP to 200 mmHg, followed by infusion of NTG, which decreased MAP to 50 mmHg. The diabetic rats had a significantly higher PWV compared to the control rats across a supraphysiological range of MAP (170–200 mmHg), but not at a lower MAP range. The non-linear PWV-MAP curve for the diabetic treated group was similar to that of diabetic untreated group, indicating that aortic stiffness was similar at every given MAP (Figure 5).

Table 4. Resting anaesthetised haemodynamic parameters obtained before the administration of vasoactive substances.

Group	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	PP (mmHg)	HR (beats/min)	PWV (m/s)
Control	140 ± 30	106 ± 27	118 ± 28	34 ± 6	364 ± 96	5.0 ± 0.6
Diabetes	135 ± 15	103 ± 18	114 ± 17	31 ± 4	343 ± 53	5.2 ± 0.3
Diabetes + vitamin D	136 ± 14	104 ± 12	115 ± 12	32 ± 3	348 ± 107	4.9 ± 0.3

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate; PWV, pulse wave velocity.
P >0.05 between all groups.

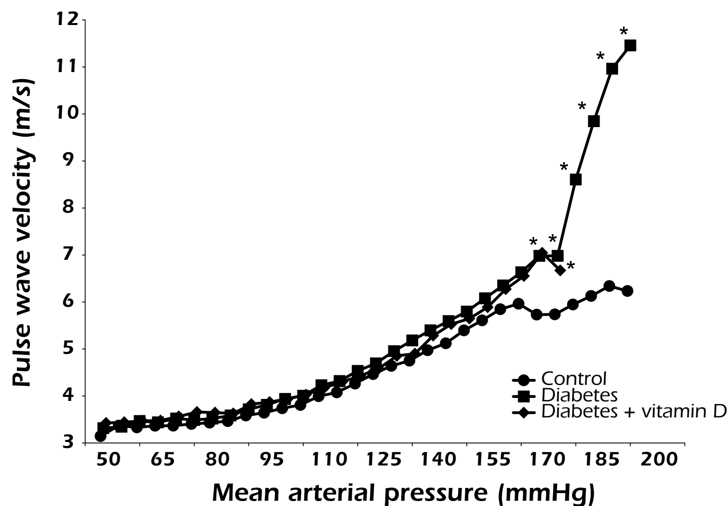

Figure 5. Isobaric PWV-MAP curves in the control, untreated diabetic, and diabetes + vitamin D groups, averaged over 5 mmHg pressure steps. * P <0.05 vs Control.

5.1.2.2. Telmisartan experiment

Prior to administration of vasoactive substances, SBP, DBP, PP, MAP, HR, PWV, and maximal change in BP (dP/dt) were recorded (Table 5). There were no differences in BPs between the control and untreated diabetic group. HR was significantly decreased in the untreated diabetic group, compared to the control group. Peak dP/dt, a surrogate index of systolic function, was significantly decreased in the untreated diabetic rats.

The means and standard deviations of coefficients of the second order polynomial curve fits to individual rats in $PWV = a + b \times MAP + c \times MAP2$ are provided in Table 6. Analysis of variance on the intercepts and coefficients is provided in Tables 7, 9, and 11. There was no significant difference in the intercept (a) between the groups, but there was a significant difference in the coefficients (b) and (c) between the untreated diabetics and controls (Tables 8, 10, and 12) but not among the other groups. A second order polynomial was fitted on PWV-MAP curve for characterisation of pressure-independent PWV across the full MAP range (Figure 6).

Table 13 gives an ANCOVA with interaction terms for MAP and Group, and MAP2 and Group. It shows that there was a significant interaction between MAP and Group ($P < 0.001$) but not MAP2 and Group ($P = 0.21$). As there was a significant interaction term, and slopes therefore unequal, robust methods ANCOVA was used to test between group differences. This showed differences between untreated diabetes and control groups at all levels other than 60, 100, and 110 mmHg MAP (Table 14).

Table 5. Resting anaesthetised haemodynamic parameters obtained before the administration of vasoactive substances.

Group	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	PP (mmHg)	HR (beats/min)	Peak dP/dt (mmHg/s)	PWV (m/s)
Control	155 ± 15[#]	113 ± 15[#]	130 ± 28[#]	41 ± 3	462 ± 46	4024 ± 617	4.5 ± 0.5[#]
Diabetes	142 ± 13[#]	105 ± 13[#]	124 ± 14[#]	38 ± 9	386 ± 48[*#]	2186 ± 198[*#]	4.6 ± 0.9[#]
Diabetes + telmisartan	110 ± 14	75 ± 14	92 ± 12	35 ± 5	321 ± 26[*]	3294 ± 183[*]	3.6 ± 0.3

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate; dP/dt,
maximal change in pressure over time; PWV, pulse wave velocity.
[*] P <0.01 vs Control; [#] P <0.01 vs Diabetes + telmisartan.

Table 6. Means and standard deviations of coefficients of the second order polynomial curve fits to individual rats in $PWV = a + b \times MAP + c \times MAP2$.

Group	a (mean ± SD)	b (mean ± SD)	c (mean ± SD)
Control	3.8 ± 0.9	–0.02 ± 0.02	0.00023 ± 0.00009
Diabetes	6.0 ± 2.1	–0.07 ± 0.04	0.00045 ± 0.00018
Diabetes + telmisartan	5.1 ± 1.5	–0.05 ± 0.03	0.00038 ± 0.00014

SD, standard deviation; MAP, mean arterial pressure.

Table 7. Analysis of variance for the intercept, (*a*), in $PWV = a + b \times MAP + c \times MAP2$.

	Df	Sum Sq	Mean Sq	F value	P value
Group	2	21.10	10.55	4.21	0.03
Residuals	24	60.20	2.51		

Df, degrees of freedom; Sum Sq, sum of squares; Mean Sq, mean of squares.

Table 8. Bonferroni adjusted, unpaired *t*-test results for the intercept, (*a*).

Group 1	Group 2	P value	P adjusted
D	DT	0.34	1.00
C	DT	0.04	0.12
C	D	0.02	0.06

D, Diabetes; DT, Diabetes + telmisartan; C, control.

Table 9. Analysis of variance for the coefficient, (b), in $PWV = a + b \times MAP + c \times MAP2$. There is a significant difference in the coefficient, (b).

	Df	Sum Sq	Mean Sq	F value	P value
Group	2	0.01	0.00	4.89	0.02
Residuals	24	0.02	0.00		

Df, degrees of freedom; Sum Sq, sum of squares; Mean Sq, mean of squares.

Table 10. Bonferroni adjusted, unpaired *t*-test results for the coefficient, (b).

Group 1	Group 2	P value	P adjusted
D	DT	0.36	1.00
C	DT	0.03	0.08
C	D	0.01	0.04

D, Diabetes; DT, Diabetes + telmisartan; C, Control.

Table 11. Analysis of variance for the coefficient, (c), in $PWV = a + b \times MAP + c \times MAP2$. There is a significant difference in the coefficient, (c).

	Df	Sum Sq	Mean Sq	F value	P value
Group	2	0.00	0.00	5.48	0.01
Residuals	24	0.00	0.00		

Df, degrees of freedom; Sum Sq, sum of squares; Mean Sq, mean of squares.

Table 12. Bonferroni adjusted, unpaired *t*-test results for the coefficient, (c).

Group 1	Group 2	P value	P adjusted
D	DT	0.39	1.00
C	DT	0.02	0.05
C	D	0.01	0.02

D, Diabetes; DT, Diabetes + telmisartan; C, Control.

Table 13. Analysis of covariance with an interaction term between MAP and Group, and MAP2 and Group. Significant interaction between Group and MAP indicates that the slopes of different groups are significantly different.

	Df	Sum Sq	Mean Sq	F value	P value
MAP	1	849.91	849.91	3953.44	<0.001
MAP2	1	47.35	47.35	220.27	<0.001
Group	2	13.41	6.70	31.18	<0.001
MAP:Group	2	5.58	2.79	12.97	<0.001
MAP2:Group	2	0.67	0.34	1.56	0.2
Residuals	571	122.75	0.21		

MAP, mean arterial pressure; Df, degrees of freedom; Sum Sq, sum of squares; Mean Sq, mean of squares.

Table 14. Robust method analysis of covariance, Control (C) *vs* Diabetes (D).

MAP	n1	n2	DIF	TEST	SE	CI.low	CI.hi	P value	crit.val
60	23	10	–0.13	1.04	0.13	–0.63	0.37	0.03	3.95
70	42	21	–0.18	3.65	0.05	–0.34	–0.03	<0.001	3.07
80	45	28	–0.17	3.64	0.05	–0.31	–0.03	<0.001	3.08
90	46	32	–0.15	2.94	0.05	–0.31	0.01	0.01	3.05
100	45	36	–0.11	1.98	0.06	–0.28	0.06	0.05	3.00
110	45	38	–0.12	1.50	0.08	–0.35	0.12	0.14	3.02
120	45	39	–0.18	1.91	0.09	–0.46	0.10	0.06	3.00
130	45	38	–0.26	2.33	0.11	–0.58	0.07	0.02	2.99
140	45	39	–0.35	2.22	0.16	–0.82	0.12	0.03	3.01
150	45	38	–0.44	2.15	0.20	–1.05	0.18	0.04	3.03
160	45	38	–0.55	2.50	0.22	–1.21	0.12	0.02	3.04
170	44	35	–0.72	3.20	0.23	–1.41	–0.03	0.001	3.05

MAP, mean arterial pressure; SE, standard error; CI, confidence interval.

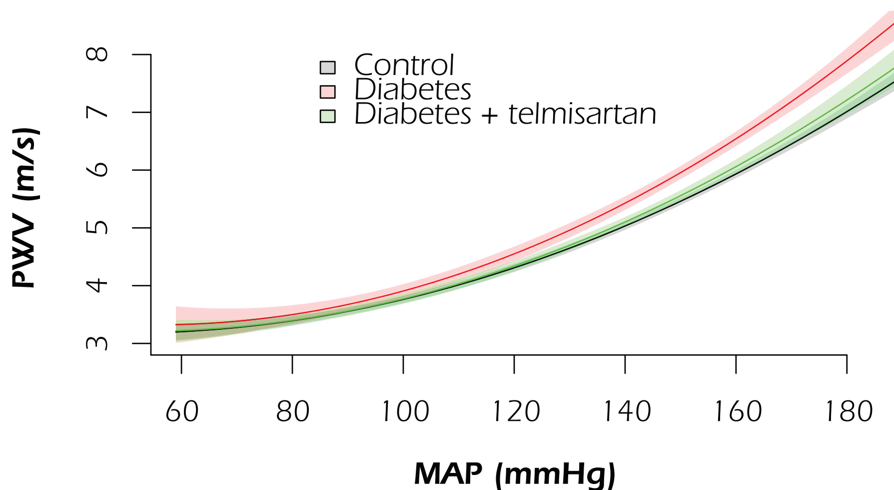

Figure 6. A second order polynomial fitted on PWV-MAP curve, with the 95% confidence interval shaded. PWV is significantly higher in the untreated diabetic group over the high pressure range, compared to the treated and control groups. No differences were observed among the treated and control groups across the full MAP range. MAP, mean arterial pressure.

5.1.3. Histological analysis and morphometric parameters of the aortic wall

5.1.3.1. Vitamin D experiment

Aortic samples in the control group showed a regular vascular morphology, while several alterations were noted in the aortic wall structure in the untreated diabetic group. In the aortas of the untreated diabetic group focal irregular arrangement of elastic fibres was noted together with decreased staining intensity of elastic fibres and increased internal diameter of the aorta (Table 15, Figures 7 and 8). Changes in the medial thickness and in collagen staining were not statistically significant compared to the control group (Table 3). Untreated DM was also associated with reduced ratio of elastin to collagen (Table 15).

Table 15. Morphometric parameters and estimations of staining intensities of connective tissue fibres in the medial and adventitial layers of the thoracic aorta.

Parameter	Control	Diabetes	Diabetes + vitamin D
Internal diameter of aorta (mm)	1.40 ± 0.27	1.65 ± 0.18*	1.55 ± 0.21
Thickness of media (µm)	86.41 ± 8.15	79.09 ± 14.61	82.93 ± 11.29
Elastic fibres in media (arbitrary units)	2.44 ± 0.33	1.84 ± 0.33¶;#	2.29 ± 0.26
Collagen fibres in media (arbitrary units)	1.20 ± 0.38	1.38 ± 0.46	1.24 ± 0.41
Collagen fibres in adventitia (arbitrary units)	2.90 ± 0.41	2.56 ± 0.40	2.52 ± 0.43
Elastin/collagen ratio in media (%)	1.72 ± 0.55	1.33 ± 0.43*;#	1.88 ± 0.47

Values are expressed as means ± SD.
* P <0.05 vs Control; ¶ P <0.01 vs Control; # P <0.05 vs Diabetes + vitamin D

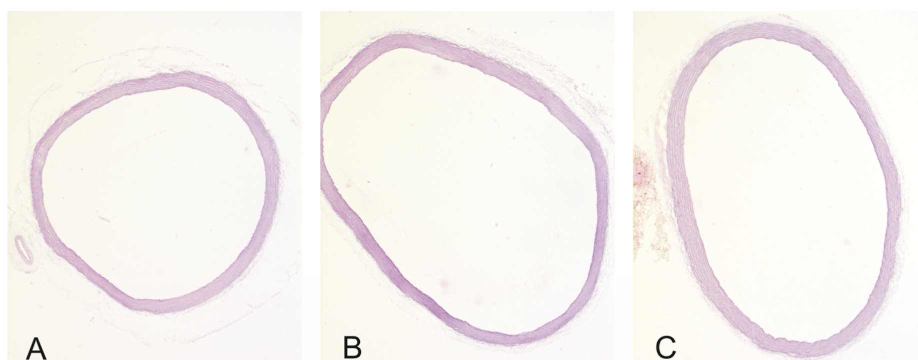

Figure 7. Micrographs of the transverse aortic sections in the control group (A), untreated diabetic group (B), and diabetes + vitamin D group (C). Note the enlarged internal diameter of the aorta in the diabetic group (B). Resorcin-fuchsin. Original magnification ×26.

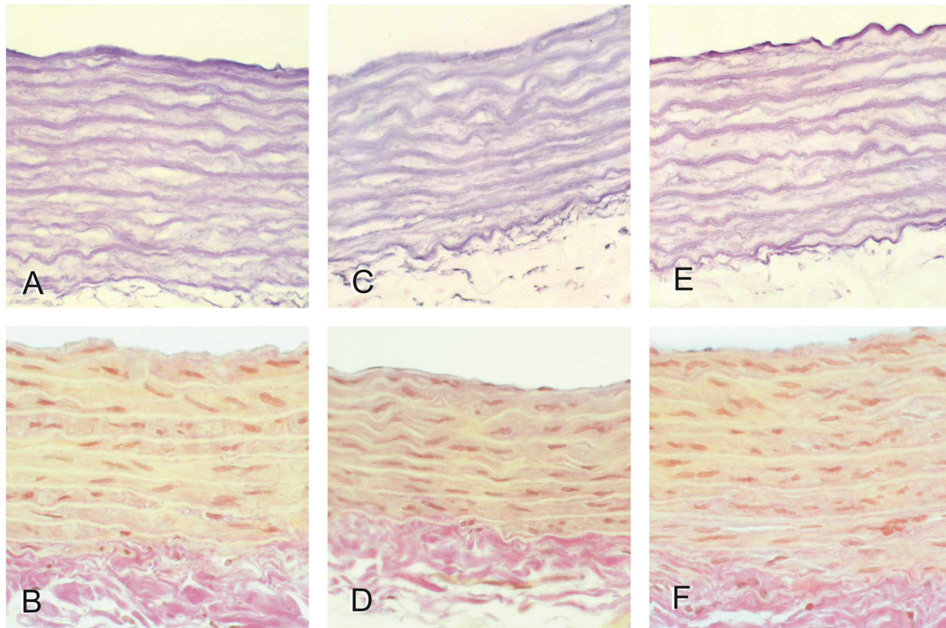

Figure 8. A panel of micrographs of the aortic sections in the control group (A and B), untreated diabetic group (C and D), and diabetes + vitamin D group (E and F). Reduction of the thickness of the medial layer (C and D) and disorganisation of elastic lamellae (C) was observed in diabetic untreated rats, while in diabetes + vitamin D group the aortic wall morphology was more similar to the control group samples. Resorcin-fuchsin (A, C, E) and van Gieson (B, D, F). Original magnification ×360.

5.1.3.2. Telmisartan experiment

The lumen diameter of the thoracic aorta and the width of the medial layer were significantly smaller in the untreated diabetic group compared to the control group (Table 16). The staining intensity of medial elastic fibres in the untreated diabetic group was decreased compared to the control group (Table 16). Furthermore, focal irregularities in the arrangement of elastic fibres were noted in the untreated diabetic group (Figure 9). Even more pronounced differences were seen in the staining of collagen fibres in the medial layer as higher collagen concentrations were present in the untreated diabetes group compared to the control group (Table 16, Figure 9). This resulted in significantly lower medial ratio of elastin to collagen in the untreated diabetic group (Table 16). No differences between groups were seen in the count of nuclei of smooth muscle cells (Table 16).

Table 16. Histomorphometric parameters of the thoracic aortas.

Parameter	Control	Diabetes	Diabetes + telmisartan
Internal diameter of aorta (mm)	1.65 ± 0.18	1.23 ± 0.19[¶]	1.54 ± 0.22[#]
Width of media (μm)	117.39 ± 16.30	82.62 ± 9.32[¶]	87.68 ± 11.11[¶]
Elastic fibres in media (arbitrary units)	2.84 ± 0.23	2.54 ± 0.34[*]	2.65 ± 0.27
Collagen fibres in media (arbitrary units)	0.66 ± 0.19	1.43 ± 0.31[¶]	1.30 ± 0.10[¶]
Elastin/collagen ratio in media (%)	4.28 ± 0.57	1.79 ± 0.035[¶]	2.07 ± 0.50[*]
Smooth muscle cell nuclei	78.86 ± 15.01	94.10 ± 21.12	89.17 ± 24.90

Values are expressed as means ± SD.
[*]P <0.05 vs Control; [¶]P <0.001 vs Control; [#]P <0.05 vs Diabetes

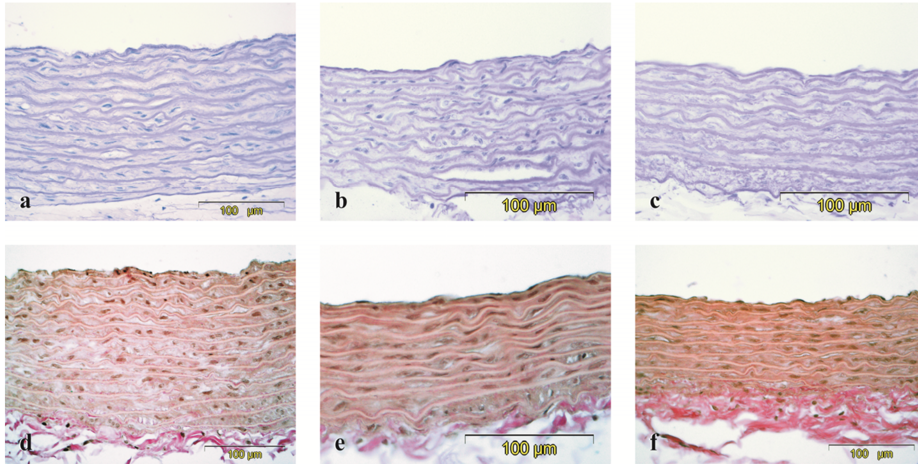

Figure 9. Representative micrographs of the aortic walls from the control group (a and d), untreated diabetes group (b and e) and diabetes + telmisartan group (c and f). Decreased thickness of the medial layer was noted in both untreated diabetes and diabetes + telmisartan group, while focal disorganisation of elastic lamellae were seen in untreated diabetes group (b). Resorcin-fuchsin (a-c) and van Gieson (d-e).

5.1.4. Advanced glycation end-products within the aortic wall (Paper III)

Untreated DM produced significantly higher levels of CML in the aortic wall homogenates (Figure 10). Immunohistochemical detection of CML demonstrated strong immunostaining in all layers of the aortic wall in the untreated diabetic group (Figure 11). In the control group, immunostainings were moderate in the intimal and adventitial layers, while the medial layer stained weakly (Table 17, Figure 11).

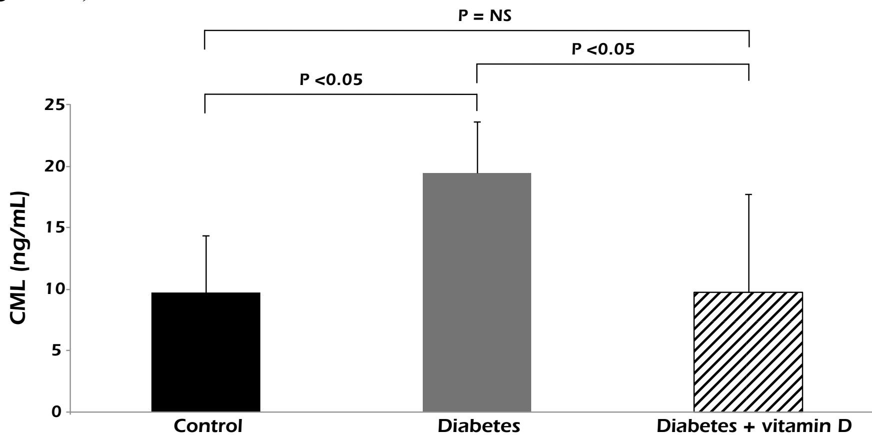

Figure 10. Untreated diabetes mellitus was associated with significantly higher levels of *N*ε-(carboxymethyl)lysine in the aortic wall that was prevented by vitamin D supplementation. CML, *N*ε-(carboxymethyl)lysine. Data are mean ± SD.

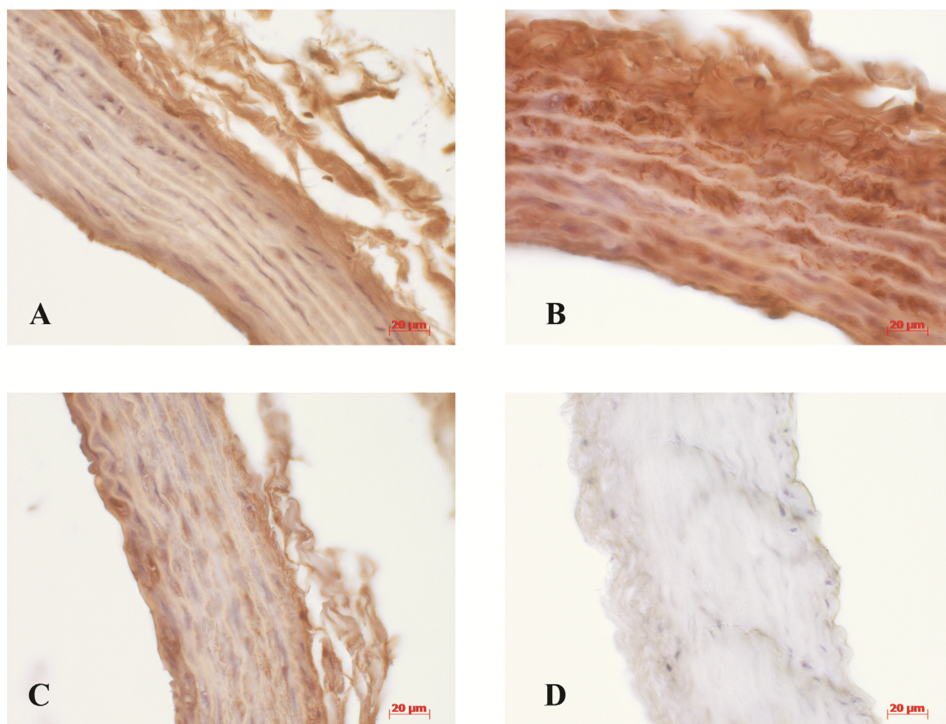

Figure 11. Immunohistochemical localisation of *N*ε-(carboxymethyl)lysine in the aortic wall of the diabetes + vitamin D group (A), untreated diabetic group (B) and control group (C). Strong immunostaining was found in all layers of the aorta in the diabetic group (B), while immunostaining in the medial layer was moderate in the diabetes + vitamin D group (A), similar to the staining intensity of the control group (C). Staining was not seen in negative controls, where the primary antibody was omitted (D). Diaminobenzidine + hemalaun.

Table 17. Estimation of the immunostaining intensity of *N*ε-(carboxymethyl)lysine in the layers of the aortic wall.

Group	Intima	Media	Adventitia
Control	1.40 ± 0.41	0.79 ± 0.37	1.96 ± 0.39
Diabetes	$2.79 \pm 0.39^{\#}$	$2.07 \pm 0.45^{\#}$	$2.86 \pm 0.24^{\#}$
Diabetes + vitamin D	$2.79 \pm 0.39^{\#}$	$1.30 \pm 0.27^{*}$	2.50 ± 0.35

The staining intensity was expressed by a scale ranging from 0 to 4 (0 – no staining, 1 – weak staining, 2 – moderate staining, 3 – strong staining, 4 – very strong staining). The scores were summed and the values are presented as means ± SD.
$^{\#}$ P <0.01 vs Control; * P <0.01 vs Diabetes.

5.2. Markers of endothelial dysfunction and oxidative stress in diabetes mellitus (Paper III)

Serum levels of ADMA were significantly higher in the untreated diabetic group compared to the control group (Table 2). TPX levels in the liver homogenates were significantly higher in the untreated diabetic group than in the control group (Figure 12). Liver TAC levels were significantly lower and, as a result, OSI was significantly higher in the untreated diabetic rats, compared to the control rats (Figures 13 and 14). The levels of serum TAC were significantly decreased in untreated diabetic rats compared to non-diabetic rats (Figure 15).

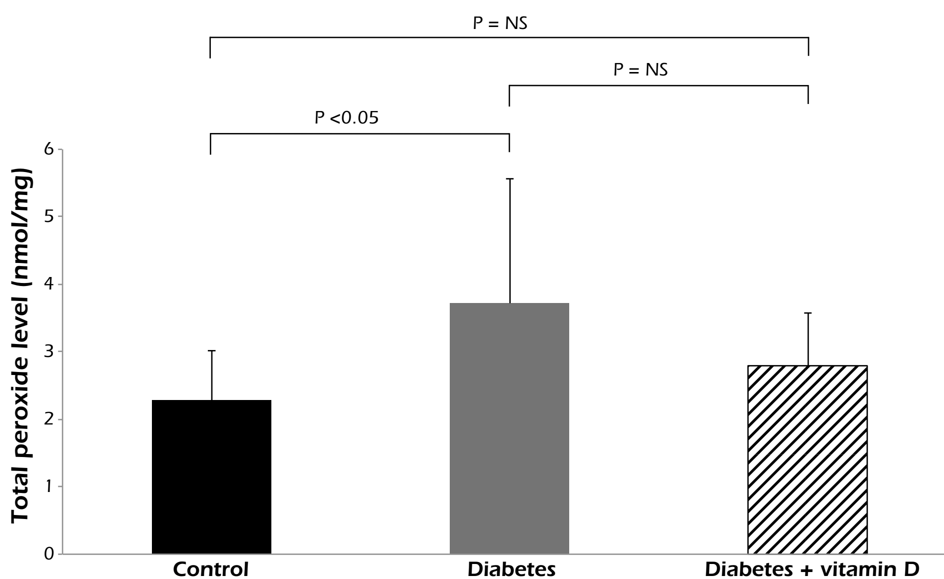

Figure 12. Total peroxide levels in the liver were significantly increased in untreated diabetes. Data are mean ± SD. TPX, total peroxide level. NS = non-significant difference.

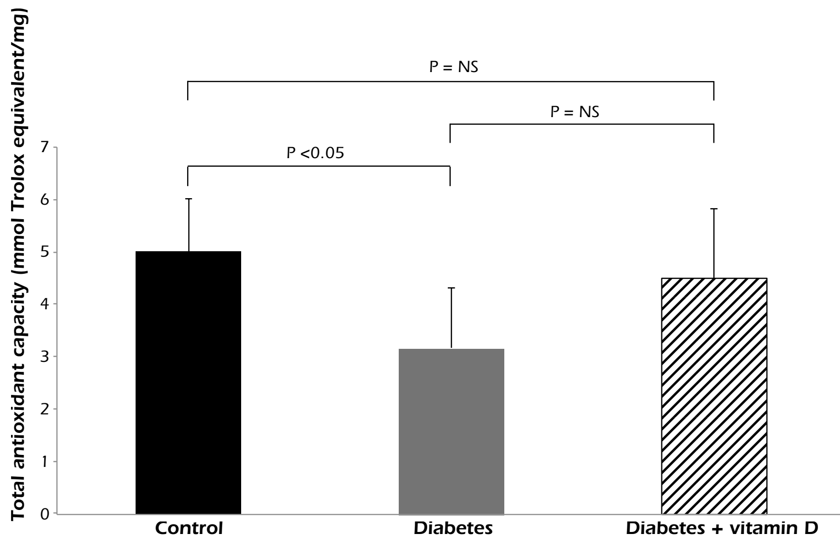

Figure 13. Untreated diabetes was associated with reduced total antioxidant capacity in the liver. Data are mean ± SD. TAC, total antioxidant capacity.

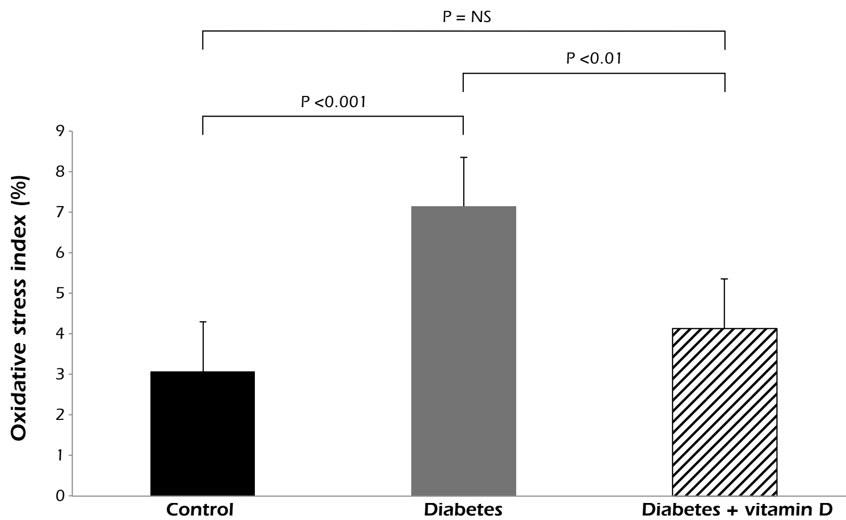

Figure 14. Oxidative stress index in the liver was significantly increased in untreated diabetes. Oxidative stress index is expressed as the per cent ratio of TPX to TAC. Data are mean ± SD. TPX, total peroxide level; TAC, total antioxidant capacity. NS = non-significant difference.

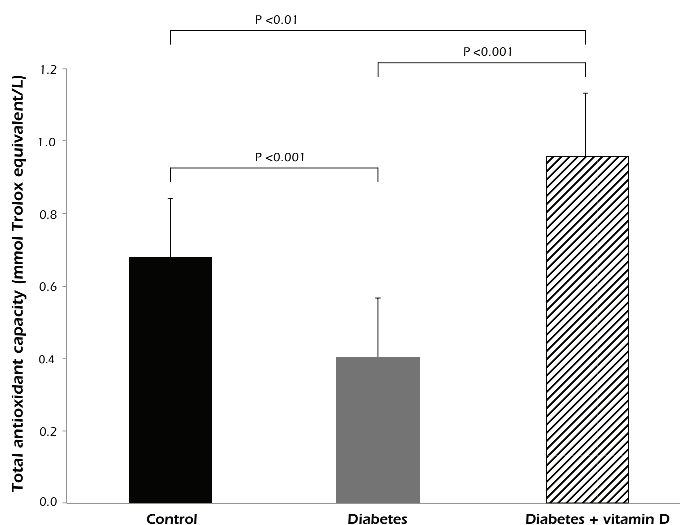

Figure 15. Serum total antioxidant capacity was significantly decreased in untreated diabetic rats. Data are mean ± SD. TAC, total antioxidant capacity.

5.3. Vascular protection by vitamin D and telmisartan (Papers I–III)

5.3.1. Effect of vitamin D on diabetic vascular complications (Papers I and III)

Administration of vitamin D resulted in significantly higher serum 25(OH)D levels compared to the control group (Table 2). However, vitamin D supplementation did not prevent the elevation of serum ADMA concentration (Table 2). Serum calcium levels were similar between all groups (Table 2).

The non-linear PWV-MAP curve for the diabetic treated group was similar to that of diabetic untreated group, indicating that aortic stiffness was similar at every given level of MAP (Figure 5). Although all three groups received similar doses of PE, MAP above 170 mmHg was not achieved in vitamin D treated rats. The reason for this effect is unknown, but may possibly include a lower sensitivity to phenylephrine.

Compared to the untreated diabetic group, milder changes within the aortic wall, particularly regarding medial elastic fibres with no focal disarrangements, were noted in the diabetic treated group (Table 15, Figure 8). Treatment with vitamin D also restored the reduced ratio of elastin to collagen seen in the aortic wall of the untreated diabetic rats (Table 15). No focal thickenings or other significant alterations of the intimal layer were found neither in the untreated nor treated diabetic groups.

Vitamin D effectively prevented CML accumulation in the aortic tissue (Figure 10), and reduced the levels of OSI in the liver, compared to untreated

diabetic rats (Figure 14). Furthermore, significant improvement of serum TAC was observed in the diabetic treated group where TAC was restored to a higher level than that in the control group (Figure 15).

Strong immunostaining for CML was still seen in the intimal and adventitial layers of the aortic wall in the vitamin D-treated diabetic group, but the thickest layer, the media, showed moderate staining, resembling the staining pattern in the control group (Table 17, Figure 11).

5.3.2. Attenuation of diabetes-induced aortic stiffening and remodelling by telmisartan (Paper II)

Resting anaesthetised SBP, DBP, and MAP were significantly lower in the telmisartan-treated diabetic group, compared to the other two groups. HR was significantly lower than that in the untreated diabetic group. Decreased dP/dt associated with untreated DM was partially attenuated by telmisartan. As a result of significantly lower resting MAP in the telmisartan group, pressure-dependent PWV values were also significantly lower in that group.

The intercepts and coefficient in $PWV = a + b \times MAP + c \times MAP2$ were not significantly different between the treated and control groups (Tables 8, 10, and 12). Robust methods ANCOVA revealed that the untreated DM and treated diabetic groups differed at all MAP levels other than 60, 110, 120, 130, and 150 mmHg (Table 18). Controls were not significantly different to treated animals at any MAP value (Table 19).

Table 18. Robust method analysis of covariance, Diabetes (D) vs Diabetes + telmisartan (DT).

MAP	n1	n2	DIF	TEST	SE	CI.low	CI.hi	P value	crit.val
60	10	21	0.12	0.98	0.12	–0.38	0.62	0.36	4.12
70	21	35	0.19	3.95	0.05	0.04	0.34	<0.001	3.10
80	28	40	0.21	4.51	0.05	0.07	0.36	<0.001	3.07
90	32	43	0.23	3.98	0.06	0.06	0.41	<0.001	3.01
100	36	45	0.16	2.08	0.08	–0.07	0.40	0.04	3.01
110	38	44	0.11	1.07	0.11	–0.20	0.43	0.29	2.99
120	39	42	0.16	1.43	0.11	–0.17	0.48	0.16	2.99
130	38	40	0.25	2.01	0.12	–0.12	0.62	0.05	3.00
140	39	40	0.36	2.13	0.17	–0.15	0.86	0.04	3.00
150	38	39	0.35	1.70	0.21	–0.27	0.98	0.10	3.02
160	38	33	0.53	2.37	0.22	–0.15	1.21	0.02	3.03
170	35	21	0.88	3.80	0.23	0.17	1.58	<0.001	3.06

MAP, mean arterial pressure; SE, standard error; CI, confidence interval.

Treatment with telmisartan significantly inhibited the diabetes-induced reduction of thoracic aorta lumen diameter but did not affect the width of the medial layer

(Table 16). The staining intensity of medial elastic fibres was not different from the controls. Furthermore, treatment with telmisartan preserved the regularity of the arrangement of elastic fibre arrangement (Figure 9). Collagen concentration was not statistically different between the untreated and treated diabetic groups and the ratio of elastin to collagen tended to increase in the telmisartan group but still remained lower than that in the control group (Table 16).

Table 19. Robust method analysis of covariance, Diabetes + telmisartan (DT) *vs* Control (C).

MAP	n1	n2	DIF	TEST	SE	CI.low	CI.hi	P value	crit.val
60	23	21	–0.01	0.20	0.06	–0.20	0.18	0.85	3.12
70	42	35	0.01	0.15	0.05	–0.13	0.14	0.88	3.00
80	45	40	0.05	1.34	0.03	–0.06	0.15	0.19	2.99
90	46	43	0.08	1.62	0.05	–0.06	0.22	0.11	2.99
100	45	45	0.05	0.69	0.08	–0.18	0.28	0.49	3.02
110	45	44	–0.00	0.04	0.09	–0.29	0.28	0.97	3.02
120	45	42	–0.02	0.21	0.10	–0.32	0.27	0.83	3.00
130	45	40	–0.01	0.06	0.12	–0.37	0.36	0.95	3.00
140	45	40	0.01	0.05	0.13	–0.40	0.41	0.96	3.00
150	45	39	–0.09	0.56	0.15	–0.54	0.37	0.57	2.99
160	45	33	–0.02	0.11	0.16	–0.49	0.45	0.92	3.00
170	44	21	0.15	0.90	0.17	–0.37	0.68	0.37	3.06

MAP, mean arterial pressure; SE, standard error; CI, confidence interval.

5.4. Association of vitamin D status with aortic stiffness in professional soldiers (Paper IV)

5.4.1. Basic characteristics and laboratory values

Of the 65 soldiers who completed pre-deployment testing, 10 soldiers did not complete post-deployment testing due to injury, incomplete deployment period, non-deployment, or no-shows for testing. The baseline characteristics are presented in Table 20.

There were significant changes in various markers of inflammation during the mission (Table 21). Plasma 25(OH)D was increased by 2.6 times in comparison with baseline values (40 ± 15 *vs* 104 ± 24 (nmol/L), $P < 0.001$).

5.4.2. Haemodynamic parameters

The post-deployment values for the indices of arterial stiffness (e.g., AIx, PWV) did not differ significantly from the baseline values. Brachial and aortic BP, MAP, and other haemodynamic parameters remained unchanged when compared with pre-deployment values (Table 22).

5.4.3. Association between the change of 25(OH)D and the change of aortic PWV

There was a significant negative correlation between the change of aortic PWV and the change 25(OH)D (Δ25[OH]D) in soldiers with Δ25(OH)D above median (>64 nmol/L, r=–0.44, P=0.02; Figure 16). This correlation was even stronger in the 4th quartile of Δ25(OH)D (>82.5 nmol/L, r=–0.6, P=0.02). However, we did not find similar correlations between 25(OH)D and other parameters of arterial stiffness, i.e., AIx and AIx@75.

Table 20. Baseline characteristics of the study group before the mission.

Variable	Value
Age (years)	26 ± 4
Weight (kg)	79 ± 10.6
Height (cm)	182 ± 7
Body mass index (kg/m^2)	23.8 ± 2.7
Smoking (%)	60
VO_2max/kg (mL/kg/min)	53.8 ± 6.1
Glucose (mmol/L)	4.6 ± 0.6
Total cholesterol (mmol/L)	4.1 ± 0.8
HDL (mmol/L)	1.3 ± 0.3
LDL (mmol/L)	2.4 ± 0.8
Triglycerides (mmol/L)	1.1 ± 0.6
Peripheral SBP (mmHg)	124.2 ± 9
Peripheral DBP (mmHg)	65.6 ± 7.6
Peripheral PP (mmHg)	56.7 ± 11.1
MAP (mmHg)	82.3 ± 8.5
Central SBP (mmHg)	102.9 ± 9
Central DBP (mmHg)	67.1 ± 8.4
Central PP (mmHg)	35.8 ± 6.4
Heart rate (beats/min)	60.8 ± 10.7
AIx (%)	–3.6 ± 9.6
AIx@75 (%)	–10.8 ± 9.7
aPWV (m/s)	6.4 ± 1.2

VO_2max/kg, maximal oxygen consumption per kg body weight; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; AIx, augmentation index; AIx@75, augmentation index corrected for a heart rate of 75 beats per minute; aPWV, aortic pulse wave velocity.

Table 21. Serum markers of inflammation before and after the mission.

Variable	Before	After	P value	Reference range[#]
Leukocyte count (× 10^9/L)	5.4 ± 1.2	6.2 ± 1	<0.001	3.5 – 8.8 × 10^9/L
Platelet count (× 10^9/L)	253.3 ± 51.3	305.9 ± 65.6	<0.001	145 – 390 × 10^9/L
TNF-α (pg/mL)	3.5 ± 1.4	2.9 ± 1.3	0.008	N/A
MCP-1 (pg/mL)	150.8 ± 60.1	228.8 ± 95	<0.001	N/A
hsCRP (mg/L)	0.4 (0.21 – 0.63)*	0.5 (0.34 – 1)*	0.030	<5 mg/L
IL-1α (pg/mL)	0.1 (0.05 – 0.14)*	0.1 (0.07 – 0.17)*	<0.001	N/A
IL-2 (pg/mL)	1.8 (1.32 – 2.30)*	2.2 (1.6 – 3)*	0.006	N/A
IL-8 (pg/mL)	9.6 (6.83 – 16.8)*	8.0 (5.74 – 11.36)*	0.002	N/A
IL-10 (pg/mL)	0.6 (0.52 – 0.79)*	0.8 (0.62 – 1.02)*	<0.001	N/A
IFN-γ (pg/mL)	1.8 (1.05 – 3.16)*	4.8 (3.14 – 6.6)*	<0.001	N/A

* indicates medians and interquartile ranges.
TNF-α, tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; IFN-γ, interferon gamma.
Reference ranges at the local clinical laboratory (Tartu University Hospital, United Laboratories). N/A, not applicable.

Table 22. Haemodynamic variables before and after the mission.

Variable	Before	After	P value
Peripheral SBP (mmHg)	124.2 ± 9.0	123.1 ± 9	0.3
Peripheral DBP (mmHg)	65.6 ± 7.6	66.0 ± 61	0.8
Peripheral PP (mmHg)	56.7 ± 11.1	57.1 ± 6.6	0.2
MAP (mmHg)	82.3 ± 8.5	84.1 ± 7.5	0.1
Central SBP (mmHg)	102.9 ± 9.0	104.0 ± 7.5	0.5
Central DBP (mmHg)	67.1 ± 8.4	68.3 ± 7.2	0.3
Central PP (mmHg)	35.8 ± 6.4	35.7 ± 7.8	0.7
Heart rate (beats/min)	60.8 ± 10.7	62.3 ± 8.5	0.3
AIx (%)	–3.6 ± 9.6	–3.8 ± 9.1	0.8
AIx@75 (%)	–10.8 ± 9.7	–9.9 ± 8.3	0.6
aPWV (m/s)	6.4 ± 1.2	6.3 ± 0.8	0.2

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; AIx, augmentation index; AIx@75, augmentation index corrected for a heart rate of 75 beats per minute; aPWV, aortic pulse wave velocity.

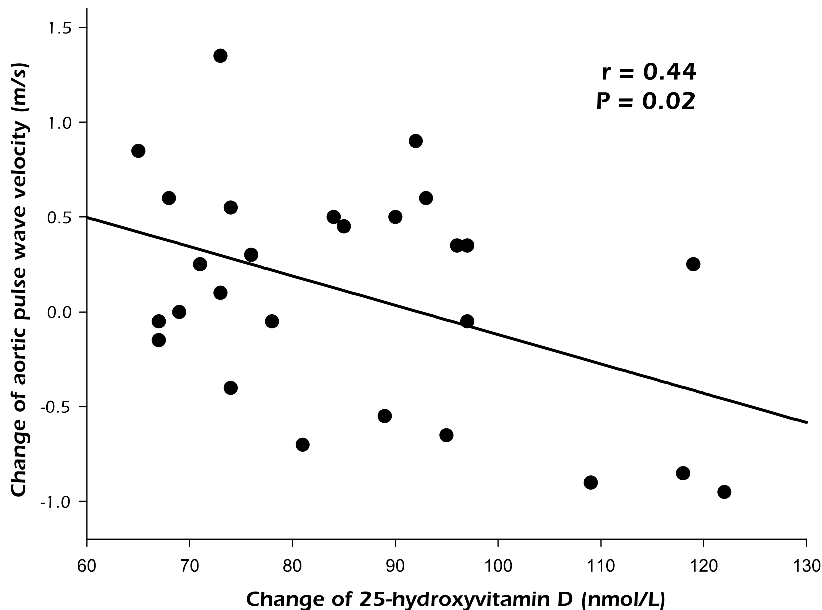

Figure 16. Correlation between the change of aortic PWV and the change of 25(OH)D in soldiers with the change of 25(OH)D above median (>64 nmol/L, n=27).

6. DISCUSSION

6.1. Characterisation of pressure-dependent aortic stiffness in experimental diabetes mellitus

Diabetes mellitus is associated with complex metabolic abnormalities in carbohydrate and lipid homeostasis that, among others, lead to major debilitating CV complications which are inadequately explained by classic risk factors (Lehto *et al.* 1999). Microvascular changes are a well-known complication of DM (Parving *et al.* 1983) but pathological changes in the large vessels are also an important feature of DM. These include stiffening of the aortic wall which is a significant contributing factor to the target organ damage leading to impaired coronary perfusion and left ventricular dysfunction (Weber *et al.* 2004; O'Rourke and Hashimoto 2007). Pulse wave velocity is a well-established measure of aortic stiffness and large-scale clinical studies have demonstrated its utility in predicting the CV risk in patients with DM (Cruickshank *et al.* 2002; Blacher *et al.* 2012). Current ESC/ESH guidelines for the management of arterial hypertension regard aortic PWV greater than 10 m/s as a marker of asymptomatic target organ damage (Mancia *et al.* 2013).

Major determinants of the development of diabetic macrovascular complications are the severity and duration of DM. Several authors have investigated the elastic properties of large arteries in patients with DM and, in general, stiffness of the aorta was found to be increased (Pillsbury *et al.* 1974; Thordarson *et al.* 1986; Oxlund *et al.* 1989). However, the study populations are often indistinctly defined, including both T1DM and T2DM patients, with a large age distribution, with or without complications, and with different durations of disease, making these data difficult to interpret and compare across different studies. Therefore, it is not well established how early appearance of hyperglycaemia is translated into deterioration of large artery function since most studies investigating diabetic vascular complications have focused on T2DM where it is difficult to separate the impact of hyperglycaemia from that of age and confounding factors (e.g., obesity and dyslipidaemia) (Salomaa *et al.* 1995). Studies in T1DM patients have demonstrated that increased arterial stiffness is an early phenomenon that occurs before the onset of overt macrovascular disease (Romney and Lewanczuk 2001; Sommerfield *et al.* 2007) whereas others have shown no difference (Kool *et al.* 1995; Yu *et al.* 2012) or even lower arterial stiffness, compared with healthy subjects (Lehmann *et al.* 1992). These discrepancies may relate to methodologies used and lack of adjustment for confounding factors.

In the present study (Papers I and II), aortic stiffness was studied in a rat model of experimental T1DM by measuring aortic PWV under BP-independent conditions. Streptozotocin-induced DM is a well-established standard experimental model for studying the pathophysiology of uncontrolled T1DM and its complications (Junod *et al.* 1969). Experimental studies on the effects of duration of STZ-diabetes have shown that increased aortic wall stiffness may be

established as early as 8 weeks after induction of DM (Zhao *et al.* 2000; Chang *et al.* 2003; Chang *et al.* 2006). Impaired aortic elastic properties have also been demonstrated in rats following 9 weeks of STZ-induced DM (Wolffenbuttel *et al.* 1998). In accordance with previous studies we demonstrate increased aortic stiffness in STZ-diabetic rats compared to nondiabetic rats following 10 weeks of uncontrolled hyperglycaemia.

It is important to note that as PWV is strongly dependent on BP (Asmar *et al.* 1995), measurements of PWV can be accurately and independently compared only if obtained over a range of BP that is often not feasible to induce in patients. Our study is the first to characterise the aortic stiffness profile measured as PWV across a wide range of blood pressures (MAP 50 to 200 mmHg) in STZ-diabetic rats. The method used for assessment of aortic stiffness in this study enables to compare PWV between the experimental groups independently of blood pressure. Furthermore, characterisation of the curvilinear relationship between PWV and BP may provide additional means for studying structural changes implicated in the pathogenesis of DM-induced aortic stiffness.

We assessed PWV over a wide range of MAP using a vasoconstrictor PE and a vasodilator NTG to raise and lower MAP, respectively. Earlier experiments have shown that changes in PWV following intravenous administration of vasoactive agents (PE and SNP) result in similar changes in PWV following venous occlusion (Butlin 2007). This indicates that PE and NTG used in our experiment affect aortic stiffness via regulation of peripheral resistance and do not act on the aortic segment being studied. Therefore, the aortic stiffness assessed by PWV is only dependent on the effects of DM and not influenced by the active effects of infused vasoactive agents.

In our experiments, differences in isobaric PWV only became evident at the high MAP range which demonstrates that intrinsic aortic stiffness was unaltered in diabetic animals across the blood pressure range assessed under resting anaesthetised conditions. By dissociating PWV and BP, our results indicate that during the course of DM, early changes in the arterial integrity are reflected in the increased aortic stiffness that can remain undetected since BP may not be elevated in that stage as was the case in our experiment. Our findings of similar resting BP between the control and diabetic animals are supported by experimental studies showing that STZ-diabetic rats may be normotensive at the early stage of the disease (Kusaka *et al.* 1987; Hicks *et al.* 1998; Chang *et al.* 2003). Furthermore, clinical studies have demonstrated that while the early course of DM may not be associated with elevated BP (Tarn and Drury 1986; Sommerfield *et al.* 2007), changes in the arterial integrity can already be observed as increased large artery stiffness (Giannattasio *et al.* 2001; Manolis *et al.* 2005; Sommerfield *et al.* 2007). Therefore, in clinical settings, it is possible that increased aortic stiffness may be established only when diabetic patients develop hypertension, a common complication later in the course of DM. The results from our experiments highlight the importance of adequate and early BP control for prevention of diabetic macrovascular complications. The 2007 ESC/ESH guidelines for treating hypertension in patients with DM put an

emphasis on aggressive BP lowering (<130/80 mmHg) to prevent or reduce the risk of vascular organ damage (e.g., microalbuminuria and retinopathy) (Mancia *et al.* 2007). However, the evidence favouring aggressive intervention is not convincing as demonstrated in the Action to Control Cardiovascular Risk in Diabetes trial where no significant reduction in CV morbidity in diabetic patients whose SBP was reduced to an average of 119 mmHg, compared with patients whose average SBP was 133 mmHg (Cushman *et al.* 2010). As a result, the 2013 ESC/ESH guidelines for treating hypertension recommend that all patients be treated to <140 mmHg SBP and patients with DM to <85 mmHg DBP (Mancia *et al.* 2013). The findings from our experiments are in agreement with this recommendation in that we did not evidence differences in aortic stiffness assessed at lower BP, but with increasing pressure, significant differences in aortic stiffness were observed between groups, indicating asymptomatic target organ damage.

6.2. Diabetes-induced structural and functional abnormalities in the aortic wall

The pathogenetic determinants of DM-induced vascular complications are incompletely understood. Macroscopic atherosclerotic lesions are more prevalent in coronary arteries and aortas from diabetic subjects than in arteries from non-diabetic subjects (Robertson and Strong 1968) but large-scale studies have shown that classic risk factors for atherosclerosis, including hypertension and dyslipidaemia, do not completely explain the incidence of CVD in patients with DM (Garcia *et al.* 1974; Lehto *et al.* 1999). In another study in T1DM patients, isolated aortic rings without visible atherosclerotic lesions were found to be significantly stiffer than those obtained from healthy control subjects (Oxlund *et al.* 1989). Therefore, it his highly probable that primary changes in the arterial geometry and morphology, rather than secondary changes due to accelerated atherosclerosis, are major factors in the pathogenesis of large artery disease among T1DM patients. These disturbances are probably related directly or indirectly to persistently elevated levels of blood glucose. In the current study we have investigated the role of hyperglycaemia in the genesis of structural and functional alterations in the aortic wall (Papers I and II).

Elastic properties of the aortic wall are strongly dependent on the content of two dominating extracellular matrix proteins, collagen and elastin, and their orderly arrangement. In our study, a decrease in the amount of elastic lamellae in the aortic media was noted in the untreated diabetic group. Furthermore, the appearance of elastic lamellae was fragmented and disorderly, compared to normal intact wave-like organisation of the elastic lamellae in the control rats. Our results are supported by earlier findings of decreased elastin content within the aortic wall in rats following 10 days of alloxan-induced T1DM (Kwan *et al.* 1988). Importantly, the diabetic rats in their study were normotensive implying that modifications of the aortic wall morphology were solely due to the diabetic

state, as was also the case in our study. Similar results were produced in another study showing a time-dependent loss of elastin within the aortic medial layer in normotensive STZ-diabetic rats over a course of 150 days (Searls *et al.* 2012). Our findings are also consistent with observations of deformed structure of elastic fibres and a relative increase in collagen content within the aortic wall, coupled with reduced aortic distensibility in STZ-diabetes (Sun *et al.* 2009).

Differential impairment of the load-bearing elements, elastin and collagen, within the aortic wall is concordant with the aortic stiffness profile in the untreated diabetic group. With increasing pressure, decreased amount of elastin and a relative increase in collagen content cause premature recruitment of stiffer collagen fibres, as demonstrated by increased aortic stiffness at the high BP range. This hypothesis is supported by *in vitro* studies on the biomechanical properties of elastin and collagen showing that in elastase-digested arteries, with increasing stretching, the engagement of collagen fibres occurs more rapidly, in contrast to gradual and smooth recruitment in the normal arteries (Fonck *et al.* 2007; Kochova *et al.* 2012). The findings from these studies imply that efficient transition from low to high pressure region is dependent on the interaction of both collagen and elastin fibres, and loss of elastin may also be responsible for abnormal function of collagen fibres.

There is no consensus in the literature as to whether the concentration of collagen is increased, decreased, or unchanged within the diabetic aortic wall. Indeed, there are conflicting reports of increased collagen (Reddy 2004), decreased collagen and elastin contents (Andreassen and Oxlund 1987), and no changes in collagen levels within the aortic wall of diabetic rats (Andreassen *et al.* 1981). These discrepancies are possibly related to methodological differences in the collagen quantification and the duration of STZ-diabetes. Furthermore, several authors have implied that alterations in the collagen network rather than total collagen content, contribute significantly to the biomechanical integrity of the vessel wall (Brüel and Oxlund 1996; Wolffenbuttel *et al.* 1998).

Diabetes-induced arterial wall remodelling is a well-established complication and characterised by thickening of intimal and medial layers. Increased carotid artery intima-media thickness has frequently been reported in patients with T1DM (Frost and Beischer 1998; Yamasaki *et al.* 1994) and is considered an independent CV risk factor (Mancia *et al.* 2007). In this regard, our findings of reduced width of aortic media in the untreated diabetic group are at variance with observations from human studies. However, experimental studies with different durations of STZ-diabetes have shown that the width of the aortic medial layer may be decreased at an early stage (Searls *et al.* 2012; Akgün-Dar *et al.* 2007) and gradually increase with the ongoing disease due to vascular smooth muscle cell proliferation (Fukuda *et al.* 2005). The structural alterations occurring during the course of DM may involve differential transcriptional regulation of matrix metalloproteinases (Song and Ergul 2006) and transforming growth factor-beta (Rumble *et al.* 1997; Kanzaki *et al.* 1997).

We studied the possible causes for changes in the functional and structural properties of the aortic wall in STZ-diabetes by examining the modification of

extracellular matrix through non-enzymatic glycation and oxidation (Paper III). Glycoxidation of proteins within the vascular wall has been proposed as an important factor influencing arterial compliance in DM. The proteins undergoing glycoxidation are preferably long-lived, and collagen and elastin are the main targets within the aortic tissue (Meng *et al.* 1996; Winlove *et al.* 1996).

Advanced glycation end-products represent a heterogeneous family of protein adducts with different properties. For example, pentosidine, the first AGE characterised, is known to form fluorescent cross-links in collagen and other proteins within the vascular wall (Winlove *et al.* 1996). While normal cross-linking is necessary to provide distensibility and mechanical strength to the tissue, excessive cross-links may lead to increased stiffness and functional disturbances of the vessel wall. *N*ε-(carboxymethyl)lysine is a compound which does not have fluorescence and neither forms cross-links in protein. However, increased tissue levels of CML have been found in subjects with DM, as CML is known to accumulate in collagen and elastin and irreversibly affect their properties (Dyer *et al.* 1993; Mizutari *et al.* 1997). Moreover, immunochemical methods have identified CML as a dominant AGE antigen (Reddy *et al.* 1995) in various tissues, including the arterial wall. *N*ε-(carboxymethyl)lysine has also been found to be a major AGE recognised by RAGE or scavenger receptors involved in the binding of AGEs and eliciting intracellular damage due to their co-localisation in various tissues, including vascular endothelium and VSM cells (Kislinger *et al.* 1999; Soulis *et al.* 1997). In the current study, we have used CML as a global marker of AGE formation. Our findings of increased deposition of CML within the aortic wall in untreated STZ-diabetes are consistent with earlier reports from animal (Meng *et al.* 1996; Soulis *et al.* 1997) and human studies (Nakamura *et al.* 1993) describing increased deposition of AGEs in the arterial wall in the settings of DM. We confirmed our findings with an immunohistochemical method using monoclonal anti-CML antibody which localised CML in the intima, media, and adventitia, with prominent stainings in the intimal and adventitial layers.

Endothelial dysfunction is regarded as an important early pathophysiological event contributing to the development of diabetic vascular damage. Moreover, endothelium-derived NO regulates arterial stiffness (Wilkinson *et al.* 2002). Increased deposition of AGEs in the aortic wall may disturb endothelial function by inhibiting NO production (Xu *et al.* 2003) or acting directly to chemically inactivate NO and inhibit NO-mediated vasodilation (Bucala *et al.* 1991). Another important regulator of NO bioavailability is endogenously produced ADMA. In our study, the serum levels of ADMA, a marker of endothelial dysfunction, were significantly elevated in untreated STZ-diabetic rats. These findings are accordance with studies showing that endothelial dysfunction occurs early in the course of DM, as evidenced by functional assessment of the endothelium (Serizawa *et al.* 2011) and concurrently elevated levels of circulating ADMA (Xiong *et al.* 2005; Altinova *et al.* 2007).

The cause for the increase of serum ADMA levels in diabetic rats is not clear. First, the renal function should be considered because ADMA is excreted

via the kidneys and has been shown to accumulate in patients with chronic renal failure (Vallance *et al.* 1992). Although we did not assess renal function in this study, it is well-established that diabetic individuals are susceptible to renal damage (Warram *et al.* 1996) and it is possible that renal ADMA clearance was decreased in STZ-diabetic rats in our study. In contrast, there have also been reports on similar serum ADMA levels in renal disease patients with normal renal function, moderate renal failure, and advanced renal failure (Kielstein *et al.* 2002). This raises a possibility that an increase in serum ADMA may be due to stimulated production or impaired degradation rather than reduced excretion. Indeed, the expression and activity of dimethylarginine dimethylamino-hydrolase, an enzyme responsible for ADMA degradation, has been found to be decreased in the aortas of STZ-diabetic rats (Lin *et al.* 2002; Lu *et al.* 2010).

Importantly, it has been suggested that serum ADMA levels may be dependent on the severity of DM rather than the duration of the disease (Xiong *et al.* 2005). This view is supported by an earlier study showing higher risk of developing CV complications in diabetic patients with poor glycaemic control, compared to patients with good glycaemic control (Andersson and Svardsudd 1995). Furthermore, ADMA levels and endothelial dysfunction can be reversed after chronic insulin treatment in STZ-diabetes (Xiong *et al.* 2003). Collectively, these results support the use of ADMA as an early marker of endothelial dysfunction in the development of diabetic vascular complications.

6.3. Significance of oxidative stress and antioxidant status in experimental diabetes mellitus

The role of antioxidant defence/protection and OxS in DM has been studied extensively and mounting evidence supports the concept that impaired antioxidant defence in concert with increased OxS has a strong impact on the pathogenesis of diabetic vascular disease. Sustained hyperglycaemia promotes a pro-oxidant environment that is exacerbated by concurrent generation of AGEs. Indeed, all AGEs accumulated in the tissue proteins require pro-oxidative environment for their formation (Baynes 1991). This implies that the levels of AGEs in the target organ may not only reflect long-term cumulative metabolic stress (i.e., hyperglycaemia) but also serves as a combined measure of both OxS and carbonyl stress.

Under normal and pathological conditions, mitochondria are considered to be the major source of endogenously produced ROS (Nohl 1994). The liver is heavily dependent on mitochondrial oxidative metabolism for its ATP requirements, and elevated levels of ROS cause mitochondrial damage that can generate further elevated OxS in the cells. Furthermore, the liver is a central organ in glucose homeostasis, and many important metabolic pathways involved in the pathogenesis of metabolic disorders in DM are localised in the liver. We assessed the level of OxS and total antioxidant status in the untreated STZ-

diabetic rats by measuring the concentrations of TPX and TAC in the liver and TAC in the serum samples (Paper III).

The assay for measuring TPX in the target tissue provides information about the levels of all derivatives of peroxides produced in the organism and can be regarded as reliable index of OxS status because it is indicative of oxidative products of lipids, peptides, and amino acids. The capacity of plasma and tissues to counteract the effects of OxS may be assessed by measuring the activities of individual antioxidants, whereas quantifying the combined capacity of all antioxidants provides additional information about the synergistic effects of different antioxidants to neutralise reactive species. The total antioxidant capacity assay is used widely to assess the contribution of different antioxidants to neutralise reactive species (Re *et al.* 1999; Erel 2004).

In agreement with previous studies (Kakkar *et al.* 1998; Feillet-Coudray *et al.* 1999; Sun *et al.* 1999), we show that the susceptibility to oxidation is significantly increased in the liver of untreated STZ-diabetic rats, as evidenced by increased levels of TPX. However, we found that TAC in the liver was unchanged which is probably the result of markedly improved self-protection against OxS, possibly by increased levels of bilirubin (Sudnikovich *et al.* 2007), vitamin E and coenzyme Q (Santos *et al.* 2001; Feillet-Coudray *et al.* 1999), and GSH (Raza *et al.* 2004). To gain more information regarding the redox balance between oxidation and antioxidation we also calculated OSI, which is the ratio of TPX to TAC. As expected, OSI was significantly higher in the untreated diabetic rats, compared to healthy rats. Thus, increased production of peroxides in the diabetic liver indicates increased susceptibility to oxidation in spite of activated antioxidant system. These findings are at variance with those reported in an earlier study, demonstrating decreased lipid peroxidation in diabetic rats paralleled by increased levels of vitamin E and coenzyme Q in the liver (Santos *et al.* 2001). However, the rats used in that study were a model of T2DM with considerably lower blood glucose levels, compared to untreated STZ-diabetic rats in our study (11 mmol/L *vs* 28 mmol/L, respectively), and were studied at a very early stage of DM reportedly not associated by complications.

Significantly lower levels of serum TAC observed in the untreated diabetic rats reflect a severely compromised systemic antioxidant response to the elevated OxS. Our results are in accordance with those reported by other investigators demonstrating decreased plasma radical-trapping potential in experimental (Feillet-Coudray *et al.* 1999) and clinical DM (Tsai *et al.* 1994; Santini *et al.* 1997). Major contributors to serum TAC are albumin, uric acid, and ascorbate, largely due to their relatively high concentrations compared to other antioxidants in the blood such as bilirubin, α-tocopherol, and β-carotene (Miller *et al.* 1993; Erel 2004). Lower serum TAC may be linked to hypo-albuminaemia as observed previously (Asayama *et al.* 1994; Feillet-Coudray *et al.* 1999); this is also consistent with the reported contribution of albumin to TAC (Cao and Prior 1998). Uric acid constitutes about 33% of the mea-sured serum TAC in healthy subjects (Erel 2004). In patients with DM,

hypouricaemia may contribute to decreased serum TAC (Marra *et al.* 2002). Reduced serum uric acid levels are probably related to increased fractional excretion of urate as a result of osmotic diuresis caused by hyperglycaemia. Decreased plasma ascorbate concentrations have been reported in clinical (Jennings *et al.* 1987) and experimental DM (Young *et al.* 1995) which may result from increased consumption by increased OxS or failed regeneration of oxidised dehydroascorbic acid (DHA) to ascorbate. Excessive consumption of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the hyperglycaemia-activated polyol pathway may also retard the reduction of DHA to ascorbate.

6.4. Vitamin D improves aortic remodelling and oxidative stress in streptozotocin-induced diabetes

A limited number of interventional studies have investigated the role of vitamin D in T1DM. Most data regarding the association between vitamin D intake and the risk of developing T1DM has been obtained from cross-sectional studies. Randomised controlled trials have been scarce and have yielded inconsistent results. Vitamin D supplementation is often recommended in pregnant women and children for prevention of vitamin D deficiency. Data from case-control studies have shown decreased risk of childhood-onset T1DM after vitamin D supplementation during the first year of life or during pregnancy (Hyppönen *et al.* 2001; Fronczak *et al.* 2003). However, it remains unclear whether these observations were due to increasing vitamin D to supraphysiological levels or were simply the result of preventing vitamin D deficiency. Recent studies examining the association between maternal intake of vitamin D and the development of T1DM in their children have failed to demonstrate a decreased risk of the disease as a result of vitamin D supplementation (Marjamäki *et al.* 2010; Simpson *et al.* 2011). These discrepancies may be due to various vitamin D doses, baseline 25(OH)D levels, achieved 25(OH)D levels, and various study designs. More information is required from prospective studies determining both the intake of vitamin D and vitamin D status in the organism to test the hypothesis whether only high levels of circulating vitamin D are protective or, conversely, only low levels are an increased risk for the disease. Observations from animal models of T1DM have shown that vitamin D supplementation in neonatal and early period of life do not protect against T1DM in nonobese diabetic (NOD) mice or in BioBreeding rats (Mathieu *et al.* 2004), whereas hypovitaminosis D in the early life doubles the occurrence of DM in NOD mice (Giulietti *et al.* 2004). The aetiology of STZ-induced DM is different from the phenotype of NOD mice in that STZ administration results in dose-dependent toxic β-cell necrosis, whereas in NOD mice, DM develops as autoimmune insulitis. Although some studies have reported improvement of glucose homeostasis in STZ-diabetic rats receiving vitamin D (Del Pino-Montes *et al.* 2004; de Souza Santos and Vianna 2005), there are also studies that show no

improvement of metabolic control with vitamin D supplementation (Noyan *et al.* 2005; Calle *et al.* 2008). Differences in the doses and forms of vitamin D (cholecalciferol or calcitriol), and severity and duration of DM may explain these discrepancies. In our study, very high levels of blood glucose and HbA_{1c} in both treated and untreated STZ-diabetic rats indicated severely compromised glucose homeostasis, and the lack of protective effect of vitamin D supplementation may be attributed to a limited number of surviving insulin-producing β-cells incapable of correcting this profound metabolic derangement.

Suboptimal vitamin D status in patients with DM has been associated with increased arterial stiffness (Lee *et al.* 2012) and endothelial dysfunction (Yiu *et al.* 2011) independent on other CV risk factors. However, data on the impact of vitamin D supplementation on the vascular complications of DM are conflicting due to a small number of randomised controlled trials and heterogeneity between studies. A single dose of 100,000 IU vitamin D has been shown to improve BP and endothelial function in vitamin D-deficient (25[OH]D <50 nmol/L) T2DM patients after 8 weeks (Sugden *et al.* 2008). However, a follow-up study in T2DM patients with higher 25(OH)D levels (<100 nmol/L) did not show any improvement of endothelial function 16 weeks following a single dose of 100,000 or 200,000 IU of vitamin D (Witham *et al.* 2010). Similarly, daily supplementation of vitamin D 5000 IU for 12 weeks did not have any significant effect on endothelial function and arterial stiffness in T2DM patients with circulating 25(OH)D levels of <30 nmol/L (Yiu *et al.* 2013). In our study, pressure-independent aortic stiffness in diabetic animals receiving vitamin D was similar to that of diabetic untreated animals. Furthermore, there were no differences in serum levels of ADMA between the two diabetic groups. These findings may be attributed to the fact that the levels of blood glucose and HbA_{1c} were unaffected by significantly improved vitamin D status, suggesting that the possible favourable effects of vitamin D against DM-induced increase in aortic stiffness and endothelial dysfunction may have been offset by the sustained high-grade hyperglycaemia.

Although vitamin D supplementation could not protect from the early impairment of large artery function it still had a positive effect on the relative preservation of elastic fibre organisation in the medial layer of the aortic wall. The mechanisms involved in this process remain unclear, but may include the down-regulation of the RAS (Li *et al.* 2002; Dong *et al.* 2012) since Ang II is known to stimulate tissue remodelling in the arteries (Levy *et al.* 1996) and agents that inhibit this system have been shown to have beneficial effects on the structural properties of the arterial wall (Albaladejo *et al.* 1994; Levy *et al.* 1996). While there are reports of adverse effects of vitamin D on the elastic lamellae within the aortic wall (Niederhoffer *et al.* 1997; Norman *et al.* 2002), the doses of vitamin D used in those studies were considerably larger and are also known to induce calcification and loss of collagen within the arterial wall (Niederhoffer *et al.* 1997). Regarding the risk of inducing hypercalcaemia and soft tissue calcification, the dose of cholecalciferol 500 IU/kg used in our study

may be considered safe as indicated by similar serum calcium levels in all study groups.

Some AGEs (e.g., pentosidine) are directly derived from the non-enzymatic reactions between proteins and carbohydrates, while a combination of glycation and oxidation reactions is required for the formation of CML (Fu *et al.* 1996). The relationship between OxS and the formation of AGEs has been established by studies showing that the generation of AGEs and protein cross-linking is significantly suppressed under antioxidative conditions without an effect on overall glycation of proteins (Chace *et al.* 1991; Fu *et al.* 1992; Fu *et al.* 1994; Baynes 1991). These results suggest that inhibition of autoxidative glycation and glycoxidation, rather than glycation, may be the relevant pathogenetic mechanisms in DM. In our study, vitamin D supplementation inhibited CML deposition in the medial layer of the aortic wall in the presence of ongoing hyperglycaemia. This implies that vitamin D is involved in other important mechanisms of CML formation. To our best of knowledge, this is the first study demonstrating such an inhibitory effect of vitamin D on CML accumulation. The mechanism underlying this finding remains to be established, but it is plausible that vitamin D interferes with the formation of ROS by upregulation of antioxidant enzymes through VDR signalling (Hamden *et al.* 2009; Dong *et al.* 2012). Furthermore, *in vitro* studies have shown that vitamin D reduces endoplasmic reticulum stress (Riek *et al.* 2012) and downregulates the expression of RAGE (Talmor *et al.* 2008). Our findings underscore the importance of OxS and antioxidant status in the development of diabetic vascular damage. Indeed, among diabetic populations, a considerable variance in the rates of AGE accumulation, despite similar blood glucose and HbA_{1c} levels, has been demonstrated which may be attributed to individual variations in OxS status (McCance *et al.* 1993).

The liver is central to the vitamin D metabolism and circulation in that liver converts absorbed vitamin D into 25(OH)D, the circulating form vitamin D, which is the best indicator of vitamin D status in the organism (Zittermann 2006). Thus, we aimed to assess the impact of vitamin D pooled in the liver on the increased OxS, as seen in the untreated diabetic rats. We found that supplementation of vitamin D was able to reduce the levels of TPX and OSI to those observed in normal rats, clearly demonstrating the antioxidative potential of vitamin D. These results are in line with those by Hamden *et al.* who showed that OxS in the diabetic liver may be improved by calcitriol (Hamden *et al.* 2009). Moreover, we found that treatment of diabetic rats with vitamin D restored the serum TAC to a level that was significantly higher than that found in control rats. Detailed explanation to the antioxidative effects of vitamin D cannot be provided, but these may include stabilisation of the plasma membrane against lipid peroxidation (Wiseman 1993) or upregulation of antioxidant systems, including GSH, GSH peroxidase, and superoxide dismutase, via its nuclear receptors (George *et al.* 2012; Sezgin *et al.* 2013). Treatment with non-calcaemic vitamin D analogues has been a focus of recent research. In a rat T2DM model, it was shown that treatment with 22-oxacalcitriol, a VDR

agonist, reduced both systemic and local OxS in the aortic wall through the suppressed gene expression of NADPH oxidase to the same degree as insulin despite unaffected high blood glucose levels (Kono *et al.* 2013). The importance of VDR in regulating OxS has been demonstrated in several studies reporting significant relationship between OxS and VDR loss in VDR knockout mice (Kallay *et al.* 2001; Kallay *et al.* 2002). These observations suggest that VDR agonists, including calcitriol, may have antioxidative effects not by reducing hyperglycaemia, but through VDR signalling.

6.5. Angiotensin II type 1 receptor blocker telmisartan attenuates diabetes-induced aortic stiffening and remodelling

In patients with DM, the prevalence of hypertension is twice as high as in non-diabetic individuals (Klein *et al.* 1996). Furthermore, even in individuals with normal BP the diagnosis of DM is associated with a moderate to high added risk of a 10 year fatal or non-fatal CV event (Mancia *et al.* 2007). Among a large range of antihypertensive agents with equipotent BP lowering effects there is an increasing need for individualisation of treatment based on the underlying disease and concomitant abnormalities (Mancia *et al.* 2009). Moreover, there is increasing awareness that several antihypertensive agents may have adverse metabolic effects while some agents may be particularly beneficial for patients with metabolic syndrome or DM regarding their effects beyond BP reduction (Mancia *et al.* 2006). For these reasons, early identification of those at high risk and targeted therapy are essential for treatment and prevention of CV complications associated with DM.

Activation of the RAS is an integral mechanism in the pathogenesis of CVD in DM and large trials have demonstrated considerable benefit of the blockade of RAS for target organ protection (Ball 2003). Renin-angiotensin system inhibitors are also preferable to other antihypertensive agents to be used in the management of hypertension in patients with DM (Mancia *et al.* 2007; Mancia *et al.* 2013). Telmisartan has attracted particular interest as an ARB with significant favourable effects against tissue remodelling in addition to equipotent BP reducing effects, compared to other RAS inhibitors, captopril or losartan (Wagner *et al.* 1998). Treatment of STZ-diabetic rats with telmisartan has been shown to reduce DM-induced increase in left ventricular collagen deposition and improve left ventricular contractility (Goyal *et al.* 2008; Goyal *et al.* 2011). Furthermore, telmisartan may improve endothelial function in patients with T1DM (Ceriello *et al.* 2007) and reduce arterial stiffness in patients with T2DM (Asmar *et al.* 2002).

In accordance with previous studies we report that treatment of STZ-diabetic rats with telmisartan for 10 weeks was able to inhibit the development of DM-induced aortic stiffening in the context of improved structural properties of the aortic wall. Specifically, telmisartan preserved the amount of elastin within the

medial layer of the aorta and maintained the normal organisation of elastin network. These effects were also associated with a modest, but insignificant, increase in the ratio of elastin to collagen. The anti-fibrotic effects of telmisartan were evident despite the lack of or minimal effect on glucose control or body weight. To our best of knowledge the current study is the first to report that the ARB telmisartan modulates DM-induced aortic stiffening and remodelling independently on BP reduction. Although we did not specifically address the possible reasons for these outcomes, previous studies have demonstrated that, in addition to direct inhibition of the pro-fibrotic effects of Ang II, treatment with RAS inhibitors may have indirect pressure-independent protective effects by interfering with AGE formation via antioxidative mechanisms (Miyata *et al.* 2002). Interestingly, telmisartan has also shown inhibitory effects on vascular proliferation in cells lacking Ang II receptors, suggesting that other mechanisms may be equally important in the ARB-mediated vascular protection (Benson *et al.* 2008).

In summary, we have shown that the ARB telmisartan prevents aortic stiffening associated with untreated STZ-diabetes in parallel with favourable effects on the structural properties of the aortic wall such as preservation of the concentration and organisation of elastin network. These results provide further evidence that inhibition of the RAS has a specific role in the prevention of DM-induced vascular damage beyond BP lowering effects.

6.6. Improvement of vitamin D status is associated with decreased aortic stiffness in professional soldiers

Before deployment to Afghanistan 74% of the soldiers had vitamin D insufficiency (serum 25[OH]D <50 nmol/L) according to the current consensus (Holick 2007). The pre-deployment measurements in our study were performed at the beginning of April when the UVB radiation is still inadequate at northern latitudes for endogenous vitamin D synthesis (Barger-Lux and Heaney 2002; Robsahm *et al.* 2004). Our results are in accordance with the findings from an epidemiological study by Kull *et al.* who showed that the prevalence of vitamin D insufficiency in Estonia is 73% and 29% in winter and summer, respectively (Kull *et al.* 2009). The mean post-deployment 25(OH)D levels had increased 2.6 times to a mean value of 104 nmol/L, ranging from 54 nmol/L to 157 nmol/L. This was an anticipated result regarding that Afghanistan is situated at the latitude of 32° N where the synthesis of vitamin D in the skin occurs for most of the year owing to high UVB radiation intensity.

Prolonged strenuous exercise induces a short-term and reversible inflammatory response as indicated by increased levels of various cytokines (Nieman *et al.* 2001) and other systemic biomarkers of inflammation, e.g., CRP (Andersson *et al.* 2010). Under chronic conditions low-grade systemic inflammation is associated with increased arterial stiffness (Yasmin *et al.* 2004; Kampus *et al.* 2004). Soldiers are often engaged in prolonged difficult physical

activities during military operations. This may result in deleterious implications on the immune system (Bernton *et al.* 1995; Gomez-Merino *et al.* 2003) and induce an acute systemic inflammatory response leading to impaired vascular function (Kampus *et al.* 2008). Consistent with previous studies we observed significant changes in the spectrum of pro- and anti-inflammatory biomarkers after the mission in the absence of clinically relevant inflammation. These results support the hypothesis by Ostrowski *et al.* that the inflammatory response to stress or exercise involves both pro- and anti-inflammatory activities (Ostrowski *et al.* 1999). Furthermore, a low-grade ongoing immune activation has also been reported in studies including veterans with Gulf War Illness (Skowera *et al.* 2004; Whistler *et al.* 2009). However, despite the presence of a low-grade inflammatory response after the mission we did not evidence any significant differences in the parameters of arterial stiffness. Therefore, we hypothesise that the immunoregulatory effects of high levels of 25(OH)D in all soldiers may have attenuated the possible inflammation-induced adverse effects on the vascular function resulting in a neutral net effect on arterial stiffness. In regard of possible favourable effects of high levels of vitamin D on vascular function we assessed the correlation between the pre- and post-deployment levels of aortic PWV and 25(OH)D. We found that increased 25(OH)D levels were associated with decreased aortic PWV in a subgroup of soldiers with the highest increase in 25(OH)D levels. Although biologically plausible, the association we observed between the change of aortic PWV and the change of 25(OH)D in this subgroup does not necessarily indicate a causal link between arterial stiffness and vitamin D status in our study, and larger prospective interventional trials are needed to confirm this hypothesis.

7. CONCLUSIONS

1. Streptozotocin-induced model of type 1 diabetes mellitus (STZ-T1DM) in rats was characterised by increased aortic stiffness, whereas blood pressure was not elevated after 10 weeks of the disease. By measuring aortic pulse wave velocity across a full range of pharmacologically modulated blood pressures we found that increased aortic stiffness only became evident at a high mean arterial pressure range, while no differences were detected at a lower mean arterial pressure range. These results suggest that increased isobaric pulse wave velocity as a measure of aortic stiffness is an early indicator of diabetes-induced arterial damage.
2. Experimental T1DM was associated with a decreased number and disturbed integrity of elastin fibres in the medial layer of the aortic wall and an increase in collagen content. These changes were expressed as a decrease in the ratio of elastin to collagen fibres in the aortic media and reduced thickness of the media. High levels of *N*ε-(carboxymethyl)lysine (CML), a major product of oxidative modification of glycated proteins, were established in the intimal, medial, and adventitial layers of the aortic wall. The localisation of CML within the diabetic aortic wall in conjunction with impaired structural integrity of the aorta demonstrates the contribution of advanced glycation end-products (AGEs) to the vascular wall damage occurring in STZ-T1DM. Oxidative stress-related production of CML within the aortic wall suggests a role for CML as an integrative biomarker of long-term oxidative damage to tissue proteins.
3. Asymmetric dimethylarginine levels in the serum were increased in rats with STZ-T1DM, indicating disturbed endothelial function. Oxidative stress index (the ratio of total peroxide level to total antioxidant capacity level) was elevated in the liver in STZ-T1DM. Diabetes also resulted in decreased serum total antioxidant capacity that indicates a substantially compromised systemic antioxidant response to the elevated oxidative stress.
4. Vitamin D supplementation for 10 weeks after the induction of STZ-T1DM in rats prevented the quantitative and qualitative changes in the elastin fibres in the aortic wall, but no effect was observed on the aortic stiffness *in vivo*. Vitamin D improved serum total antioxidant capacity and decreased oxidative stress index in the liver, the key organ in the metabolism and circulation of vitamin D. The accumulation of CML in the medial layer of the aortic wall was significantly reduced by vitamin D. These findings suggest that the vasoprotective effects of vitamin D could be mediated via inhibition of the accumulation of AGEs in the aortic wall in the context of reduced oxidative stress and improved systemic antioxidant capacity.
5. Treatment of diabetic rats with an angiotensin II type 1 receptor blocker telmisartan for 10 weeks after induction of STZ-diabetes reduced aortic stiffness *in vivo* independently of blood pressure reduction and partially preserved the amount and structural integrity of the elastin fibres within the aortic wall. The protective effects of telmisartan support the concept that the

inhibition of the renin-angiotensin system has a specific role in the improvement of diabetic vascular complications beyond blood pressure reduction.

6. Long-term exposure to physical and environmental stress during a 6-month military mission did not increase aortic stiffness and peripheral or central blood pressures in young well-trained professional soldiers. Before the deployment, vitamin D insufficiency was prevalent in 74% of the soldiers. After the deployment, vitamin D status was normalised in all soldiers. In a subgroup of soldiers with the highest increase in vitamin D levels there was a significant correlation between the decrease in aortic stiffness and the increase in serum vitamin D levels. Significant changes in serum biomarkers of inflammation were also established after the mission. In the perspective of possible favourable effects of vitamin D on inflammation and vascular function we postulate that the overall neutral effect on aortic stiffness may have resulted from the interaction between high levels of vitamin D and activated inflammatory system.

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9. SUMMARY IN ESTONIAN

Vitamiin D ja angiotensiin II retseptori blokaatori mõju arterikahjustustele

Vitamiin D keskseks biofunktsiooniks on kaltsiumi ja luukoe ainevahetuse regulatsioon. Tänapäeval on kindlaks tehtud, et vitamiin D retseptor on ekspresseeritud paljudes rakkudes, sealhulgas pankrease β-rakkudes, immuunrakkudes, endoteelirakkudes, kardiomüotsüütides ja neuronites. See asjaolu viitab, et vitamiin D võib mõjutada kudede ja organite olulisi funktsioone, mis ei ole otseselt seotud kaltsiumi ainevahetusega. Kliinilistes uuringutes on leitud, et madal vitamiin D tase organismis on seotud kõrgema südame- ja veresoonkonna (SV) haiguste riskiga, mis väheneb vitamiin D taseme normaliseerudes. Vitamiin D normaalse taseme säilitamise tähtsust kinnitavad epidemioloogilised uuringud, mis on näidanud, et vitamiin D defitsiit on Põhja-Euroopas levinud kliiniline probleem.

Arterite seinte jäigenemine on vananemise ja SV haigustega kaasnev protsess, mis on tingitud arterite struktuursete ja funktsionaalsete omaduste muutustest. Suurte arterite (sh. aordi) suurenenud jäikus on iseseisev riskitegur SV haiguste kujunemisel nii tervetel kui kõrge riskiga inimestel ja spetsiifiline ravi sihtmärk. Pulsilaine leviku kiirus aordis on arterite jäikust iseloomustav parameeter, mis on ühtemoodi mõõdetav nii inimestel kui katseloomadel. Euroopa Hüpertensiooni Ühingu ja Euroopa Kardioloogide Seltsi hüpertensiooni ravijuhise järgi on aordi pulsilaine kiirus üle 10 m/s ka asümptomaatilise organkahjustuse marker.

Diabeedi korral mitmekordistub SV haiguste risk ja vaskulaarsed tüsistused on diabeediga patsientide peamisteks surmapõhjusteks. Seetõttu on väga oluline varases staadiumis tuvastada häireid arterite struktuuris ja funktsioonis ning määrata ravi. Diabeedi ravi peamiseks eesmärgiks on normaliseerida ainevahetuslikud häired, ent tüsistuste leevendamine võib sealjuures jääda puudulikuks. Diabeediga kaasnevate arterikahjustuste patogeneesi uurimine katseloomadel on väärtuslik võimalus haiguse kulu mõistmiseks ja uute ravieesmärkide leidmiseks.

Kestva raske hüperglükeemia tingimustes, mis on dekompenseeritud diabeedi peamiseks metaboolseks tunnuseks, toimub intensiivne valkude ja teiste biomolekulide mitteensümaatiline glükeerimine, mille tulemusena kuhjuvad kudedes, sealhulgas arterite seintes, glükeerimise lõpp-produktid. Rakuvälise maatriksi põhivalkude, kollageeni ja elastiini, mitteensümaatiline glükeerimine halvendab arteriseina biomehaanilisi omadusi ning vähendab arterite elastsust. Glükeerimise lõpp-produktide teket soodustab tugev kestev oksüdatiivne stress, mida antioksüdantne kaitsesüsteem ei suuda adekvaatselt vähendada. Glükeerimise lõpp-produktid koos tugeva kestva oksüdatiivse stressiga on määrava tähtsusega diabeedi vaskulaarsete tüsistuste kujunemisel ning nende taset vähendav ravi võib oluliselt parandada diabeediga kaasnevaid arterikahjustusi.

Diabeediga kaasneb reniin-angiotensiin süsteemi liigne aktivatsioon, mis tõstab arteriaalset vererõhku ja pikas perspektiivis süvendab arteriseinte remodelleerumist ja jäigenemist. Reniin-angiotensiin süsteemi blokaatorid (nt. angiotensiini konverteeriva ensüümi inhibiitorid ja angiotensiin II retseptori blokaatorid) on laialdaselt kasutusel arteriaalse hüpertensiooni ravis, ent neil on ka samavõrd olulisi toimeid, mis ei ole otseselt seotud vererõhu langetamisega. Angiotensiin II retseptori blokaatorite rühmast võib telmisartaan olla eelistatud ravim diabeediga patsientidele tänu soodsatele metaboolsetele toimetele. Seepärast võib telmisartaan pakkuda unikaalseid võimalusi diabeedist tingitud arterikahjustuste ennetamiseks ja raviks.

Uurimuse eesmärgid

Peamiseks eesmärgiks oli uurida vitamin D mõju aordiseina struktuursetele, funktsionaalsetele ja biokeemilistele näitajatele ning süsteemselt ja sihtorganis mõõdetud oksüdatiivse stressi tasemele; samuti uurida angiotensiin II retseptori blokaatori telmisartaani mõju arterikahjustuste vähendamisele roti diabeedimudelis.

Uurimuse täpsed eesmärgid olid järgmised:

1. Hinnata aordi jäikust pulsilaine leviku kiiruse abil streptosototsiin-tekitatud (STZ) roti 1. tüüpi diabeedimudelis.
2. Uurida morfoloogilisi näitajaid aordi histoloogilistel preparaatidel ja glükeerimise lõpp-produktide taset aordiseinas roti STZ-diabeedimudelis.
3. Mõõta asümmeetrilise dimetüülarginiini taset seerumis ning oksüdatiivse stressi ja totaalse antioksüdantse vastuse taset seerumis ja maksas roti STZ-diabeedimudelis.
4. Hinnata vitamiin D lisamanustamise mõju aordi jäikusele, aordiseina remodelleerumisele, oksüdatiivse stressi tasemele ja glükeerimise lõpp-produktide tasemele roti STZ-diabeedimudelis.
5. Uurida angiotensiin II retseptori telmisartaani mõju aordi jäikusele ja remodelleerumisele roti STZ-diabeedimudelis.
6. Hinnata seost aordi pulsilaine leviku kiiruse ja vitamiin D taseme vahel ning seerumi põletikunäitajate muutuseid noortel treenitud meestel kauakestva füüsilise ja keskkonnast tingitud stressi järgselt.

Meetodid

Wistar liini rottidel tekitati diabeet STZ (65 mg/kg kehakaalu kohta) ühekordse süstiga. Ühele katsegrupile manustati 10 nädala jooksul ülepäeviti suukaudselt 500 IU/kg kehakaalu kohta vitamiin D või iga päev 10 mg/kg kehakaalu kohta telmisartaani. 10. nädalal mõõdeti anesteseeritud rottidel invasiivselt pulsilaine leviku kiirust aordis. Määrati oksüdatiivse stressi biomarkerite tase ja uuriti aordi histoloogilisi preparaate.

Uuringualusteks olid 65 sõdurit, keda uuriti enne ja pärast 6 kuud kestnud sõjalist missiooni Afganistanis. Aordi jäikust mõõdeti pulsilaine leviku kiiruse abil aordis ja määrati seerumi põletikumarkerite ja vitamiin D tase.

Tulemused ja järeldused

1. Streptosototsiin-indutseeritud diabeediga rottidel suurenes aordi jäikus pärast 10 nädalat kestnud haigust, kuid arteriaalne vererõhk jäi muutumatuks. Mõõtes pulsilaine kiirust aordis farmakoloogiliselt tõstetud ja langetatud vererõhkude juures, leidsime, et aordi jäikus on oluliselt suurem kõrge rõhu vahemikus, kuid madalate rõhkude juures ei olnud uuringurühmade vahel erinevusi. Tulemused viitavad, et vererõhust sõltumatutes tingimustes mõõdetud pulsilaine kiirus aordis on diabeediga kaasnevate arterikahjustuste varane näitaja.
2. Eksperimentaalne diabeet vähendas elastsete kiudude hulka ja tõstis kollageenkiudude hulka aordiseina keskkestas, samuti oli häiritud elastsete kiudude struktuurne terviklikkus. Aordiseina kõikidesse kestadesse oli kuhjunud *N*ε-karboksümetüüllüsiin (CML), mis on valkude glükeerimise ja oksüdatiivse modifitseerimise peamine lõpp-produkt. *N*ε-karboksümetüüllüsiini kuhjumine koos aordiseina struktuursete kahjustustega näitab, et glükeerimise lõpp-produktid (AGE-d) mängivad olulist rolli STZ-diabeediga kaasnevate vaskulaarkahjustuste kujunemisel. Oksüdatiivse stressiga seotud CML teke aordiseinas viitab, et CML võib olla koevalkude pikaajaliste oksüdatiivsete kahjustuste integreeritud biomarkeriks.
3. Streptosototsiin-diabeediga rottidel oli seerumi asümmeetrilise dimetüülarginiini tase oluliselt tõusnud, mis viitab endoteeli häiritud funktsioonile. Oksüdatiivse stressi indeks (peroksiidide taseme ja antioksüdantse vastuse suhe) oli STZ-diabeediga rottide maksas oluliselt tõusnud. Samuti vähenes STZ-diabeedi korral seerumi antioksüdantne vastuse tase, mis viitab süsteemse antioksüdantse vastuse olulisele dekompensatsioonile.
4. Vitamiin D manustamine rottidele 10 nädala jooksul pärast STZ-diabeedi tekitamist vähendas aordiseina elastsete kiudude kvantitatiivseid ja kvalitatiivseid muutuseid, kuid ei avaldanud toimet *in vivo* mõõdetud aordi jäikusele. Vitamiin D parandas oluliselt seerumi antioksüdantset vastust ning vähendas oksüdatiivse stressi indeksit maksas, mis on ühtlasi vitamiin D ainevahetuses võtmetähtsusega elund. Aordiseina keskkestas vähenes oluliselt vitamiin D toimel CML kuhjumine. Antud tulemused viitavad, et vitamiin D vasoprotektiivsed toimed võivad olla vahendatud AGE-de kuhjumise takistamise kaudu aordiseinas, mille taustal on vähenenud oksüdatiivse stressi tase ja paranenud süsteemne antioksüdantne vastus.
5. Angiotensiin II retseptori blokaatori telmisartaani manustamine 10 nädala jooksul pärast STZ-diabeedi tekitamist rottidele vähendas vererõhu langetamisest sõltumatult aordi jäikust *in vivo* ja osaliselt säilitas aordiseina elastsete kiudude struktuurse terviklikkuse ja ja hulga. Telmisartaani protektiivsed toimed viitavad, et reniin-angiotensiin süsteemi inhibeerimisel on

spetsiifiline roll diabeedi vaskulaarsete tüsistuste parandamisel, mis ei ole seotud vererõhu langetamisega.

6. Pikaajaline füüsiline ja keskkonnast tingitud stress 6 kuud kestva sõjalise missiooni ajal ei tõstnud noortel treenitud professionaalsetel sõduritel aordi jäikust, perifeerset ega tsentraalset vererõhku. Enne missiooni esines 74%-l sõduritest vitamiin D defitsiit ja missioonijärgselt oli kõikidel sõduritel vitamiin D tase organismis normaliseerunud. Sõdurite alagrupis, kus oli kõige suurem vitamiin D taseme tõus, leidsime, et vitamiin D taseme tõus oli seotud aordi jäikuse vähenemisega. Missioonijärgselt esines ka olulisi muutusi seerumi põletikunäitajate spektris. Võttes arvesse vitamiin D potentsiaalseid põletikuvastaseid toimeid, oletame, et oluliselt tõusnud vitamiin D tase võis kompenseerida põletikunäitajate muutusi, mistõttu aordi jäikuses muutusi ei ilmnenud.

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11. PUBLICATIONS

CURRICULUM VITAE

Name:	Erik Salum
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Education:

1990–2001	Miina Härma Gymnasium, Tartu
2002–2009	University of Tartu, Faculty of Medicine, Medicine
2009–2013	University of Tartu, Faculty of Medicine, Department of Cardiology, PhD studies

Professional employment:

2006–2009	Translator, Translation Agency Atrenda
2009–	Specialist, Department of Cardiology, University of Tartu
2012–2012	Visiting Researcher, Australian School of Advanced Medicine, Macquarie University, Sydney, Australia (4.5 months).

Special courses:

2010	Basic course of laboratory animal science: C-category competence, Kuopio, Finland.
2010	International Conference "Artery 10", Verona, Italy
2011	International Conference "Artery 11", Paris, France
2011	Basic military course for reserve medical professionals, ensign rank
2012	24th Scientific Meeting of the International Society of Hypertension, Sydney, Australia

Scientific work:

Main research is focused on the functional measurements of arterial properties in a rodent model of diabetes associated with structural and biochemical abnormalities, and the effects of vitamin D and an angiotensin II receptor blocker in this model.

Six scientific articles in international peer-reviewed journals. Five presentations in international scientific conferences.

Membership: Estonian Society of Cardiology.

Publications in international peer-reviewed journals:

1. Salum E, Kals J, Kampus P, Salum T, Zilmer K, Aunapuu M, Arend A, Eha J, Zilmer M. Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. Diabetes Res Clin Pract 2013; 100:243–249.

2. Salum E, Kampus P, Zilmer M, Eha J, Butlin M, Avolio AP, Põdramägi T, Arend A, Aunapuu M, Kals J. Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. Cardiovasc Diabetol 2012; 11:58.
3. Kaldmäe M, Salum E, Annuk M, Kals J, Kampus P, Zilmer K, Eha J, Zilmer M. Oxidative stress status in homeless people. Oxid Antioxid Med Sci 2012; 1:35–39.
4. Veldre G, Kums T, Salum E, Eha J. Relationship between soldiers' body height-weight category and changes in their spinal column kyphotic curvature during a long-term military mission. Papers on Anthropology 2011; 20:423–428.
5. 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.
6. Serg M, Kampus P, Kals J, Zagura M, Muda P, Tuomainen TP, Zilmer K, Salum E, Zilmer M, Eha J. Association between asymmetric dimethylarginine and indices of vascular function in patients with essential hypertension. Blood Press 2011; 20:111–116.

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1990–2001	Miina Härma Gümnaasium, Tartu
2002–2009	Tartu Ülikool, Arstiteaduskond, arstiõpe
2009–2013	Tartu Ülikool, Arstiteaduskond, Kardioloogia kliinik, doktoriõpe

Erialane teenistuskäik:

2006–2009	Tõlk, OÜ Atrenda tõlkebüroo
2009–	Spetsialist, Tartu Ülikooli kardioloogia kliinik
2012–2012	Külalisteadur, Australian School of Advanced Medicine, Macquarie ülikool, Sydney, Austraalia (4,5 kuud).

Erialane täiendus:

2010	Katseloomateaduse baaskursus: C-kategooria, Kuopio, Soome.
2010	Rahvusvaheline konverents "Artery 10", Verona, Itaalia
2011	Rahvusvaheline konverents "Artery 11", Pariis, Prantsusmaa
2011	Reservtervishoiutöötajate sõjaline baaskursus, lipniku auaste
2012	Rahvusvaheline konverents "24th Scientific Meeting of the International Society of Hypertension", Sydney, Austraalia

Teadustegevus:

Teadustöö põhisuundadeks on arterite funktsionaalsed mõõtmised roti diabeedimudelil ja sellega seotud struktuursete ja biokeemiliste häirete ning vitamiin D ja angiotensiin II retseptori blokaatori mõju uurimine antud mudelis.

Ilmunud on 6 teadusartiklit rahvusvahelistes eelretsenseeritavates ajakirjades. Viis ettekannet rahvusvahelistel teaduskonverentsidel.

Eesti Kardioloogide Seltsi liige.

Artiklid rahvusvahelistes eelretsentseeritavates ajakirjades:

1. Salum E, Kals J, Kampus P, Salum T, Zilmer K, Aunapuu M, Arend A, Eha J, Zilmer M. Vitamin D reduces deposition of advanced glycation end-products in the aortic wall and systemic oxidative stress in diabetic rats. Diabetes Res Clin Pract 2013; 100:243–249.
2. Salum E, Kampus P, Zilmer M, Eha J, Butlin M, Avolio AP, Põdramägi T, Arend A, Aunapuu M, Kals J. Effect of vitamin D on aortic remodeling in streptozotocin-induced diabetes. Cardiovasc Diabetol 2012; 11:58.

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5. 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.
6. Serg M, Kampus P, Kals J, Zagura M, Muda P, Tuomainen TP, Zilmer K, Salum E, Zilmer M, Eha J. Association between asymmetric dimethylarginine and indices of vascular function in patients with essential hypertension. Blood Press 2011; 20:111–116.

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