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**Arterial stiffness and cardiovascular risk factors**

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Academic dissertation

To be presented with the permission of the Medical Faculty of the University of Helsinki, for public examination in Biomedicum Helsinki, on December 21, 2007, at 12 noon.

Helsinki 2007
To Ansku, Alvin, and Ebbe
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Abstract

Background: As the human body ages, the arteries gradually lose their elasticity and become stiffer. Although inevitable, this process is influenced by hereditary and environmental factors. Interestingly, many classic cardiovascular risk factors affect the arterial stiffness. During the last decade, accelerated arterial stiffening has been recognized as an important cardiovascular risk factor associated with increased mortality as well as with several chronic disorders.

Objectives: This thesis examines the role of arterial stiffness in relation to variations in a physiological feature in healthy individuals. In addition, the effect on arterial stiffness of an acute transitory disease and the effect of a chronic disease are studied. Furthermore, the thesis analyzes the prognostic value of a marker of arterial stiffness in individuals with chronic disease. Finally, a potential method of reducing arterial stiffness is evaluated.

Material and study design: The first study examines pulse wave reflection and pulse wave velocity in relation to muscle fibre distribution in healthy middle-aged men. In the second study, pulse wave reflection in women with current or previous preeclampsia is compared to a healthy control group. The effect of aging on the different blood pressure indices in patients with type 1 diabetes is examined in the third study, whereas the fourth paper studies the relation between these blood pressure indices and mortality in type 2 diabetes. The fifth study evaluates how intake of a fermented milk product containing bioactive peptides affects pulse wave reflection in individuals with mild hypertension.
Results and conclusions: Muscle fibre type distribution is not an independent determinant of arterial stiffness in middle-aged males. Pulse wave reflection is increased in pregnant women with preeclampsia, but not in previously preeclamptic non-pregnant women. Patients with type 1 diabetes have a higher and more rapidly increasing pulse pressure, which suggests accelerated arterial stiffening. In elderly type 2 diabetic patients, very high and very low levels of pulse pressure are associated with higher mortality. Intake of milk-derived bioactive peptides reduces pulse wave reflection in hypertensive males but not in hypertensive females.
List of original publications

This thesis is based on the following publications:


The publications are referred to in the text by their roman numerals.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AASI</td>
<td>ambulatory arterial stiffness index</td>
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<tr>
<td>AER</td>
<td>albumin excretion rate</td>
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<tr>
<td>AGE</td>
<td>advanced glycation end product</td>
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<td>AIx</td>
<td>augmentation index</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BPM</td>
<td>beats / minute</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycosylated haemoglobin A1c</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>IMT</td>
<td>intima-media thickness</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MET</td>
<td>metabolic equivalent</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>Tr</td>
<td>time to return of the reflected pulse wave</td>
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<td>WHO</td>
<td>world health organization</td>
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1. Introduction

The buffering capacity of the large arteries of the human body is essential for maintaining a steady blood flow. During systole, the arterial walls expand and absorb energy, which is released during diastole. With increasing age, the large arteries of the human body gradually lose their elasticity and become stiffer. The systolic blood pressure consequently tends to rise linearly with age, whereas the diastolic blood pressure generally starts to decline after approximately 60 years of age. This process results in a widening of the pulse pressure, which therefore can be regarded as a surrogate measure of arterial stiffness.1

The process of arterial stiffening has important clinical consequences. Arterial stiffening adds to the cardiac afterload by increasing aortic systolic blood pressure and reduces coronary perfusion by lowering the diastolic blood pressure. Arterial stiffness thereby increases the susceptibility to myocardial ischemia and increases the pressure-induced damage on coronary and cerebral arteries.

In recent years, pulse pressure and other measures of arterial stiffness have been recognized as an important risk factor for cardiovascular disease.2, 3 Additionally, many established cardiovascular risk factors, such as hypertension, diabetes, and smoking have been found to increase arterial stiffness.4, 5, 6

The beneficial effect of physical activity on cardiovascular health is well-established. Striated muscle fibre-type distribution differs between elite athletes of various types of sports and is mostly determined by genetic factors.7 A high proportion of slow-twitch
striated muscle fibres has been associated with a favourable cardiovascular risk profile including a reduced risk of hypertension. Nevertheless, it is unclear whether muscle fibre distribution has an effect on arterial stiffness.

Preeclampsia is regarded as a state of endothelial dysfunction, which has been associated with arterial stiffness. Moreover, a history of preeclampsia is an established risk factor for cardiovascular disease later in life. The role of arterial stiffness has not yet been studied in this context.

Both type 1 and type 2 diabetes are associated with an increased risk of cardiovascular disease. The risk is particularly elevated in patients with diabetic nephropathy, but is also higher in patients without diabetic kidney disease. It has also been established that both type 1 and type 2 diabetes increase arterial stiffness. However, the effect of diabetes on arterial stiffness and on the role of arterial stiffening in the pathogenesis of cardiovascular disease in patients with diabetes are still unclear.

Milk casein derived biologically active tripeptides have been shown to have antihypertensive effects in clinical trials. In vitro studies show that these peptides have a beneficial effect on arterial tone. Studies on humans in vivo are still required to verify the benefits of bioactive tripeptides in a clinical setting.

The present studies were undertaken in order to study the relationship between arterial stiffness and cardiovascular risk factors in order to assess the role of arterial stiffness in the pathogenesis of cardiovascular disease.
2. Review of the literature

2.1. Arterial stiffness

2.1.1. Definitions of arterial stiffness

Arterial stiffness is a term employed to define the arteries’ capacity to expand and contract during the cardiac cycle. Other terms such as arterial compliance, distensibility and elasticity, are all different aspects of arterial stiffness.\(^\text{16}\) Although these terms are interrelated, they are not synonymous (Table 1).\(^\text{17}\) Compliance is defined as the change in volume for a given pressure change. In the arterial system compliance relates to the change in artery diameter caused by left ventricular ejection. Distensibility is used to define compliance relative to the initial volume or diameter of an artery. A loss of arterial elasticity results in reduced arterial compliance and distensibility. When pressure increases, a point is eventually reached with less distensibility occurring at higher pressures as a consequence of the elastic properties of the arterial media.\(^\text{18}\) At low pressures elastin fibres take up pressure, whereas at higher pressures the tension is absorbed by the more rigid collagen fibres and compliance consequently decreases. Differences in arterial compliance should therefore generally be corrected for blood pressure.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Method</th>
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<tr>
<td>Compliance</td>
<td>Arterial segment volume/diameter change with pressure change</td>
<td>Ultrasonography</td>
</tr>
<tr>
<td>Distensibility</td>
<td>Compliance relative to initial volume/diameter</td>
<td>Ultrasonography</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>Difference between systolic and diastolic blood pressure</td>
<td>Blood pressure measurement</td>
</tr>
<tr>
<td>Pulse wave velocity (PWV)</td>
<td>The speed of the pulse wave over an arterial segment</td>
<td>ECG-gated tonometry, ultrasound, or doppler</td>
</tr>
<tr>
<td>Augmentation index (AIx)</td>
<td>Augmentation of aortic pulse wave by wave reflection expressed as a ratio of aortic pulse pressure</td>
<td>Carotid or radial tonometry</td>
</tr>
<tr>
<td>Capacitive (large) artery compliance (C1)</td>
<td>Change in volume throughout exponential diastolic pressure decay</td>
<td>Diastolic pulse contour analysis by radial tonometry</td>
</tr>
<tr>
<td>Oscillatory (small) artery compliance (C2)</td>
<td>Change in volume per oscillatory pressure change throughout exponential diastolic pressure decay</td>
<td>Diastolic pulse contour analysis by radial tonometry</td>
</tr>
<tr>
<td>Ambulatory Arterial Stiffness Index (AASI)</td>
<td>1 minus the regression slope of diastolic blood pressure and systolic blood pressure readings</td>
<td>Ambulatory blood pressure measurement</td>
</tr>
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</table>
2.1.2. The role of extracellular matrix

The physical properties of the arterial walls largely depend on the two extracellular proteins elastin and collagen. The proportion of elastin and collagen in the arterial wall is regulated by a slow dynamic process of formation and degradation. Elastin and collagen degradation is regulated by catabolic matrix metalloproteases. Disturbances of this balance typically lead to higher collagen content and a diminished proportion of elastin, which reduces arterial elasticity. Collagen production is also stimulated by elevated blood pressure. At the histological level arterial ageing manifests as a two- to three-fold increase of intima-media thickness during the normal life span. Histological examination of stiffened arteries shows damaged endothelium, increased collagen content, broken elastin molecules, hypertrophied vascular smooth muscle layer, inflammatory activity, and increased matrix metalloproteinases.

The tensile strength of the arterial wall is mainly made up of cross-linked collagen molecules. Due to its slow turnover rate, collagen is particularly susceptible to nonenzymatic glycation and cross-linking. This leads to a more unorganized and dysfunctional collagen fibre structure with inferior elasticity.

Elastin molecules that have a key function in providing arterial wall elasticity are also stabilized by cross-linking. Activated metalloproteases generate broken and frayed elastin molecules and disruption of the cross-links predisposes to protein mineralization and an increase in arterial stiffness.
Advanced glycation end products (AGEs) also contribute to arterial stiffening by forming irreversible cross-links between slow-turnover proteins such as collagen and elastin.\textsuperscript{31, 32, 33, 34} The non-enzymatic protein glycation process forms cross-linked molecules that are structurally more rigid and less susceptible to degradation.\textsuperscript{35} AGEs also impair endothelial function by quenching nitric oxide (NO) and by increasing the generation of oxidants.\textsuperscript{36} Furthermore, by binding to specific receptors, AGEs initiate inflammatory responses that can increase vascular stiffness via activation of metalloproteinases, a phenomenon that contributes to endothelial dysfunction and promotes atherosclerosis.\textsuperscript{37, 38, 39}

2.1.3. Endothelial function

Arterial stiffness is not only determined by the structure of extracellular matrix. Endothelial cell function and vascular smooth muscle cell tone do also have a strong influence on arterial stiffness. Arterial tone is affected by a number of factors like shear mechanical stress as well as paracrine mediators such as angiotensin II, endothelin, and NO.\textsuperscript{40, 41}

The endothelium is made up of a single layer of cells that line all the blood vessels in the body, including conduit arteries, resistance arteries, arterioles and capillaries.\textsuperscript{42} The endothelial cells provide a critical barrier between the blood and the tissues. For a long time, the endothelium was considered a passive membrane that prevented the diffusion of macromolecules. It is now known that the endothelium is an important autocrine, paracrine and endocrine organ.
The function of each vessel and the role of its respective endothelium vary according to its location in the body. In the larger arteries, a healthy endothelium provides a surface that limits the activation of clotting and inflammation, blocks the transfer of atherogenic lipid particles into the arterial wall, and prevents adhesion of platelets and monocytes. In the resistance arteries, endothelial cells contribute to the regulation of blood flow and blood pressure. In the precapillary arterioles, the endothelium plays a role in the transport of nutrients and hormones, including glucose, fat, and insulin.43

The endothelial cells produce a wide range of substances such as NO, prostacyclin, endothelin, vascular endothelial growth factor, interleukins, tissue plasminogen activator, angiotensin converting enzyme (ACE) and von Willebrand factor. Synthesis of NO occurs through enzymatic oxidation of L-arginine.

Endothelial dysfunction is characterized by loss of endothelium-dependent vasodilatation and can be considered an early phase in the pathogenesis of cardiovascular disease. Decreased production, increased degradation or decreased sensitivity to NO are involved in endothelial dysfunction. Therefore, the term ‘decreased NO bioavailability’ is often used to describe the pathophysiological processes that involve NO in endothelial dysfunction.44

Reduced bioavailability of NO impairs vascular smooth muscle relaxation and thus causes functional stiffening of the arteries. Several studies have established an effect of endothelial dysfunction on arterial stiffening.9, 45, 46, 47, 48 Interestingly, a recent study has shown evidence that arterial stiffness itself may disturb endothelial function and NO release and thereby accelerate the stiffening process.49
2.1.4. Intima media thickness

Increased intima-media thickness (IMT) from ultrasound measurements of the carotid artery is considered as a surrogate marker of generalized atherosclerosis, and has in several studies been shown to predict the cardiovascular events such as stroke and myocardial infarction. The pathophysiological concept behind carotid IMT as a marker of target organ damage is that intimal thickening at the carotid artery may be an early stage of atherosclerotic disease.

IMT of the carotid artery can be measured by B-mode ultrasound in a fairly uncomplicated manner. The assessment of IMT has emerged as one of the most popular methods of determining early atherosclerotic changes and the progression of atherosclerosis.

A close relationship of IMT with a number of cardiovascular risk factors has been found. Cholesterol, body mass index and smoking are significantly related to the annual progression of carotid IMT. Also hypertension appears to have a great effect on IMT.

A number of cross-sectional studies have shown that IMT is increased in diabetic subjects in comparison with non-diabetic subjects. Diabetic subjects without a diagnosis of CVD have similar IMT compared to non-diabetic subjects with CVD. Furthermore, the progression of IMT is approximately 25% greater in diabetic subjects, even after adjustment for established cardiovascular risk factors. Additionally, there are findings that imply that poor glycaemic control may accelerate the increase in IMT. Interestingly, the Epidemiology of Diabetes Interventions and Complications study
showed that intensive insulin therapy in type 1 diabetes slows progression of IMT suggesting that improved glycaemic control is responsible for the accelerated thickening of the intima media in diabetic subjects.59

It is however unclear to what extent IMT correlates with measures of arterial stiffness. In type 2 diabetic patients central pressure augmentation correlates with IMT independently of other risk factors.60 A study on patients in dialysis showed no correlation between IMT and PWV which suggests that aortic stiffness and carotid atherosclerosis may partially differ in their pathologic background.61 Similar results were found in a study on young adults, in which no independent association between IMT and PWV could be observed.62

2.1.5. Methods for assessment of arterial stiffness

2.1.5.1. History

For a long time it has been known that the characteristics of the arterial pulse change with age and assessment of the arterial pulse has traditionally been considered an important part of the clinical examination of a patient.63

In the 19th century the sphygmograph, which registered the arterial waveform, was invented.64, 65, 66 As the ability to interpret the shape of the pulse wave developed, it was discovered that the shape of the waveform was significantly influenced by age and disease.67 Nevertheless, when Riva-Rocci developed the mercury sphygmomanometer in the late 19th century,68 sphygmomanometry that focused on the absolute systolic and diastolic blood pressure gained clinical popularity on the expense of sphygmography. For
approximately a century arterial stiffening was considered to merely reflect ageing and the clinical significance of the shape of the arterial waveform has not been generally appreciated until quite recently.

2.1.5.2. Pulse pressure

Pulse pressure is the difference between systolic and diastolic blood pressure and is the consequence of cardiac contraction and is strongly influenced by the properties of the arterial tree. As the pulse pressure is mainly determined by cardiac output, aortic and large artery stiffness, and pulse wave reflection, it constitutes a surrogate marker for arterial stiffness.

Whereas the systolic blood pressure tends to increase linearly with age in the western population, the diastolic blood pressure generally rises during adulthood and peaks at approximately 60 years of age and thereafter starts to decline due to arterial stiffening. This naturally results in a rapidly increasing pulse pressure.

Pulse pressure is the most easily available measure of arterial stiffness since it can be assessed with a standard sphygmomanometer. Unfortunately, assessment of arterial stiffness by pulse pressure can be quite inaccurate. The brachial blood pressure is strongly determined by the phenomenon of pulse wave amplification from the aorta to the peripheral arteries. Due to pulse wave amplification, the peripheral systolic blood pressure and consequently also the pulse pressure can differ markedly between central and peripheral arteries. Pulse wave amplification decreases with age and is most
prominent in the young.\textsuperscript{76} Thus the usefulness of brachial pulse pressure as a marker of arterial stiffness is poor in the young but increases substantially with age.

In this context, it can be emphasized that it is in fact the central, not the peripheral blood pressure that contributes most to the development of the early stages of cardiovascular disease, e.g. coronary and carotid atherosclerosis and left ventricular hypertrophy.\textsuperscript{77}

Pulse pressure has been shown to be a powerful predictor of cardiovascular morbidity and mortality in a number of studies. The Framingham study demonstrated that in the elderly population pulse pressure is a stronger predictor of cardiovascular disease than systolic or diastolic pressure alone.\textsuperscript{2} This finding has been confirmed several times.\textsuperscript{78, 79, 80, 81, 82} Additionally, pulse pressure has been found to predict all-cause and cardiovascular mortality particularly in the elderly but also in the general population.\textsuperscript{83, 84, 85, 86} A meta-analysis by Gasowski showed that in hypertensive patients pulse pressure, but not the mean arterial blood pressure, is associated with an increased risk of fatal coronary heart events.\textsuperscript{87} Conflicting results were provided by the Chicago Heart Association and Health Department Study, which failed to find a relation between pulse pressure and subsequent mortality.\textsuperscript{88, 89} Another large study performed on African-Americans also challenged this view.\textsuperscript{90}

There is however satisfactory consensus that, in the young and middle-aged, diastolic pressure still is the best blood pressure index to predict coronary heart disease as was originally shown by the Framingham study.\textsuperscript{91}
2.1.5.3. Pulse wave velocity

The speed at which the pressure wave generated by cardiac contraction travels from the aorta to the peripheral arteries is mainly determined by the artery wall stiffness and lumen diameter. Pulse wave velocity (PWV) can be calculated by measuring the time for the pulse to pass between two points with known distance. The measurement usually involves taking separate recordings from two sites and relating them to the R wave of a simultaneously recorded ECG. A variety of methods can be applied to register the pulse wave such as doppler ultrasound, or applanation tonometry. Since the aorta is the major component of arterial stiffness, the carotid-femoral pulse wave velocity, which is a measure of aortic stiffness, is the most commonly used in the evaluation of regional stiffness. Carotid-radial pulse wave velocity is mainly a measure of velocity and thus of stiffness in the brachial artery and is also quite commonly used when conduit artery stiffness is examined.

Assessment of pulse wave velocity is relatively simple and the method has been widely applied and has been found to be both robust and reproducible. Studies show that pulse wave velocity is an independent predictor of cardiovascular disease and mortality in both hypertensive patients and in patients with end-stage renal disease. Furthermore, aortic pulse wave velocity is a powerful independent predictor of mortality in diabetic and elderly population samples.
2.1.5.4. Pulse wave analysis

As the left cardiac ventricle contracts it creates a forward pressure wave that travels to the periphery throughout the arterial tree. When the forward wave reaches the branching points of arteries, regions of increased arterial stiffness, and high-resistance arterioles a backward wave occurs as a consequence of wave reflection. The reflected waves are superimposed on the wave that travels forward resulting in an arterial waveform that varies throughout the arterial tree.

Arterial stiffening increases the amplitude and the velocity of the reflected waves. In elastic vessels, the reflected wave tends to arrive back to the aorta during diastole and thereby augments diastolic pressure and improves coronary perfusion. As arterial stiffness and hence pulse wave velocity increases, the reflected wave returns to the aorta at an earlier phase of the cardiac cycle thereby augmenting the systolic pressure instead of the diastolic pressure. Consequently, arterial stiffening reduces coronary perfusion and increases cardiac oxygen consumption by augmenting cardiac afterload.

Since the arterial waveform varies throughout the arterial tree, the extent of wave reflection is assessed more accurately by analyzing the central pressure waveform than the peripheral waveforms. Although the reflected waves originate predominantly at the major branches of the aorta, stiffness of the smaller arteries and arterioles has a considerable influence on the central pressure waveform. Central pulse pressure augmentation may therefore provide a better marker of systemic arterial stiffness than single large artery measures, such as pulse wave velocity or aortic ultrasound.
Pulse wave analysis (PWA) is a non-invasive method to measure arterial stiffness.\textsuperscript{101} Applanation tonometry that uses a Millar transducer is employed to record pressures at the radial or the carotid artery, and a validated generalized transfer function based upon a comparison with intra-arterial pressures in patients undergoing surgery is then applied to generate the corresponding central waveform.\textsuperscript{102, 103} The augmentation index (AIx), which is a measure of systemic arterial stiffness can then be calculated as the difference between the first and second systolic peaks expressed as a percentage of the central pulse pressure. Satisfactory waveform recordings from the radial artery are typically obtained within a few minutes by a trained examiner.

The AIx has been associated with the presence and extent of coronary artery disease.\textsuperscript{104} Increased arterial wave reflection is also a risk factor for cardiovascular events in patients with established coronary artery disease.\textsuperscript{105, 106} In renal failure patients, high AIx has been established as an independent predictor of all-cause and cardiovascular mortality.\textsuperscript{107}

However, the use of pulse wave analysis to assess arterial stiffness is associated with some problems. Since the pulse wave reflection returns to the aorta at an earlier phase of the cardiac cycle when the heart rate is high, there is an inverse association between heart rate and AIx that needs to be adjusted for.\textsuperscript{75} The AIx is only in part determined by arterial stiffness as increases in peripheral wave reflectance may also be caused by increased peripheral vascular resistance and by the distending effect of an elevated blood pressure.\textsuperscript{101} Furthermore, it seems that the generalized transfer function may be inappropriate for the derivation of central waveforms in patients with diabetes thereby making the AIx an unreliable measure of arterial stiffness these in patients.\textsuperscript{108, 109}
2.1.5.5. Diastolic pulse contour analysis

The compliance of both large and small arteries can be examined by assessment of the diastolic portion of the pressure pulse contour utilizing a modified Windkessel model.\textsuperscript{110, 111, 112} Two components of arterial compliance are obtained: large (capacitive) artery compliance, \( C_1 \), and small (oscillatory) artery compliance, \( C_2 \). Similarly to pulse wave analysis, the waveform of the radial artery can be determined non-invasively by using tonometry. In a 7 year prospective trial, \( C_2 \), but not \( C_1 \), was a predictor of cardiovascular events.\textsuperscript{113} Nevertheless, the validity of diastolic pulse contour analysis has been subject to some criticism that casts doubts over the reliability of this methodology to accurately measure arterial stiffness.\textsuperscript{114, 115}

2.1.5.6. Ultrasonography

Arterial distensibility and compliance can be measured by ultrasound examination of large arteries (brachial, femoral and carotid arteries and the abdominal aorta). Images of the arterial walls are recorded and the maximum and minimum arterial diameter is registered. Compliance and distensibility can then be calculated using a formula including blood pressure.\textsuperscript{116, 117} Ultrasound has the advantage of being non-invasive, but the equipment is expensive and the learning procedure to master this technique requires plenty of time and effort. Another problem is its high user-dependency and there have been some concerns about the reproducibility of the results obtained with this technique.\textsuperscript{118}
2.1.5.7. Magnetic resonance imaging

Magnetic resonance imaging can be used to measure arterial stiffness by relating the change in arterial diameter to the distending pressure. Most studies using this technique have been performed on the aorta. Although magnetic resonance imaging is non-invasive and quite accurate, it remains expensive, and its use will probably be limited to well-equipped research settings. Moreover, magnetic resonance imaging has been used to assess pulse wave velocity. This technique allows path length to be assessed accurately, but is as expected not only expensive, but also time-consuming and is therefore not in common use at the present time.

2.1.5.8. Ambulatory arterial stiffness index

Recently, a novel method to determine arterial stiffness based on 24-hour ambulatory blood pressure measurement has been proposed. The ambulatory arterial stiffness index (AASI) is calculated from the individual blood pressure readings as 1 minus the slope of diastolic on systolic pressure during 24-hour ambulatory monitoring. The AASI appears to be closely correlated with pulse wave velocity and with the AIx. The prospective study by Dolan et al showed that in 11 291 hypertensive patients, AASI correlated with cardiovascular mortality. In adjusted analyses, AASI was a better predictor of fatal strokes, than of cardiac events. Due to its novelty the method is yet to be evaluated in other studies.
2.1.6. Factors that affect arterial stiffness

2.1.6.1. Age

Stiffening of large arteries seems to be an inevitable consequence of the normal aging process and age is consequently the most important determinant of arterial stiffness.\textsuperscript{123, 124} The positive association between age and arterial stiffness has been confirmed in a large number of studies using various techniques.\textsuperscript{24, 125, 126, 127, 128, 129} However, whereas the large central arteries stiffen progressively with age, the elastic properties of the smaller muscular arteries change little with age.\textsuperscript{130, 131}

2.1.6.2. Hypertension

Although large artery stiffening is a strongly age-related process, it is also markedly accelerated by the presence of hypertension.\textsuperscript{4, 132} Benetos et al found that the age-induced pulse wave velocity progression was more than 3-times greater in poorly controlled hypertensive patients compared with well-controlled hypertensive patients.\textsuperscript{133} A study on 24-h blood pressure showed that impaired night time blood pressure decline is associated with increased arterial stiffness assessed by pulse wave analysis.\textsuperscript{134} Another study showed that a determinant of hypertension, low birth weight is related to increased arterial stiffness.\textsuperscript{135} Interestingly, recent results demonstrating that aortic stiffness is an independent predictor of progression to hypertension in normotensive subjects suggest that lower arterial elasticity is related to the development of hypertension.\textsuperscript{136, 137}
2.1.6.3. Physical activity and muscle fibre distribution

The beneficial effect of physical activity on cardiovascular health is undisputable. The protection from cardiovascular disease offered by physical activity appears to be mediated by modification of cardiovascular risk factors such as blood pressure, lipid profile, and body weight in a favourable manner.\textsuperscript{138, 139}

Muscle fibre-type distribution differs between elite athletes of various types of sports and is mostly determined by genetic factors.\textsuperscript{7} A high proportion of type I (slow-twitch) fibres is generally found in endurance sports athletes, while speed and power sports athletes have a preponderance of type II (fast-twitch) fibres.\textsuperscript{140, 141, 142} Endurance athletes have a substantially lower risk of developing atherosclerotic disease compared with power sport athletes and the general population.\textsuperscript{143, 144} Former endurance athletes and subjects with a high proportion of type I fibres also have a reduced risk of developing hypertension.\textsuperscript{8, 145} Hypertensive subjects show a lower proportion of type I fibres than do normotensive subjects and blood pressure in fact correlates negatively with the proportion of type I fibres in cross-sectional studies.\textsuperscript{146, 147} Furthermore, a high proportion of type I muscle fibres in skeletal muscle has been associated with a favourable cardiovascular risk profile characterised by a low prevalence of obesity, high HDL cholesterol, and low serum insulin.\textsuperscript{148, 149} It is unclear whether these relationships are a consequence of the inclination to physical activity that is caused by a high proportion of type I muscle fibres or whether the effects of muscle fibre-type distribution on cardiovascular risk are mediated by other mechanisms.\textsuperscript{150}
Age-induced stiffening of the large arteries is less pronounced in those who engage in regular endurance exercise.\textsuperscript{151} Arterial compliance can also be reduced by exercise training.\textsuperscript{152, 153, 154} It is unclear whether low-to-moderate exercise has an impact on the arterial elastic properties.\textsuperscript{155, 156} In contrast to aerobic training, resistance training increases arterial stiffness and pulse pressure.\textsuperscript{157}

\textbf{2.1.6.4. Smoking}

Smoking is an established risk factor for cardiovascular disease. An acute stiffening effect of cigarette smoking has been demonstrated in non-smokers as well as in smokers.\textsuperscript{158, 159, 160} Passive smoking has also been associated with acutely increased aortic stiffening.\textsuperscript{161} Consistent with these results, accelerated arterial stiffening has been reported in long-term smoking,\textsuperscript{6, 162} although there are also conflicting results.\textsuperscript{163} The effects of long-term active and passive smoking on arterial stiffening appear to be independent of blood pressure levels.\textsuperscript{164} No intervention study that directly determines whether smoking cessation reduces the stiffness of large elastic arteries has yet been published. Nevertheless, since smoking cessation reduces pulse pressure in hypertensive patients, it is reasonable to expect that smoking cessation would improve large arterial stiffness.\textsuperscript{165}
2.1.6.5. Other factors

The metabolic syndrome is closely related to hypertension and type 2 diabetes. Therefore it is not surprising that the metabolic syndrome is also associated with an increased acceleration of the pulse wave velocity with age. In young individuals, ultrasonically estimated carotid distensibility was associated with the metabolic syndrome. Another feature of the metabolic syndrome, dyslipidaemia has also been associated with higher central pulse pressure and higher AIx.

Aortic and brachial pulse wave velocity, pulse wave reflection, and pulse pressure, relate to the levels of inflammation in healthy individuals, suggesting that inflammation may be involved in arterial stiffening. High-sensitivity C-reactive protein, a marker of systemic inflammation, is independently related to pulse wave velocity, a marker of aortic stiffness, and AIx, a manifestation of wave reflection, in essential hypertension.

Increased arterial stiffness is a typical feature of subjects with renal insufficiency, from mild to moderate reduction of creatinine clearance to end-stage renal failure. In a study conducted in a large cohort of untreated subjects with normal or elevated blood pressure, Mourad et al. found a negative association between creatinine clearance calculated by the Cockcroft–Gault formula and the carotid–femoral pulse wave velocity. In a patients with type 2 diabetes and treated hypertension, with a normal to elevated urinary albumin-to-creatinine ratio, creatinine clearance and carotid–femoral pulse wave velocity correlate inversely, independently of age.
Moderate alcohol consumption has been associated with lower pulse wave velocity even after adjusting for blood pressure and other variables and there seems to be a J-shaped relationship between alcohol intake and pulse wave velocity.\textsuperscript{177, 178}

2.1.7. Treatment of arterial stiffness

2.1.7.1. Pharmacological therapy

2.1.7.1.1. Vasodilator agents

The dynamic component of arterial stiffness can easily be reduced by vasodilating agents that relax smooth muscle cells in muscular arteries and arterioles. Several indirect measures of arterial stiffness are improved by vasodilator therapy, such as pulse wave velocity, pulse wave reflection, and blood pressure.\textsuperscript{179, 180, 181, 182, 183} Vasodilator drugs such as ACE-inhibitors, angiotensin receptor blockers, calcium channel blockers, and nitrates cause an acute reduction in the AIX and pulse wave by actively dilating muscular conduit and resistance arteries and reducing peripheral resistance.\textsuperscript{184, 185, 186} Additionally they have passive stiffness-reducing effects on elastic arteries as a consequence of lower distending pressure. Through their effect on pulse wave reflection, vasodilator drugs lower central systolic and pulse pressure to a larger extent than they reduce brachial systolic and pulse pressures.\textsuperscript{187, 188} This mechanism could explain the observed brachial blood pressure-independent benefits of vasodilator drugs in clinical trials such as the HOPE trial and the LIFE study.\textsuperscript{189, 190}
2.1.7.1.2. Drugs affecting vessel wall structure

Whilst most antihypertensive agents reduce the dynamic vasoconstrictive component of arterial stiffness, newer therapeutic molecules modify the structure of the arterial walls. Presently, several substances that improve arterial stiffness are being studied. The mechanisms of these new potential drugs are inhibition of the formation of AGEs (aminoguanidine, pyridoxamine, and OPB-9195), non-enzymatic cleaving of existing arterial wall AGE cross-links (alagebrium), and blocking of AGE receptors. In clinical trials aminoguanidine has improved arterial stiffness and reduced AER in patients with diabetic nephropathy, but there are unsolved safety issues concerning this drug.191, 192 Pyridoxamine and OPB-9195 remain in preclinical testing. In a randomized, placebo-controlled trial in elderly patients with increased arterial stiffness, administration of an AGE cross-link breaker, (3-phenylacyl-4,5-dimethylthiazolium chloride, or alagebrium) caused a significant reduction in pulse pressure and pulse wave velocity compared with placebo.193 Soluble AGE receptor molecules acting as false AGE ligands also seem to decrease vascular inflammation and arterial stiffness.194 These agents are currently undergoing preclinical testing.

2.1.7.1.3. Other drugs

Statin therapy detectably reduces the stiffness of both the aorta and conduit arteries after several weeks of therapy.195, 196 The effect can in part be attributed to reduction of LDL
cholesterol, but statins also improve arterial stiffness in the absence of dyslipidaemia. This lipid-independent mechanism may be related to the activation of NO synthesis.

Stiffening of the arteries related to insulin resistance and diabetes can be modified by pharmacological ligands of the peroxisome proliferator activated receptors. In patients with type 2 diabetes, pioglitazone treatment reduced aortic pulse wave velocity after three months of treatment.197

2.1.7.2. Lifestyle modification

2.1.7.2.1. Sodium intake

Of all dietary factors, sodium intake probably has the most potent effect on arterial stiffness. Cross-sectional findings indicate that subjects who follow a low-sodium diet have more compliant arteries than age- and blood pressure-matched control subjects with higher sodium intake.198, 199 Moderate dietary sodium restriction improved carotid AIx and ultrasonographically measured arterial compliance in postmenopausal women independently of changes in body weight, mean blood pressure, plasma volume, and heart rate indicating a direct effect of sodium restriction on arterial stiffness.200, 201 Thus, high salt intake accelerates arterial aging, and both short-term and long-term sodium restriction decreases arterial stiffness independently from the effect on mean blood pressure.
2.1.7.2.2. Weight loss

A number of intervention studies have investigated the short-term effects of weight loss on arterial stiffness and have shown that a reduction in body weight is associated with reductions in large artery stiffness. But the effect of weight reduction on arterial stiffness may be merely an epiphenomenon of concomitant decreases in blood pressure. However, a recent intervention study showed that moderate weight loss reduces aortic pulse wave velocity in patients with type 2 diabetes independently of blood pressure.

2.1.7.2.3. Dietary modification

Several dietary supplements seem to have an effect on arterial stiffness independently of their effect on the body weight. Supplementation of n-3 polyunsaturated fatty acids found in fish oil improves systemic arterial compliance in dyslipidaemic subjects, most likely by lowering triglycerides and LDL concentrations. In an intervention study on healthy volunteers, administration of isoflavones that bind to human estrogen receptors reduced pulse wave velocity after six weeks. Three weeks of folic acid supplementation increased systemic arterial compliance in a placebo-controlled study.

The antioxidant vitamin C, ascorbic acid has been reported to reduce pulse wave reflection acutely and after four weeks of oral administration. Similarly, vitamin E intake induced a substantial decrease in systemic arterial stiffness in middle-aged subjects. These results could however be a consequence of reduced peripheral
resistance induced by antioxidative vitamins. Contrary to these results a recent study involving both short-term and long-term administration of ascorbic acid did not show any effect on carotid arterial stiffness.\textsuperscript{215}

2.2. Preeclampsia

Approximately 4\% of pregnancies are complicated by preeclampsia, making the disease accountable for a substantial part of maternal and perinatal mortality. Many of the underlying mechanisms of the pathophysiology of preeclampsia are still unclear, but one hypothesis suggests that insufficient invasion of the uterine spiral arteries by placental cytотrophoblasts causes placental ischemia that leads to the release of yet unknown vasoactive factors. These substances damage the maternal endothelium causing impairment of endothelial function and the clinical symptoms hypertension and proteinuria.\textsuperscript{216, 217}

Several biochemical markers of endothelial dysfunction have been shown to be elevated in preeclampsia\textsuperscript{218} and in vitro tests performed on arteries from preeclamptic women have revealed increased vessel wall thickness and impaired endothelial NO synthesis.\textsuperscript{219} However, due to the practical and ethical difficulties of performing invasive testing of endothelial function in pregnant women, endothelial dysfunction has had to be studied using indirect methods.

Impaired endothelial function has also been observed in non-pregnant women with a history of preeclampsia.\textsuperscript{220} This implies that the endothelial dysfunction of the affected
women is not confined to pregnancy and that subclinical alterations in vascular function may still exist several years after a preeclamptic pregnancy. Since endothelial dysfunction is a central element in the pathogenesis of atherosclerosis, it is not surprising that women with a history of preeclampsia have an increased risk of coronary heart disease.\textsuperscript{10, 221, 222}

2.3. Type 1 diabetes

Type 1 diabetes mellitus is caused by autoimmune destruction of the insulin-producing pancreatic beta-cells. This process gradually results in a total loss of insulin secretion. The disease generally manifests at an early age and is characterized by hyperglycaemia, ketoacidosis, polyuria, weight loss, and dehydration. Until the discovery of insulin and development of insulin therapy by Banting and Best in the 1920’s the disease was invariably lethal. Type 1 diabetes accounts for about 10\% of all patients with diabetes. Finland has the highest incidence of type 1 diabetes mellitus in the world. It is approximated that more than 30000 patients with type 1 diabetes currently reside in Finland.\textsuperscript{223}

Patients with type 1 diabetes present an excess of atherosclerosis resulting in increased cardiovascular morbidity and mortality. The risk is particularly elevated in patients with diabetic nephropathy, but is also higher in patients without diabetic kidney disease.\textsuperscript{12} It has been established that patients with type 1 diabetes have stiffer arteries than age-matched non-diabetic control subjects, and that the process of arterial stiffening is
initiated before any signs of micro- or macrovascular disease can be detected. Furthermore, the arterial stiffening process seems to be accelerated in type 1 diabetes and the arterial stiffness consequently correlates with the duration of diabetes independently of age.

2.4. Type 2 diabetes

Type 2 diabetes is characterized by a combination of insulin resistance and relatively insufficient insulin secretion. The diagnosis is usually made in adult age. The majority patients with type 2 diabetes exhibit the metabolic syndrome, which in addition to insulin resistance includes abdominal obesity, hypertension, and dyslipidaemia. It seems that interaction between complex genetic and environmental factors like obesity and physical inactivity play a central role in the intricate pathogenesis of the disease.

Patients with type 2 diabetes have a 2-4 fold increased risk of dying from cardiovascular disease. The hypertension that frequently accompanies type 2 diabetes further increases the cardiovascular risk. Several large studies have established the beneficial effect of effective blood pressure lowering therapy on cardiovascular mortality in patients with type 2 diabetes.

Premature arterial stiffening is typical for type 2 diabetes and increased pulse pressure has been found to predict cardiovascular mortality and progression of renal failure in type 2 diabetes. In 2005 Cockcroft et al showed that pulse pressure was superior to
systolic or diastolic blood pressure in predicting coronary heart disease in patients with type 2 diabetes.\textsuperscript{242}

Type 2 diabetes is associated with a greater age-related stiffening of the aorta in women compared with men.\textsuperscript{243} Impaired glucose tolerance and impaired insulin sensitivity have been associated with increased arterial stiffness measured by common carotid arterial distensibility.\textsuperscript{244, 245, 246} Furthermore, it has been demonstrated that insulin resistance is independently associated with pulse wave velocity in non-diabetic subjects.\textsuperscript{247, 248, 249}

\section*{2.5. Milk-derived biologically active peptides}

The milk-derived tripeptides isoleucine-prolyl-prolyl and valine-prolyl-prolyl (Evolus®) have been shown to dose-dependently lower blood pressure after oral administration in spontaneously hypertensive rats.\textsuperscript{250} Continuous feeding of these peptides to spontaneously hypertensive rats has also attenuated the development of hypertension.\textsuperscript{251, 252}

The mechanisms behind the antihypertensive effects are still unknown, but it seems that it is partially based on inhibition of the renin-angiotensin-aldosterone system.\textsuperscript{253, 254} Additionally, in vitro studies show improved endothelial release of NO in spontaneously hypertensive rats after administration of isoleucine-proline-proline and valine-proline-proline.\textsuperscript{11, 255}
Some studies on humans have demonstrated a blood pressure lowering effect of the peptides in mildly hypertensive subjects. In these studies biologically active peptides were generated during milk fermentation by enzymes produced by *Lactobacillus helveticus*. 
3. Aims of the present study

The present studies that focused on arterial stiffness were performed in order to answer the following questions:

I. Are physical activity and muscle fibre-type distribution determinants of endothelial function and arterial stiffness?

II. Is systemic arterial stiffness measured by pulse wave reflection increased in pregnant preeclamptic and previously preeclamptic normotensive non-pregnant women?

III. Can an altered age-related blood pressure pattern, suggestive of accelerated arterial aging be detected in patients with type 1 diabetes?

IV. Is pulse pressure superior to systolic and diastolic blood pressure in predicting all-cause and cardiovascular mortality in elderly patients with type 2 diabetes?

V. Can arterial stiffness and endothelial function be improved by intake of bioactive milk-derived peptides?
4. Subjects and study design

4.1. Ethical aspects

All studies were approved by the ethics committee of the Hospital District of Helsinki and Uusimaa. All subjects gave informed consent prior to participation.

4.2. Healthy men (I)

Fifty-four apparently healthy men who underwent a muscle biopsy for determination of muscle fibre distribution in 1984 were re-studied in 2003. Aortic pulse wave velocity and pulse wave reflection were assessed by applanation tonometry. Endothelial function was evaluated by examining the effects of salbutamol and nitroglycerin on pulse wave reflection. Physical activity was assessed using the Kuopio Ischemic Heart Disease Study 12-month physical activity questionnaire, which was self-administered by the subjects with a subsequent interview.

4.3. Women with current or previous preeclampsia (II)

In this cross-sectional case-control study pulse wave reflection was assessed by applanation tonometry at the radial artery. Twenty-six currently pregnant women with preeclampsia, 26 currently pregnant control subjects, 22 normotensive non-pregnant previously preeclamptic women, and 22 non-pregnant control subjects were studied.
4.4. Patients with type 1 diabetes (III)

This study was part of the Finnish Diabetic Nephropathy Study (FinnDiane), a multi-centre, nationwide study of diabetic late complications. A total of 3025 patients with a diagnosis of type 1 diabetes with an age at onset < 36 years and insulin therapy initiated within one year had, in a consecutive manner, been recruited at 59 hospitals and health care centres by October 31, 2002. All patients underwent a thorough physical examination during 1998-2002. Their medical history regarding diabetes, diabetic complications, cardiovascular disease, and data on the latest HbA1c measurement were acquired from medical records. The analyses were limited to the 2988 patients aged 18-64 years.

4.5. Control subjects (III)

The control group was obtained from a national population-based health survey, Health 2000. This survey consists of a randomly drawn nationally representative sample of persons aged 30 years or over, who attended a comprehensive health examination in the local health centre or comparable premises during the time period 2000-2001.257 All 5486 subjects younger than 75 years without self-reported diabetes were included in the analyses.
4.6. Patients with type 2 diabetes (IV)

The Botnia Study was initiated in 1990 with the aim to identify risk factors for type 2 diabetes. Patients with type 2 diabetes from five primary health care centres in Finland were invited to participate together with their family members. Diagnosis of diabetes was based upon existing WHO criteria. The current study represents 1294 consecutive patients with type 2 diabetes that were examined between 1990 and 1997. Subjects with a diagnosis of type 2 diabetes (n=1173) and previously non-diagnosed family members whose results from an oral glucose tolerance test (OGTT) met the most recent WHO diabetes criteria (n=121) were included. Patients with glutamic acid decarboxylase antibodies or maturity-onset diabetes of the young were excluded.

Data on the subjects’ vital status was obtained from the national population registry on May 31st 2004. In order to classify the cause of mortality, death certificates of the deceased subjects were obtained from the central death-certificate registry. Additionally, medical records were acquired if the cause of death was unclear. Cardiovascular mortality was classified using the 9th revision of the International Classification of Disease (codes 399-459) before 1997 and the 10th revision (codes I 10-99) thereafter.

4.7. Subjects with mild hypertension (V)

Eighty-nine subjects participated in this double blind randomized placebo-controlled study. Subjects with systolic blood pressure in office blood pressure measurement between 140 and 155 mm Hg and diastolic blood pressure between 85 and 99 mm Hg
were included. Exclusion criteria were smoking, blood pressure-lowering medication, secondary hypertension, unstable coronary artery disease, diabetes mellitus, malignant diseases, alcohol abuse, milk allergy, and pregnancy. A total of 396 research subjects responded to recruitment advertisements in newspapers in the Helsinki area. Of 216 subjects that entered the run-in period 122 did not fulfil the inclusion criteria and were excluded. One subject from the intervention group and four subjects from the control group withdrew from the study during the intervention periods. The final analysis included 89 subjects.

After a 4-week run-in period the subjects were randomly allocated to an active group that received bioactive tripeptides or to a control group that received a similar placebo product without bioactive tripeptides. During the first 12 weeks of intervention the subjects were given a two daily 100 ml doses of a fermented milk product containing bioactive tripeptides in a low concentration (Isoleucine-Prolyl-Prolyl 1.2 mg/100 g and Valine-Prolyl-Prolyl 1.3 mg/100 g) or placebo. For the following 12 weeks the active treatment group received two daily 200 ml doses of a similar product containing a high concentration of bioactive tripeptides Isoleucine-Prolyl-Prolyl 5.8 mg/100 g and Valine-Prolyl-Prolyl-Prolyl 6.6 mg/100 g). Subjects were asked to fill out a report form concerning their daily use of the test products and any adverse events. Pulse wave analysis and endothelial function testing was performed at the beginning and at the end of each intervention period. The entire study was performed in a double-blinded fashion.
5. Methods

5.1. Definitions

5.1.1. Hypertension

Essential hypertension was defined as a systolic blood pressure \( \geq 140 \) mmHg or a diastolic blood pressure \( \geq 90 \) mmHg or antihypertensive medication in control subjects and in diabetic patients with a normal albumin excretion rate (III). Isolated systolic hypertension was defined as a systolic blood pressure \( \geq 140 \) mmHg and a diastolic blood pressure <90 mmHg, irrespective of antihypertensive medication.

Subjects with a systolic blood pressure in the office blood pressure measurements between 140 and 155 mm Hg and a diastolic blood pressure between 85 and 99 mm Hg were classified as having mild hypertension (V).

5.1.2. Preeclampsia

Preeclampsia was defined as the onset of proteinuria (\( \geq 300 \) mg/24h, or 2+ with dipstick) and elevated blood pressure (\( \geq 140/90 \) mmHg or an increase \( \geq 30/15 \) mmHg from the first trimester of pregnancy) during the last trimester of pregnancy (II).
5.1.3. Diabetes

Type 1 diabetes was defined as a diagnosis of type 1 diabetes with an age at onset below 36 years, insulin therapy initiated within one year, and C-peptide negativity (III). In previously diagnosed patients in study IV, type 2 diabetes was defined according to existing WHO criteria at the time of diagnosis.259 In subjects with previously undiagnosed diabetes, a diagnosis of diabetes was based upon the results in an oral glucose tolerance test according to the most recent WHO criteria.260

5.2. Blood pressure measurement

In Study III and IV blood pressure was measured by trained nurses using mercury sphygmomanometers. Systolic blood pressure was recorded at phase I and diastolic blood pressure at phase V of Korotkoff sounds. Two blood pressure recordings were obtained from the right arm of a sitting patient after at least 5 minutes of rest. The mean of the two recordings was used in the studies. In Study II, blood pressure was measured with an aneroid sphygmomanometer using a similar protocol. In Study I and V, blood pressure was recorded in the dominant arm using a validated oscillometric technique (Omron M4, Omron Matsusaka Co., Ltd., Kyoto, Japan). Recordings were taken in a supine position after at least 5 minutes of rest.
5.3. Pulse wave velocity (I)

Carotid-femoral (aortic) pulse wave velocity was measured using the SphygmoCor (AtCor Medical Pty. Ltd., Sydney, Australia) device by sequentially recording ECG-gated carotid and femoral artery waveforms with a high-fidelity micromanometer (SPC-301; Millar Instruments, Texas, U.S.A.) for 30 seconds. The timing of these waveforms was compared with that of the R wave on the simultaneously recorded ECG. Pulse wave velocity was determined by calculation of the difference in carotid to femoral path length divided by the difference in R wave to waveform foot times. The difference in carotid to femoral path length was estimated from the distance from the sternal notch to the femoral and carotid pulse respectively measured in a direct line.

5.4. Pulse wave analysis (I, II, and V)

After at least 5 minutes of rest, brachial blood pressure was recorded from the dominant arm using an aneroid sphygmomanometer or a validated oscillometric manometer (M4-I, Omron Corporation, Japan). Pulse wave reflection was assessed with the SphygmoCor pulse wave analysis System (AtCor Medical Pty. Ltd., Sydney, Australia). Radial artery waveforms were recorded non-invasively at the wrist of the dominant arm with an applanation tonometer (SPT-301B, Millar Instruments, Houston, Texas, U.S.A.). The waveforms were collected into a personal computer and the SphygmoCor software was used to generate the corresponding aortic waveforms using a validated transfer function. The aortic AIx was calculated as the augmentation of the aortic systolic
pressure by the reflected pulse wave expressed as a percentage of the aortic pulse pressure. Because AIx is influenced by heart rate, it was adjusted to a heart rate of 75 bpm by the software. Central and mean arterial blood pressure was calculated from the digitized brachial blood pressure curve. The time to return of the reflected wave (Tr) was calculated as the time from the beginning of the derived aortic systolic pressure waveform to the inflection point. Tr can be used as a substitute for pulse wave velocity (a higher pulse wave velocity will result in a shorter Tr). All measurements were subjected to quality control by the software and only high-quality recordings, defined as a quality index ≥80% were included in the analyses.

5.5. Endothelial function testing (I and V)

Vascular endothelial function was studied using the pulse wave analysis method developed by Wilkinson et al. This method examines the effects of the β2-adrenergic receptor agonist salbutamol and nitroglycerin on the AIx. Salbutamol activates the L-arginine pathway in endothelial cells and causes endothelial release of NO. Nitroglycerin is a donor of NO when it is degraded in the blood stream. NO causes vasodilation by relaxing vascular smooth muscle cells. The reduction of AIx induced by inhalation of salbutamol thus reflects the endothelial NO production capacity, while the reaction to nitroglycerin is a measure of vasodilatory capacity in response to supraphysiologic NO stimulus.
After the baseline recordings of the AIX, a 500 μg tablet of nitroglycerin (Nitro, Orion, Finland) was administered sublingually. AIX was measured after 3, 5, 10, 15, and 20 minutes. Thirty to sixty minutes later two 200 μg inhalations of salbutamol (Ventoline Evohaler, GlaxoSmithKline) were given with a spacer device (Volumatic). AIX was measured 5, 10, 15, and 20 minutes later. The response to nitroglycerin and salbutamol was defined as the maximum change from baseline after drug administration. An endothelial function index (EFI) was calculated as the absolute change induced by salbutamol divided by the absolute change induced by nitroglycerin expressed as a percentage.

5.6. Assessment of physical activity (I)

In Study I, leisure-time physical activity (LTPA) was assessed by the Kuopio Ischemic Heart Disease Study 12-month physical activity questionnaire, which was self-administered by the subjects with a subsequent interview to ensure completeness. The questionnaire included a list of activities for which the subjects reported the mean intensity and duration and the frequency for each month. Specific MET values of different intensities of each type of activity have been assessed. The volume of exercise activities was expressed in metabolic equivalents multiplied by hours per week (MET/h/wk), a relative measure of energy expenditure, where 1 MET corresponds to energy expenditure at rest. The reliability and validity of the questionnaire has been confirmed by several studies.
5.7. Verification of mortality data (IV)

Data on the subjects’ vital status was obtained from the national population registry. In order to classify the cause of mortality, death certificates of the deceased subjects were obtained from the central death-certificate registry. Additionally, medical records were acquired if the cause of death was unclear. Cardiovascular mortality was classified using the 9th revision of the International Classification of Disease (codes 399-459) before 1997 and the 10th revision (codes I 10-99) thereafter.

5.8. Assays

Biochemical analyses of blood samples were included in Study I, II, IV, and V. Total cholesterol, HDL cholesterol and triglycerides were measured enzymatically, and LDL was calculated according to the Friedewald formula (Study I, IV, and V). Urine albumin concentration was measured by an immunoturbidometric method (study III and IV). HbA1c measurements were acquired from medical records (Study III and IV). Fasting plasma glucose was measured by a hexokinase method, (Study IV). Glutamic acid decarboxylase antibodies were determined by a modified radiobinding assay (Study IV). C-reactive protein was determined using an immunoturbidometric method (Study V).
5.9. Statistical methods

In general, all analyses were performed with SPSS version 11.0 or 12.0.1 (SPSS Inc, Chicago, IL, U.S.A.). Results are expressed as the mean ± SEM or ± SD for normally distributed variables, and as the median (range or interquartile range) for non-normally distributed variables. P-values <0.05 were considered statistically significant.

Normally distributed variables were analyzed with ANOVA or Student’s t-test and non-normally distributed variables with Mann-Whitney U-test. Simple linear regression (Pearson) was used to examine univariate correlations. More complex correlations were analyzed by means of multiple regression analysis using stepwise backward elimination.

In study III, the difference in blood pressure levels between diabetes patients and control subjects was analyzed in matching age groups. When analyzing males and females together, a statistical model giving both sexes equal weight in each age group was used.

In study IV, relevant clinical variables were entered into a forward stepwise Cox regression model in order to determine mortality risk factors. AER was not included in the model due to missing data on a large number of patients. Variables that contributed significantly were added to the model in order of significance, except gender, which was forced into the model. Hazard ratios were calculated by merging the results of separate analyses for medicated and non-medicated patients in order to reduce confounding effects caused by antihypertensive medication.

The analyses in Study V were performed according to the intention to treat principle. Statistical comparison of outcome measurements was performed using paired t-test. The
changes in measurements between groups were analyzed using analysis of covariance with the baseline value as the covariable.
6. Results

6.1. Study I

Table 2 describes the characteristics of the studied subjects in Study I. Administration of nitroglycerin reduced the A1x by 24±5 percentage units and administration of salbutamol reduced the A1x by 17±6 percentage units. Figure 1 illustrates the relationships between type I% and the measures of arterial stiffness and endothelial function.

We performed univariate linear regression analyses, in which aortic pulse wave velocity correlated positively with BMI (R=0.43, P=0.001), alcohol consumption (R=0.33, P=0.018), and diastolic blood pressure (R=0.42, P=0.002). In corresponding analyses A1x correlated positively with plasma triglycerides (R=0.31, P=0.022), smoking status (R=0.40, P=0.002, never smoker = 1, previous smoker = 2, current smoker = 3), and mean arterial pressure (R=0.35, P=0.009), and inversely with type I% (R=0.29, P=0.033).

The endothelial function index correlated inversely with total plasma cholesterol (R=0.36, P=0.008), LDL cholesterol (R=0.37, P=0.005), and leisure-time physical activity (R=0.33, P=0.015).
Table 2. Characteristics of the subjects (Study I). N=54.

<table>
<thead>
<tr>
<th>Variable</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>58</td>
<td>(52-78)</td>
</tr>
<tr>
<td>Type I muscle fibers, %</td>
<td>58 ± 15</td>
<td></td>
</tr>
<tr>
<td>Exercise, MET-hours/week</td>
<td>31</td>
<td>(0-100)</td>
</tr>
<tr>
<td>Exercise in 1984, MET-hours/week</td>
<td>30</td>
<td>(0-106)</td>
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<td>Smoking, current/previous/never</td>
<td>8/23/23</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption, g/day</td>
<td>14</td>
<td>(0-83)</td>
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<tr>
<td>AHT, yes/no</td>
<td>18/36</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>140 ± 16</td>
<td></td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>85</td>
<td>(67-98)</td>
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<tr>
<td>Mean arterial blood pressure, mmHg</td>
<td>106</td>
<td>(84-124)</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>55</td>
<td>(40-100)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.5 ± 0.9</td>
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</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 ± 4.1</td>
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</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.5 ± 0.4</td>
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<tr>
<td>Triglycerides, mmol/l</td>
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<td>(0.7-5.5)</td>
</tr>
<tr>
<td>Aortic PWV, m/s</td>
<td>7.9</td>
<td>(5.4-13.7)</td>
</tr>
<tr>
<td>AIX, %</td>
<td>21 ± 6</td>
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<tr>
<td>AIX reduction by nitroglycerin, %-units</td>
<td>24 ± 5</td>
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</tr>
<tr>
<td>AIX reduction by salbutamol, %-units</td>
<td>17 ± 6</td>
<td></td>
</tr>
<tr>
<td>Endothelial function index, %</td>
<td>73 ± 25</td>
<td></td>
</tr>
</tbody>
</table>

Normally distributed data is presented as mean±SD, non-normally distributed data as median (range). AHT indicates antihypertensive therapy; PWV, pulse wave velocity; AIX, augmentation index.
Figure 1. (Study I) Aortic pulse wave velocity (PWV), basal augmentation index (AIx), and endothelial function index (EFI) in subjects with a type I% below (diagonal stripes) and above (black) the median value of 58.5%. Columns represent median (PWV) and mean (AIx, EFI) values. Error bars indicate SD for normally distributed variables.

Stepwise backward multiple regression analyses with aortic pulse wave velocity, AIx, and endothelial function index as dependent variables were performed. Age, BMI, leisure-time physical activity, type I%, smoking, alcohol consumption, diastolic blood pressure, LDL cholesterol, HDL cholesterol, and triglycerides were entered into the models. Table 3 shows the final models. Pulse wave velocity was positively associated with age, BMI, and systolic blood pressure. A higher AIx was associated with smoking, high LDL cholesterol, and high diastolic blood pressure. Inverse correlations were
Table 3. Multiple regression analyses with measurements of arterial stiffness and endothelial function as dependent variables (Study II)

<table>
<thead>
<tr>
<th>Variable</th>
<th>R²</th>
<th>β</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aortic PWV, m/s</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>0.26</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>0.37</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption, g/day</td>
<td>0.21</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>0.33</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td><strong>AIx, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>-0.19</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Smoking*</td>
<td>0.52</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>0.29</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>0.31</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td><strong>EFI, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>-0.25</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>LTPA, MET/week</td>
<td>-0.36</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption, g/day</td>
<td>-0.17</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>-0.30</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>-0.27</td>
<td>0.027</td>
<td></td>
</tr>
</tbody>
</table>

Final models after backward elimination. Age, BMI, leisure-time physical activity (LTPA), type I%, smoking, alcohol consumption, LDL cholesterol, HDL cholesterol, triglycerides, and blood pressure were entered as determinants of aortic pulse wave velocity (PWV), augmentation index (AIx), and endothelial function index (EFI). *Never smoked = 1, previous smoker = 2, current smoker = 3.
observed between endothelial function index and age, LDL cholesterol, triglycerides and leisure-time physical activity.

Pulse wave velocity, A1x, or endothelial function index did not differ between subjects with and without antihypertensive medication.

6.2. Study II

The clinical characteristics of the studied subjects in study II are presented in Table 4. Women with preeclampsia had a higher body weight compared with pregnant control subjects. The weight prior to 10 weeks of gestation did not differ between the two currently pregnant groups. With regard to current age, age at delivery, and height, the non-pregnant groups were similar. A higher body weight was observed in previously preeclamptic women in comparison with the non-pregnant control subjects.

Hemodynamic characteristics of the subjects studied in Study II are presented in Table 5. With the exception of brachial pulse pressure, all blood pressure indices were significantly higher in subjects with preeclampsia, whereas heart rate was significantly lower in comparison with the pregnant control subjects (76 ± 1 vs. 84 ± 2 bpm, P < 0.001). Compared with the pregnant control subjects, A1x was higher in the currently preeclamptic group (23 ± 2 vs. 4 ± 1%, P < 0.001). When the A1x was adjusted to a heart rate of 75 bpm, the difference diminished slightly, but remained significant (23 ± 1% vs. 8 ± 1%, P < 0.001). No significant differences in any of the hemodynamic characteristics
Table 4. Clinical characteristics of subjects (Study II)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Current Preeclampsia (n = 26)</th>
<th>Pregnant Control (n = 26)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32 ± 1</td>
<td>31 ± 1</td>
<td>0.21</td>
</tr>
<tr>
<td>Gestational age at examination (days)</td>
<td>252 ± 4</td>
<td>256 ± 1</td>
<td>0.48</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168 ± 1</td>
<td>166 ± 1</td>
<td>0.16</td>
</tr>
<tr>
<td>Present weight (kg)</td>
<td>80 (68-119)</td>
<td>72 (60-105)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Weight &lt; 10 weeks of pregnancy (kg)</td>
<td>63 (50-95)</td>
<td>60 (50-85)</td>
<td>0.13</td>
</tr>
<tr>
<td>Maximal proteinuria in pregnancy (g/24h)</td>
<td>4.2 (0.3-9.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gestational age at delivery (days)</td>
<td>263 (221-290)</td>
<td>276 (216-297)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Previous Preeclampsia (n = 22)</th>
<th>Non-pregnant Control (n = 22)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36 ± 1</td>
<td>37 ± 1</td>
<td>0.93</td>
</tr>
<tr>
<td>Age at delivery (years)</td>
<td>31 ± 1</td>
<td>30 ± 1</td>
<td>0.28</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165 ± 1</td>
<td>164 ± 1</td>
<td>0.69</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72 (45-103)</td>
<td>64 (47-96)</td>
<td>0.03</td>
</tr>
<tr>
<td>Maximal proteinuria in pregnancy (g/24h)</td>
<td>8.5 (0.8-33)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gestational age at delivery (days)</td>
<td>230 ± 5</td>
<td>282 ± 2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Normally distributed variables are presented as mean ± SEM. Non-normally distributed variables are presented as median (range).

Table 5. Hemodynamic characteristics (Study II)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Current Preeclampsia (n = 26)</th>
<th>Pregnant Control (n = 26)</th>
<th>Previous Preeclampsia (n = 22)</th>
<th>Non-pregnant Control (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial SBP (mmHg)</td>
<td>145 ± 3*</td>
<td>119 ± 2</td>
<td>125 (108 - 162)</td>
<td>120 (104 - 168)</td>
</tr>
<tr>
<td>Brachial DBP (mmHg)</td>
<td>91 ± 2*</td>
<td>69 ± 2</td>
<td>77 ± 2</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>Brachial PP (mmHg)</td>
<td>54 (38 - 80)</td>
<td>50 (36 - 69)</td>
<td>54 ± 2</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>Brachial MAP (mmHg)</td>
<td>111 ± 2*</td>
<td>85 ± 2</td>
<td>97 ± 3</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>Aortic SBP (mmHg)</td>
<td>133 ± 2*</td>
<td>101 ± 2</td>
<td>114 (92 - 148)</td>
<td>105 (90 - 158)</td>
</tr>
<tr>
<td>Aortic DBP (mmHg)</td>
<td>92 ± 2*</td>
<td>71 ± 2</td>
<td>78 ± 3</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>Aortic PP (mmHg)</td>
<td>39 (28 - 65)*</td>
<td>29 (23 - 45)</td>
<td>39 (26 - 55)</td>
<td>36 (29 - 88)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>76 ± 1*</td>
<td>84 ± 2</td>
<td>66 ± 2</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>AIx (%)</td>
<td>23 ± 2*</td>
<td>4 ± 1</td>
<td>8 ± 2</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Normally distributed variables are presented as mean ± SEM. Non-normally distributed variables are presented as median (range). * P < 0.001 vs. corresponding control group. No P-values occurred in the range 0.001-0.05. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; bpm, beats per minute; AIx, augmentation index.
could be observed between the previously preeclamptic group and the non-pregnant control subjects.

In currently pregnant subjects, the non-adjusted AIx correlated positively with mean arterial blood pressure \( (r = 0.71, P < 0.001) \) and inversely with heart rate \( (r = -0.62, P < 0.001) \). We did not observe any correlation with age, gestational age, height, or early pregnancy weight. In the preeclamptic group the amount of proteinuria did not correlate with the AIx. A multiple linear regression analysis with the AIx as the dependent variable was performed. Current preeclampsia was independently associated with increased AIx in a model that also included heart rate and mean arterial blood pressure (Table 6).

A corresponding multiple regression model including non-pregnant subjects was performed. Mean arterial pressure, heart rate, and age contributed to the AIx independently of each other (Table 6). A history of preeclampsia was not associated with pulse wave reflection in these subjects.

### 6.3. Study III

Patients with type 1 diabetes were on average younger, had a lower body-mass index (BMI), and had more cardiovascular disease than control subjects (Table 7). Smoking was equally common in both groups, whereas the use of antihypertensive medication was more frequent in the diabetic group.

Patients with diabetes had a higher systolic blood pressure than control subjects in most age categories (Figure 2-4). In comparison with control subjects, diabetic males under 35
Variables with significant bivariate correlation with the dependent variable were entered into the models. B indicates the non-standardized regression coefficient; MAP, mean arterial pressure.

years and diabetic females under 40 years had a higher diastolic blood pressure, while the diastolic pressure was lower in males and females with diabetes in the age groups over 45 years (Figure 2-4). In diabetic patients, the highest diastolic pressure was observed in the age group 40-44 years (82±1 mmHg). In the control group, the highest diastolic blood pressure was observed in the age group 55-59 years (85±0 mmHg) (Figure 2-4).

Diabetic patients showed a higher pulse pressure was in all age categories (Figure 2-4). Comparable pulse pressure readings were observed at 15-20 years younger age in diabetic than in control subjects. Pulse pressure in diabetic patients stratified by AER is demonstrated in Figure 5. Patients with macroalbuminuria had the highest pulse pressure, followed by microalbuminuric and normoalbuminuric diabetes patients. Furthermore, it can be noted that control subjects had significantly lower pulse pressure than normoalbuminuric diabetes patients in all age categories. In a stratification of the diabetic patients by age at onset of diabetes, pulse pressure differed between diabetic subgroups in

<table>
<thead>
<tr>
<th>Pregnant Subjects</th>
<th>B</th>
<th>SE of B</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preeclampsia</td>
<td>14.3</td>
<td>2.92</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Brachial MAP (mmHg)</td>
<td>0.05</td>
<td>0.09</td>
<td>0.59</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>-0.39</td>
<td>0.09</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$R^2 = 0.76$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-pregnant Subjects</th>
<th>B</th>
<th>SE of B</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous preeclampsia</td>
<td>-0.65</td>
<td>1.77</td>
<td>0.72</td>
</tr>
<tr>
<td>Brachial MAP (mmHg)</td>
<td>0.37</td>
<td>0.06</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>-0.26</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.76</td>
<td>0.20</td>
<td>0.001</td>
</tr>
<tr>
<td>$R^2 = 0.57$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7. Clinical characteristics (Study III)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetic subjects</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>2988</td>
<td>5486</td>
<td>-</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>2164</td>
<td>4755</td>
<td>-</td>
</tr>
<tr>
<td>Males, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>51.7</td>
<td>46.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>52.0</td>
<td>47.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body-mass index, kg/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>25.1±0.1</td>
<td>26.8±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>25.3±0.1</td>
<td>26.6±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smokers, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>24.7</td>
<td>23.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>23.7</td>
<td>25.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Antihypertensive medication, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>40.1</td>
<td>14.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>49.6</td>
<td>11.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cardiovascular disease, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>7.2</td>
<td>6.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>9.7</td>
<td>3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of diabetes, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>22.4±0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>26.2±0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>8.5±0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age 30-64 years</td>
<td>8.4±0.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ±SEM.
Figure 2. (Study III) Age-specific mean blood pressure indices in diabetic and control males. Filled symbols indicate diabetic males; hollow symbols, control males; squares, systolic blood pressure; circles, diastolic blood pressure; triangles, pulse pressure. **P < 0.001 for systolic blood pressure, †P < 0.001 for diastolic blood pressure. P < 0.001 for pulse pressure between all corresponding age categories.
Figure 3. (Study III) Age-specific mean blood pressure indices in diabetic and in control females. Filled symbols indicate diabetic females; hollow symbols, control females; squares, systolic blood pressure; circles, diastolic blood pressure; triangles, pulse pressure. *P < 0.05 for systolic blood pressure. **P < 0.01 for systolic blood pressure. †P < 0.05 for diastolic blood pressure. ‡P < 0.001 for diastolic blood pressure. †† < 0.001 for pulse pressure between all corresponding age categories.
Figure 4. (Study III) Age-specific mean blood pressure indices in diabetic and in control males and females. Filled symbols indicate diabetic subjects; hollow symbols, control subjects; squares, systolic blood pressure; circles, diastolic blood pressure; triangles, pulse pressure. *P < 0.05 for systolic blood pressure. **P < 0.001 for systolic blood pressure. †P < 0.001 for diastolic blood pressure. ‡P < 0.001 for pulse pressure between all corresponding age categories.
Figure 5. (Study III) Age-specific mean pulse pressure stratified by the AER. Non-classifiable and ESRF diabetic subjects are excluded. *P < 0.05 between diabetic subgroups. **P < 0.01 for diabetic subjects with a normal AER vs. controls.
all age groups between 25 and 59 years (Figure 6). No significant pulse pressure
differences were observed between age- and sex-specific tertiles of HbA1c within the
diabetic group. We noted that there were no differences in pulse pressure between
smokers and non-smokers.

Clinically significant were entered into a backward multiple linear regression analysis
with pulse pressure as the dependent variable. In the final model, male gender, age,
duration of diabetes, and AER, were independently associated with pulse pressure in
patients with diabetes (Table 8).

Essential hypertension was significantly but marginally more frequent in diabetic patients
with a normal AER compared to control subjects (Figure 7). Isolated systolic
hypertension was approximately three times more common in normoalbuminuric diabetic
subjects of all ages (Figure 8).

<table>
<thead>
<tr>
<th>Table 8. Multiple linear regression analysis of diabetic subjects with pulse pressure as dependent variable (Study III)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B</strong></td>
</tr>
<tr>
<td>Male sex</td>
</tr>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Duration of diabetes, years</td>
</tr>
<tr>
<td>AER, mg/24h*</td>
</tr>
</tbody>
</table>

R² = 0.274. Coefficient values are ±SEM. *After logarithmic transformation.
**Figure 7.** (Study III) Prevalence of essential hypertension in normoalbuminuric diabetic subjects (black) and controls (gray). *P < 0.05 between groups. **P < 0.01 between groups.

**Figure 8.** (Study III) Prevalence of isolated systolic hypertension in normoalbuminuric diabetic subjects (black) and controls (gray). **P < 0.001 between groups.
6.4. Study IV

The characteristics of the type 2 diabetic cohort at baseline are described in Table 9-11. During a median follow-up time of 9.5 years (range 6.5-14.4 years) 192 (35% of deaths) patients died of coronary heart disease, 68 (12%) of cerebrovascular disease, 72 (13%) of other cardiovascular disease, 102 (18%) of neoplasms, 100 (18%) of other disease, and 12 (2%) of accidents or suicide. Six (1%) of the total 552 deaths could not be classified. Patients, who were still alive at follow-up were younger, had a shorter duration of diabetes, had lower pulse pressure and lower AER. Furthermore, survivors had higher diastolic blood pressure and body-mass index than the patients that died. Additionally, patients who died of cardiovascular disease had higher systolic blood pressure, fasting plasma glucose, and triglycerides, but lower HDL cholesterol compared with the survivors.

The Cox regression analyses showed that the unadjusted relationship with all-cause and cardiovascular mortality was negative for diastolic blood pressure, but positive for pulse pressure (Table 12 and 13). After adjusting for other risk factors, systolic blood pressure and diastolic blood pressure correlated negatively with both all-cause and cardiovascular mortality. No association between pulse pressure and mortality was observed in the adjusted analyses.

A corresponding Cox regression analysis including only patients with available AER measurements was performed (N = 666). When AER was added to the model, the hazard ratio for all blood pressure indices declined by 0.01-0.03.
Table 9. Baseline anamnestic characteristics according to outcome (Study IV)

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Entire group</th>
<th>Survivors</th>
<th>Cardiovascular death</th>
<th>Other death</th>
</tr>
</thead>
<tbody>
<tr>
<td>N =</td>
<td>1294</td>
<td>742</td>
<td>332</td>
<td>220</td>
</tr>
<tr>
<td>Sex, % males</td>
<td>46</td>
<td>45</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Age, years</td>
<td>69.1 (61.1-75.7)</td>
<td>64.0 (56.7-71.4)</td>
<td>75.7 (70.5-80.9)*</td>
<td>74.8 (69.4-80.2)*</td>
</tr>
<tr>
<td>Duration of diabetes, years</td>
<td>6.3 (2.4-11.6)</td>
<td>4.6 (1.4-9.8)</td>
<td>8.2 (4.4-13.9)*</td>
<td>7.7 (3.1-13.2)*</td>
</tr>
<tr>
<td>Diabetes treatment, diet/oral agents/insulin/combination %</td>
<td>39/28/11/22</td>
<td>41/31/11/18</td>
<td>38/25/12/27†</td>
<td>37/24/10/29†</td>
</tr>
<tr>
<td>Antihypertensive medication, %</td>
<td>51</td>
<td>46</td>
<td>62*</td>
<td>52</td>
</tr>
<tr>
<td>Stroke, %</td>
<td>8</td>
<td>4</td>
<td>16*</td>
<td>7†</td>
</tr>
<tr>
<td>Coronary heart disease, %</td>
<td>29</td>
<td>21</td>
<td>48*</td>
<td>28†</td>
</tr>
<tr>
<td>Total CVD, %</td>
<td>32</td>
<td>23</td>
<td>55*</td>
<td>30†</td>
</tr>
<tr>
<td>Current smokers, %</td>
<td>8</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Normally distributed values are presented as mean±SD, non-normally distributed values as median (interquartile range). CVD indicates cardiovascular disease. *P<0.01 vs survivors. †P<0.05 vs survivors.

Table 10. Clinical baseline characteristics according to outcome (Study IV)

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Entire group</th>
<th>Survivors</th>
<th>Cardiovascular death</th>
<th>Other death</th>
</tr>
</thead>
<tbody>
<tr>
<td>N =</td>
<td>1294</td>
<td>742</td>
<td>332</td>
<td>220</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>149±21</td>
<td>148±20</td>
<td>151±21†</td>
<td>150±20</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>83±11</td>
<td>85±11</td>
<td>80±11*</td>
<td>82±10*</td>
</tr>
<tr>
<td>Pulse pressure, mmHg</td>
<td>66±18</td>
<td>63±18</td>
<td>71±19*</td>
<td>69±18*</td>
</tr>
<tr>
<td>Mean arterial BP, mmHg</td>
<td>105±10</td>
<td>106±12</td>
<td>104±12</td>
<td>104±12</td>
</tr>
<tr>
<td>Body-mass index, kg/m²</td>
<td>28.3±4.7</td>
<td>28.8±4.7</td>
<td>27.5±4.5*</td>
<td>27.5±4.6*</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.93±0.08</td>
<td>0.93±0.08</td>
<td>0.93±0.09</td>
<td>0.93±0.07</td>
</tr>
</tbody>
</table>

Normally distributed values are presented as mean±SD, non-normally distributed values as median (interquartile range). BP indicates blood pressure. *P<0.01 vs survivors. †P<0.05 vs survivors.
Table 11. Baseline biochemical characteristics according to outcome (Study IV)

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Entire group</th>
<th>Survivors</th>
<th>Cardiovascular death</th>
<th>Other death</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 1294</td>
<td>742</td>
<td>332</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/l</td>
<td>8.4 (7.1-11.0)</td>
<td>8.1 (7.0-10.7)</td>
<td>9.1 (7.2-11.6)*</td>
<td>8.3 (6.9-10.7)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.5±1.5</td>
<td>7.4±1.6</td>
<td>7.7±1.4</td>
<td>7.4±1.5</td>
</tr>
<tr>
<td>AER, µg/min</td>
<td>5.5 (2.8-11.7)</td>
<td>4.7 (2.5-9.3)</td>
<td>6.8 (3.7-22.2)*</td>
<td>7.1 (3.1-13.7) *</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.85±1.20</td>
<td>5.84±1.13</td>
<td>5.96±1.26</td>
<td>5.72±1.30</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.80±1.05</td>
<td>3.75±1.01</td>
<td>3.89±1.05</td>
<td>3.70±1.15</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.18±0.31</td>
<td>1.19±0.30</td>
<td>1.12±0.29*</td>
<td>1.23±0.35</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.70 (1.25-2.38)</td>
<td>1.55 (1.26-2.27)</td>
<td>1.83 (1.32-2.54)*</td>
<td>1.58 (1.15-2.45)</td>
</tr>
</tbody>
</table>

Normally distributed values are presented as mean±SD, non-normally distributed values as median (interquartile range). AER indicates albumin excretion rate. *P<0.01 vs survivors.

Table 12. Cox regression models for all-cause mortality after adjustment for other risk factors (Study IV)

<table>
<thead>
<tr>
<th>Variable added</th>
<th>Hazard ratio*</th>
<th>SBP</th>
<th>DBP</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure per 10 mmHg</td>
<td>1.00 (0.96-1.05)</td>
<td>0.70 (0.64-0.76)</td>
<td>1.12 (1.07-1.18)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>1.11 (1.09-1.12)</td>
<td>0.92 (0.88-0.97)</td>
<td>0.86 (0.78-0.94)</td>
<td>0.95 (0.91-1.00)</td>
</tr>
<tr>
<td>Gender, male</td>
<td>1.51 (1.24-1.83)</td>
<td>0.92 (0.88-0.97)</td>
<td>0.82 (0.75-0.90)</td>
<td>0.96 (0.92-1.01)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>2.40 (1.62-3.52)</td>
<td>0.92 (0.88-0.96)</td>
<td>0.82 (0.75-0.90)</td>
<td>0.96 (0.91-1.01)</td>
</tr>
<tr>
<td>Diabetes duration, years</td>
<td>1.02 (1.01-1.04)</td>
<td>0.92 (0.88-0.97)</td>
<td>0.83 (0.76-0.91)</td>
<td>0.96 (0.91-1.01)</td>
</tr>
<tr>
<td>Previous CVD</td>
<td>1.39 (1.15-1.69)</td>
<td>0.93 (0.89-0.97)</td>
<td>0.85 (0.77-0.93)</td>
<td>0.96 (0.91-1.01)</td>
</tr>
</tbody>
</table>

Hazard ratios refer to a pressure increase of 10 mmHg. Analyses were performed with antihypertensive medication as strata variable. Values in parenthesis are 95% confidence intervals. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; CVD, cardiovascular disease. *Hazard ratio in final model not including blood pressure indices.
Table 13. Cox regression models for cardiovascular mortality after adjustment for other risk factors (Study IV)

<table>
<thead>
<tr>
<th>Variable added</th>
<th>Hazard ratio*</th>
<th>SBP</th>
<th>DBP</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure per 10 mmHg</td>
<td>1.00 (0.96-1.05)</td>
<td>0.65 (0.58-0.72)</td>
<td>1.14 (1.07-1.20)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>1.12 (1.10-1.14)</td>
<td>0.92 (0.87-0.98)</td>
<td>0.80 (0.71-0.90)</td>
<td>0.96 (0.91-1.03)</td>
</tr>
<tr>
<td>Gender, male</td>
<td>1.49 (1.13-1.97)</td>
<td>0.92 (0.88-0.97)</td>
<td>0.77 (0.68-0.86)</td>
<td>0.98 (0.91-1.04)</td>
</tr>
<tr>
<td>Previous CVD</td>
<td>1.82 (1.38-2.40)</td>
<td>0.93 (0.88-0.99)</td>
<td>0.81 (0.72-0.91)</td>
<td>0.97 (0.91-1.04)</td>
</tr>
<tr>
<td>Diabetes duration, years</td>
<td>1.03 (1.01-1.04)</td>
<td>0.93 (0.87-0.98)</td>
<td>0.82 (0.73-0.93)</td>
<td>0.96 (0.90-1.03)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>2.19 (1.25-3.81)</td>
<td>0.93 (0.88-0.99)</td>
<td>0.81 (0.72-0.91)</td>
<td>0.97 (0.91-1.04)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>0.55 (0.33-0.89)</td>
<td>0.92 (0.87-0.98)</td>
<td>0.82 (0.73-0.92)</td>
<td>0.96 (0.90-1.03)</td>
</tr>
</tbody>
</table>

Hazard ratios refer to a pressure increase of 10 mmHg. Analyses were performed with antihypertensive medication as strata variable. Values in parenthesis are 95% confidence intervals. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; CVD, cardiovascular disease. *Hazard ratio in final model not including blood pressure indices.

Figure 9-11 show cardiovascular mortality in relation to the age- and gender-adjusted blood pressure indices. The relationships between all-cause mortality and blood pressure were similar to those shown in these figures.

A negative association between systolic blood pressure and mortality was observed in patients with a positive history of cardiovascular disease and systolic blood pressure levels < 140 mmHg were associated with increased mortality in this group. In patients older than the median age of 69.1 years systolic blood pressure correlated with mortality in a U-shaped fashion, whereas no such relationship in patients below median age was observed. Patients with and without antihypertensive medication showed an inverse association between systolic blood pressure and mortality.
Figure 9. (Study IV) Cardiovascular mortality in relation to age- and gender-adjusted systolic blood pressure (SBP). Left: Previous cardiovascular disease (hollow squares, dotted line), no previous cardiovascular disease (filled circles, solid line). Center: Age > median (69.1 years) (hollow squares, dotted line), age < median (filled circles, solid line). Right: Antihypertensive medication (hollow squares, dotted line), no antihypertensive medication (filled circles, solid line). Vertical bars indicate 95% confidence intervals.

Figure 10. (Study IV) Cardiovascular mortality in relation to age- and gender-adjusted diastolic blood pressure (DBP). Left: Previous cardiovascular disease (hollow squares, dotted line), no previous cardiovascular disease (filled circles, solid line). Center: Age > median (69.1 years) (hollow squares, dotted line), age < median (filled circles, solid line). Right: Antihypertensive medication (hollow squares, dotted line), no antihypertensive medication (filled circles, solid line). Vertical bars indicate 95% confidence intervals.
Cardiovascular mortality correlated negatively with diastolic blood pressure in patients with previous cardiovascular disease. In patients with antihypertensive medication, diastolic blood pressure correlated with mortality in an inverse manner.

The association between pulse pressure and mortality was U-shaped in both patients with and without previous cardiovascular disease with the highest risk found in the lowest and highest pulse pressure category. In patients older than the median age of 69.1 years pulse pressure correlated with mortality in a U-shaped fashion, whereas the relationship tended to be positive in patients below the median age.

All-cause mortality stratified by systolic blood pressure and diastolic blood pressure levels is shown in Figure 12. Mortality increases towards the upper right corner, where the combination of a high systolic blood pressure and a low diastolic blood
pressure results in a high pulse pressure. A similar peak in mortality can be observed in the lower left corner, where a low systolic blood pressure and a high diastolic blood pressure result in a low pulse pressure. A corresponding analysis for cardiovascular mortality displayed a very similar pattern.

Figure 12. (Study IV) All-cause mortality stratified by age- and gender-adjusted systolic (SBP) and diastolic blood pressure (DBP)
6.5. Study V

Table 14 shows the characteristics of the study subjects at baseline. Data on blood pressure, arterial stiffness and endothelial function and the change in these parameters to 24 weeks are presented in Table 15. At follow-up, AIx had declined in the peptide group in comparison to the placebo group (Figure 13). The differences in change between the groups were clearly more pronounced in males. In peptide group males, AIx decreased significantly compared to placebo group males, -2.30% (95%CI -4.31 to -0.28) vs. 1.74% (95%CI 0.44 to 3.04), P=0.004. On the other hand, no divergence in the change of AIx were seen in females, -0.39% (95%CI -2.34 to 1.56) vs. 0.35% (95%CI -1.77 to 2.48), P=0.90 (Figure 13).

Table 14. Baseline demographic and clinical data at baseline (Study V)

<table>
<thead>
<tr>
<th></th>
<th>Placebo (N=44)</th>
<th>Peptide (N=44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males, n (%)</td>
<td>27 (61)</td>
<td>27 (60)</td>
</tr>
<tr>
<td>Age, years</td>
<td>49±5</td>
<td>49±5</td>
</tr>
<tr>
<td>Body mass index, kg/cm²</td>
<td>28.5±3.9</td>
<td>27.6±3.6</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.94±0.08</td>
<td>0.90±0.10</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>155±14</td>
<td>151±15</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>94±9</td>
<td>95±12</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>114±10</td>
<td>117±10</td>
</tr>
<tr>
<td>CRP, pg/ml</td>
<td>1.4 (0.8-2.6)</td>
<td>1.2 (0.6-3.1)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.9±0.9</td>
<td>5.9±1.0</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>1.5±0.4</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.2 (0.9-1.8)</td>
<td>1.4 (0.9-2.0)</td>
</tr>
</tbody>
</table>

Normally distributed values are presented as mean±SD and non-normally distributed values as median (interquartile range).

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Table 15. Hemodynamic characteristics at baseline and change to 6 months

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Change to 6 months</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Peptide</td>
<td>Placebo</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean (95% CI)</td>
</tr>
<tr>
<td>AIx,%</td>
<td>20.0±8.4</td>
<td>24.3±8.4</td>
<td>1.20 (0.09 to 2.32)</td>
</tr>
<tr>
<td>Tr, ms</td>
<td>147±8</td>
<td>143±9</td>
<td>-1.9 (-3.7 to -0.2)</td>
</tr>
<tr>
<td>EFI</td>
<td>0.31±0.16</td>
<td>0.32±0.16</td>
<td>0.04 (-0.04 to 0.12)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>151±15</td>
<td>155±14</td>
<td>-2.6 (-5.7 to 0.6)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>95±12</td>
<td>94±9</td>
<td>-1.7 (-3.6 to 0.08)</td>
</tr>
</tbody>
</table>

Analysis of covariance (ANCOVA) with body mass index and baseline measure as covariates. P-values refer to differences in change. AIx indicate augmentation index; Tr, time to return of pulse wave; EFI, endothelial function index; SD, standard deviation; CI, confidence interval.
Figure 13. (Study V) Augmentation index (AIx) change to 6 months. Bars represent 95% confidence intervals.
The Tr did not change in the peptide group. In the placebo group a decrease in Tr was observed. However, the difference between the groups was insignificant. In males, the groups differed significantly regarding Tr change, 1.7 ms (95%CI: -0.44 to 3.9) vs. -2.1 ms (95%CI -4.1 to -0.1), P=0.022. The corresponding results for females were not significant -1.4 ms (95%CI -3.7 to 0.9) vs. -1.6 ms (95%CI -5.2 to 1.9), P=0.58.

No differences between the groups regarding endothelial function index or change in endothelial function index could be observed. The baseline AIx and the responses to nitroglycerin and salbutamol are shown in Figure 14.

At follow-up after 24 weeks, both systolic and diastolic blood pressure had declined in both groups. The difference in change between the groups was insignificant. In peptide group, the decrease in systolic and diastolic blood pressure were significant compared to the baseline level, systolic blood pressure -4.6 mmHg (95%CI -8.4 to -0.8), P=0.018, diastolic blood pressure -3.7 mmHg (95%CI -6.1 to -1.3), P=0.004. In the placebo group, no significant changes compared to the baseline level were observed. Notably, no significant changes in any of the hemodynamic parameters were observed after 12 weeks of low-dose treatment.
Figure 14. (Study V) Augmentation index (AIx) during endothelial function testing at baseline, at 12 weeks, and at 24 weeks. AIx I indicates baseline reading, GTN, reading after administration of nitroglycerin; AIx II, second baseline reading after 60 minutes, SAL, reading after administration of salbutamol.
7. Discussion

7.1. Methods

Arterial stiffness was assessed by three different methods. Pulse pressure, which is considered a surrogate measure of arterial stiffness, was the index of interest in the studies performed on patients with diabetes (Study III and IV). While pulse pressure has the advantage of being easily measured, it has some limitations. Particularly in younger subjects, pulse pressure is an unreliable measure of arterial stiffness on the individual level due to the phenomenon of pulse wave amplification. This circumstance does not however constitute a major problem in these studies. Study III examined the age-induced pressure changes in a cohort of type 1 diabetic patients and thus compared pulse pressure patterns between large groups. Such a study design is not likely to be seriously confounded by pulse wave amplification. Study IV, in turn, was performed on rather old type 2 diabetic patients with a median age of 69 years. In this age group pulse pressure reflects arterial stiffness quite accurately. The fact that Study III and IV used blood pressure recordings from a single occasion will most likely result in an overestimation of hypertension when compared with measurements on several occasions.\textsuperscript{271} In Study III, this circumstance is nevertheless unlikely to generate any considerable differences between the groups as both groups were examined in the same manner.

Carotid-femoral pulse wave velocity was employed as one of the arterial stiffness indices in Study I. This method is considered very robust and reliable. Nevertheless, the method is limited by the fact that it measures the elastic properties of a single arterial segment and not the entire arterial system.
Pulse wave analysis by radial artery tonometry provides a simple, technically easy, quick, and reproducible non-invasive means of determining systemic arterial stiffness. In Study I, II, and V, the commercially available SphygmoCor system was used to assess arterial stiffness by calculation of the AIx. The SpghygmoCor device uses a generalized arterial transfer function to reconstruct central aortic from radial pressure waveforms. The radial artery has been considered the best site for non-invasive assessment of pulse wave reflection because optimal applanation may be easier to achieve at this site than at other sites. The transfer function has previously been validated in a number of studies.\(^{272, 273, 274, 275}\) Since a high blood pressure increases the AIx by distending the arterial walls, the ambient blood pressure had to be considered in the interpretation of the pulse wave analysis results in all three studies. In Study I, the observed correlation between muscle fibre type and AIx turned out to be a consequence of the association between blood pressure and muscle fibre type, which thus was not independently associated with AIx.

The method for assessment of endothelial function by pulse wave velocity used in Study I and V was developed by Wilkinson et al in 2002.\(^{266}\) This method examines the effects of the \(\beta_2\)-adrenergic agonist salbutamol and nitroglycerin on AIx. The reduction of the AIx induced by inhalation of salbutamol reflects activation of the L-arginine/NO pathway in endothelial cells, while the reaction to nitroglycerin is a measure of vasodilatory capacity. It has previously been demonstrated that the vasodilator effect of salbutamol is to a large extent mediated by the L-arginine/NO pathway.\(^{276}\) Importantly, the salbutamol-induced AIx response has also been found to correlate with the change in forearm blood flow after intra-arterial acetylcholine infusion, which is considered the gold standard of endothelial function measurement. The validity of the method is strengthened by the fact that the
endothelial function index of Study I correlated strongly with established determinants of endothelial function, such as age, LDL cholesterol, and triglycerides.

7.2. Study I

Arterial stiffness and endothelial dysfunction are important risk factors for cardiovascular disease. Notably, physically active individuals have a reduced risk of atherosclerotic disease. A high proportion of type I (slow-twitch) muscle fibres in skeletal muscle is associated with a favourable cardiovascular risk profile. In Study I, we tested physical activity and muscle fibre-type distribution as determinants of endothelial function and arterial stiffness in middle-aged men. The results show that muscle fibre-type distribution does not have a major effect on arterial stiffness or endothelial function. In contrast, an association between physical activity and impaired endothelial function was observed.

Although the AIx correlated inversely with the type I% in a univariate analysis, the result of the multiple regression analysis suggests that this was merely an effect of the lower blood pressure associated with a high type I%. In other words, high blood pressure increases the functional stiffness of the large arteries and thus aortic pulse wave reflection by distending the arterial walls.\textsuperscript{101}

Previous studies have shown that the age-induced stiffening of the large arteries is less pronounced in subjects who engage in regular endurance exercise.\textsuperscript{151} Furthermore, it has been shown that arterial compliance can be reduced by exercise training.\textsuperscript{153} It is unclear whether low-to-moderate exercise has an impact on the arterial elastic properties. In
contrast to aerobic training, resistance training seems to increase arterial stiffness and pulse pressure. The lack of correlation between leisure-time physical activity and pulse wave velocity or pulse wave reflection observed in Study I suggests that physical activity is unlikely to be a major determinant of arterial stiffness in middle-aged men.

Notably there was an inverse relationship between exercise and endothelial function. Stimulation of endothelial NO release by salbutamol reduced the AIx less in physically active subjects, whereas no such correlation was observed in the response to the NO donor nitroglycerin.

Heavy exercise increases free radical formation and oxidative stress is known to decrease the bioavailability of NO. The study by Bergholm et al that demonstrated a harmful effect of intense aerobic training on endothelial function in forearm vessels also reported reduced concentrations of circulating antioxidants after 3 months of intense exercise training. Oxidative stress could thus be one of the factors behind this finding. Another possible explanation for the negative association between leisure-time physical activity and endothelial function could be that a large part of the physically active subjects performed exercise during the days prior to examination, which could impair the endothelial function.

The vascular bed that was studied in Study I differs from that of the previous cross-sectional studies showing enhanced endothelial function in athletes. These previous studies measured alterations in local blood flow in the arteries of the upper limb, whereas changes in pulse wave reflection reflect endothelial function in the entire arterial tree. It is possible that exercise affects the vascular beds in a dissimilar manner.
Some limitations of Study I should be noted. Muscle fibre type distribution was determined almost 20 years before the assessment of vascular properties, which could affect the results. On the other hand, the prevailing perception is that muscle fibre distribution is genetically determined and is thus relatively constant.\textsuperscript{7,280} It is not possible to draw any definite conclusions on the specific effects of exercise based on Study I. Since the study design was cross-sectional, the associations observed in Study I could result from unknown confounding factors. Intervention studies are needed to clarify how exercise affects arterial stiffness and endothelial function.

One third of the studied subjects were on antihypertensive medication. In order to minimize any confounding effects, all antihypertensive medication was withheld for at least 24 hours. No significant differences in arterial stiffness or endothelial function were observed between treated and non-treated subjects. Thus, any major confounding effect due to blood pressure medication seems unlikely.

The results of Study I indicate that muscle fibre type distribution is unlikely to have a major effect on arterial stiffness and endothelial function. Therefore, other mechanisms linking a high proportion of slow muscle fibres to low cardiovascular risk should be sought.
7.3. Study II

Disturbed maternal endothelial function is believed to be central in the pathogenesis of preeclampsia and has been observed to persist for several years following the preeclamptic pregnancy. Endothelial dysfunction has been reported to cause increased pulse wave reflection, a measure of systemic arterial stiffness. Study II tested the hypothesis that preeclampsia and a history of preeclampsia are associated with increased pulse wave reflection. The results of Study II demonstrate that pulse wave reflection and thus systemic arterial stiffness are increased in preeclampsia compared with normal pregnancy. Pulse wave reflection in previously preeclamptic normotensive non-pregnant women did not differ from that of the non-pregnant control subjects. Accordingly, Study II shows that the increased arterial stiffness induced by preeclampsia is transitory and not permanent.

General vasodilatation and decreased arterial stiffness are features of normal uncomplicated pregnancies. Enhanced NO release by the endothelium is considered a central mechanism in the pregnancy-induced hemodynamic changes. Preeclampsia, on the other hand, is characterized by endothelial dysfunction and diminished endothelial release of NO. Moreover, reduced arterial compliance during the first trimester of pregnancy in previously preeclamptic women has been observed. Considering the previously reported association between arterial stiffness and endothelial dysfunction, impaired endothelial NO release seems to be a potential cause of the increased pulse wave reflection in preeclamptic women.
Prior studies have observed endothelial dysfunction in previously preeclamptic women. Women with a history of preeclampsia could thus be expected to present increased pulse wave reflection. The results of Study II do not support this hypothesis. However, the study excluded women on antihypertensive medication and therefore did not include the subjects most likely to have impaired endothelial function and increased arterial stiffness. It is also possible that the arterial stiffening effect of endothelial dysfunction does not manifest at the relatively young age at which the subjects were studied. Furthermore, pulse wave reflection is affected by many additional factors that may obscure the effect of endothelial dysfunction.

Since the functional stiffness of the arteries is affected by the stretching effect of blood pressure, pulse wave reflection generally correlates positively with blood pressure. In Study II, mean arterial pressure correlated with the AIx in both pregnant and non-pregnant subjects. In the multiple regression analysis of the pregnant subjects, preeclampsia came out as a strong independent determinant of AIx, whereas the association between blood pressure and pulse wave reflection was overshadowed by the massive effect of preeclampsia. Thus, the increased pulse wave reflection observed in preeclampsia must be considered independent of the distending effect of higher blood pressure.

No conclusions on the pathophysiological mechanisms behind the elevated systemic arterial stiffness in preeclampsia can be drawn based on the study. It can be speculated that any major changes of the elastic properties of the arterial walls are unlikely to develop during the short duration of preclampsia. The reduced arterial compliance would
instead be a result of increased vascular tone caused by endothelial dysfunction. Alternative pathophysiological mechanisms behind the vascular dysfunction in preeclampsia have been presented in other studies. The possibility, that preeclampsia increases pulse wave reflection by a NO-independent mechanism, can therefore not be excluded. Study II nevertheless shows that regardless of the underlying mechanism, preeclampsia impairs arterial compliance.

The results of Study II support the view that the hemodynamic consequences of preeclampsia are not limited to hypertension. Preeclampsia should be regarded as a state of generalized vascular dysfunction including elevated systemic arterial stiffness.

### 7.4. Study III

Pulse pressure increases with age as a result of arterial stiffening and is a powerful predictor of cardiovascular disease. Type 1 diabetes is associated with excessive cardiovascular mortality and increased arterial stiffness. Study III examined whether the age-related blood pressure changes in type 1 diabetic patients differ from those of the non-diabetic background population. Study III is the first report showing that the age-induced blood pressure changes of patients with type 1 diabetes are shifted to a younger age. Patients with type 1 diabetes generally have a higher systolic blood pressure and a diastolic blood pressure that starts to decline at a younger age than in the non-diabetic population. As a consequence of this, patients with diabetes have a higher and more rapidly increasing pulse pressure. It seems that this premature rise in pulse pressure is
strongly related to the duration of diabetes and to the development of diabetic kidney
disease. However, it also characterizes type 1 diabetic patients without signs of kidney
disease. The findings are indicative of accelerated arterial stiffening and may explain the
higher cardiovascular risk associated with type 1 diabetes.

Aging appears to affect blood pressure in diabetic and non-diabetic subjects in a parallel
manner. But the changes are shifted to a younger age by 15-20 years in type 1 diabetes.
This shift denotes accelerated stiffening. Study III is purely observational and does not
explore the mechanisms behind the findings. According to an established hypothesis,
deposition of advanced glycation end-products and cross-linking of collagen molecules in
the arterial walls may contribute to the increased arterial stiffness in patients with
diabetes.\textsuperscript{286, 287} Another mechanism that could be involved is endothelial dysfunction,
which has been associated with both diabetes and arterial stiffness.\textsuperscript{288}

The observation that patients with a young onset of diabetes showed an earlier increase in
pulse pressure than patients with a later onset indicates that the duration has a
considerable impact on arterial stiffness. The similar finding in the analysis including
only normoalbuminuric patients and the results of the multiple regression analysis
showed that the duration of diabetes was associated with elevated pulse pressure
independently of age and diabetic kidney disease. Thus, although the recent glycaemic
control measured by HbA1\textsubscript{c} was not associated with pulse pressure, the cumulative
exposure to hyperglycaemia seems to play an important role in the process of arterial
stiffening.
In contrast to the situation in type 2 diabetes, blood pressure derangements associated with type 1 diabetes have generally been considered to be the result of diabetic kidney disease. This belief is largely based on the influential work by Nørgaard et al, which reported a prevalence of essential hypertension in type 1 diabetic patients without diabetic kidney disease that was similar to that of the general population.\textsuperscript{289} Study III challenges this view by demonstrating that type 1 diabetes is, even in the absence of diabetic kidney disease, associated with a detrimental blood pressure pattern. The observations provided by the study are in accordance with the greatly elevated risk of cardiovascular disease in type 1 diabetic patients with overt diabetic kidney disease. The premature rise in pulse pressure could also explain the increased risk of cardiovascular disease in type 1 diabetic patients without diabetic kidney disease. This is supported by two recent studies that have identified pulse pressure as a powerful independent risk factor for cardiovascular morbidity and mortality in patients with type 1 diabetes.\textsuperscript{290, 291}

The finding that not only pulse pressure, but also essential hypertension is more common in type 1 diabetic patients is in conflict with the prevailing perception that the prevalence of essential hypertension of type 1 diabetic patients is similar to that of the non-diabetic population. Previous studies have generally defined hypertension by considerably higher blood pressure values than those presently used. As the higher prevalence of essential hypertension in our diabetic patients was largely due to a higher prevalence of isolated systolic hypertension, one can speculate that this discrepancy with previous results is caused by the fact that a large proportion of the patients with moderate isolated systolic hypertension would not have been identified by the more restrictive criteria used
previously. Any comparison with earlier studies will however be complicated by the more aggressive blood pressure treatment practiced today.

A potential confounder is the higher rate of antihypertensive medication in the diabetic group. However, as most of the commonly used antihypertensive agents reduce pulse pressure, this circumstance will in fact cause an underestimation of the difference. This will to a lesser degree affect the results of normoalbuminuric patients with diabetes, whose rate of antihypertensive medication was only slightly higher than that of the control subjects, but may be a considerable confounder in patients with diabetic kidney disease. Since the pulse pressure of untreated diabetic patients was higher than that of untreated control subjects in all age groups, we can safely exclude the possibility that the difference between the groups is caused by blood pressure lowering medication.

Study III is the first study to show that, even in the absence of any signs of diabetic kidney disease, patients with type 1 diabetes have a higher pulse pressure that increases earlier and more rapidly compared to the non-diabetic background population. This finding is indicative of accelerated arterial stiffening and it may also explain the increased risk of cardiovascular disease associated with type 1 diabetes.

7.5. Study IV

The presence of hypertension aggravates the high cardiovascular risk in patients with type 2 diabetes. Pulse pressure is a marker of arterial stiffness and constitutes a risk factor for cardiovascular mortality. Study IV examined the relationship between different blood
pressure indices and mortality in a cohort of patients with type 2 diabetes. The study demonstrated that a U-shaped association between pulse pressure and mortality exists in elderly patients with type 2 diabetes. In addition, low blood pressure is associated with poor survival in patients with type 2 diabetes, who have a history of previous cardiovascular disease.

The study did not assess the cardiac function or vascular properties of the patients. Thus, one can only speculate about the mechanisms underlying the finding that patients with high or low pulse pressure had a strikingly elevated mortality compared to patients with intermediate pulse pressure.

Pulse pressure and arterial stiffness constitute risk factors for cardiovascular disease and mortality in both diabetic and non-diabetic subjects. In view of the increased arterial stiffness in type 2 diabetes, the high mortality in the highest pulse pressure categories are likely to be linked to increased arterial stiffness. As well as indicating vascular aging, arterial stiffness increases cardiac afterload by augmenting central systolic blood pressure and reduces coronary perfusion by decreasing diastolic blood pressure, thus sensitizing the heart to ischemia.

Study IV demonstrated that low blood pressure constitutes a risk factor for cardiovascular and all-cause mortality in patients with type 2 diabetes. The finding is somewhat controversial considering the large amount of evidence of the deleterious effects of hypertension and the well-established benefit of treating hypertension in type 2 diabetes. On the other hand, most major trials have examined middle-aged hypertensive patients without cardiovascular disease. Study IV examined a cohort
recruited with type 2 diabetes as the only criteria, and is therefore likely to represent a more typical type 2 diabetic population.

The poor prognosis associated with a low blood pressure in patients with previous cardiovascular disease is most probably related to cardiac output failure. Recent reports show a greatly increased prevalence of heart failure in type 2 diabetes.\textsuperscript{295, 296} Low blood pressure has previously been associated with heart failure in patients with type 2 diabetes.\textsuperscript{297} In these patients, low blood pressure is likely to be an indicator of poor cardiovascular health rather than the cause of it. Subclinical heart failure could also explain the high mortality associated with low pulse pressure in patients without previously known cardiovascular disease.

Study IV provides the first evidence that low blood pressure is a risk factor for mortality in patients with type 2 diabetes. Previously, similar associations have been observed in elderly non-diabetic populations.\textsuperscript{298, 299} Since the negative associations between blood pressure and mortality were confined to patients with older age or previous cardiovascular disease, the results of Study IV supports the view that the positive association between blood pressure and survival is limited to the elderly population. The observations clearly underline the need to further clarify the impact of blood pressure treatment in the elderly diabetic population.

The fact that a substantial proportion of the studied subjects were taking antihypertensive medication at the time of the baseline visit would certainly have influenced the results. High risk patients with previous cardiovascular disease and diabetic complications are more likely to use medication. An artificially low blood pressure in high risk patients
could therefore seriously confound the results. On the other hand, any effects of blood pressure on the circulatory system would be mediated by the actual blood pressure rather than a theoretical naive blood pressure. Given that the main findings of the study can be observed when analyzing medicated and non-medicated patients separately, a major confounding effect caused by antihypertensive medication seems unlikely.

Study IV shows that both low and high pulse pressure are risk factors for mortality in elderly type 2 diabetic patients. Low systolic and low diastolic blood pressure are predictive of elevated all-cause and cardiovascular mortality in type 2 diabetic patients with previous cardiovascular disease. In contrast, no blood pressure index contributed significantly to mortality in young type 2 diabetic patients without cardiovascular disease. The results of Study IV suggest that there is a need to re-evaluate the role of blood pressure as a risk marker in certain high risk subgroups of type 2 diabetic patients.

7.6. Study V

The results of this paper indicate that fermented milk containing bioactive peptides reduces arterial stiffness measured by pulse wave reflection in mildly hypertensive males. Endothelial function defined as the hemodynamic response to β2-adrenergic stimulation remained unchanged, which suggests that enhanced endothelial NO release involved in the reduction in pulse wave reflection and blood pressure.

Given that the intervention in Study V lasted for merely 24 weeks, it is doubtful that a substantial change in the structural properties of the arterial walls would have occurred
during this rather short time. It is more likely that diminished vascular tone in arteries and arterioles causes an increased arterial compliance as a result of reduced tension in the arterial walls.

It must also be remembered that the functional arterial stiffness is influenced by the distending effect of blood pressure on the arterial walls. The reduction in AIx can probably to some extent be explained by lowered distending pressure on the arterial walls, since the blood pressure decreased in the peptide group.

Milk protein-derived peptides have been shown to reduce arterial tone. Previous studies have demonstrated a vasodilating effect of isoleucyl-prolyl-proline and valyl-prolyl-proline in spontaneously hypertensive rats in vitro. The mechanisms behind this effect are still unknown, but it seems that it is partially based on inhibition of the renin-angiotensin-aldosterone system. Since ACE-inhibitors have been shown to reduce arterial stiffness, this hypothesis consistent with the findings of Study V.

The hemodynamic response to stimulation of endothelial NO release was not affected by administration of bioactive tripeptides. However, since basal NO release by the endothelium is an important determinant of pulse wave reflection, it is possible that bioactive tripeptides enhance basal endothelial NO release although the maximal capacity of NO release does not change.

The favorable effect on arterial stiffness could also be partly mediated through the mineral composition of Lactobacillus helveticus fermented milk. Calcium, potassium,
and magnesium have all been shown to enhance vasorelaxation and vascular function in experimental trials.301, 302, 303, 304, 305, 306

The results of Study V indicate that intake of *Lactobacillus helveticus* fermented milk containing high doses of isoleucine-prolyl-proline and valyl-prolyl-proline tripeptides reduce arterial stiffness measured by pulse wave analysis in mildly hypertensive males.
8. Summary and conclusions

I. Muscle fibre type distribution is not an independent determinant of arterial stiffness or endothelial function. Impaired endothelial function was observed in physically active men underlining the need for further research.

II. Pulse wave reflection reflecting systemic arterial stiffness are increased in pregnant women with preeclampsia, but not in normotensive non-pregnant women with a history of preeclampsia. The results support the concept of generalized vascular dysfunction in preeclampsia.

III. As a result of a higher systolic blood pressure and an earlier decrease in diastolic blood pressure, patients with type 1 diabetes have a higher and more rapidly increasing pulse pressure. The finding indicates accelerated arterial aging and may explain the higher cardiovascular morbidity and mortality in type 1 diabetes.

IV. In elderly type 2 diabetic patients pulse pressure correlates with mortality in a U-shaped fashion. Systolic and diastolic blood pressure correlated negatively with mortality after adjustment for other risk factors. In patients with previous cardiovascular disease, low systolic blood pressure and diastolic blood pressure are predictive of elevated mortality. The role of blood pressure as a risk marker in elderly type 2 diabetic patients with cardiovascular disease is complex and needs to be studied further.

V. Intake of *Lactobacillus helveticus* fermented milk, which contains the tripeptides isoleucine-prolyl-prolyl and valine-prolyl-prolyl, reduces arterial stiffness measured
by pulse wave reflection in hypertensive males. On the other hand, the NO release capacity of the arterial endothelium is not improved by intake of this milk product.
9. Implications and significance

Methods, study design, and patient material differ quite substantially between the five substudies of this thesis. The common denominator of the five manuscripts has been arterial stiffness, which has been examined from several angles. The first paper studies arterial stiffness in relation to a physiological variation in healthy subjects, whereas the second paper looks at arterial stiffness in patients with an acute illness. The research on diabetes has focused on the long-term effects on arterial stiffness of a chronic disease and on the importance of arterial stiffening on the outcome in the context of that chronic disease. Finally, the last study evaluated whether arterial stiffness can be improved by a simple dietary modification.

It is an established truth that people who exercise have less cardiovascular disease than their sedentary counterparts. The physiological mechanisms that are responsible for this association are yet to be clarified. It has been speculated that a high proportion of muscle fibres would account for a part of this association. Study I shows a lack of association between muscle fibre distribution and arterial stiffness and endothelial function, which are well-known characteristics of poor vascular health. Future researchers will thus know to look elsewhere for links between muscle fibre distribution and cardiovascular health.

Women who develop preeclampsia during pregnancy have an increased risk of cardiovascular disease later in life. Many of the factors that increase the susceptibility of preeclampsia are also established cardiovascular risk factors. In this sense, one can consider pregnancy as a window to women’s future health. The results of study II added arterial stiffening as a new aspect to this view. We now know that the arterial stiffness is
elevated during acute preeclampsia, whereas no signs of increased arterial stiffness can be detected a few years after a preeclamptic pregnancy. We can only speculate that in another twenty or thirty years, the arteries of the previously preeclamptic women will once again display increased stiffness.

In addition to confirming previous reports that showed increased arterial stiffness in patients with type 1 diabetes, study III had important clinical implications. Previously, it was thought that the prevalence of essential hypertension in patients with type 1 diabetes was similar to that of the general population. The new findings of this study clearly show that even in the absence of diabetic kidney disease, patients with type 1 diabetes, have a higher prevalence of hypertension. Notably, the rate of essential isolated systolic hypertension was 3-fold in the diabetic population. This study sends a clear message to physicians who treat patients with type 1 diabetes. Look for systolic hypertension!

Although hypertension has been known to be an important risk factor for cardiovascular disease and mortality for a considerable time, pulse pressure has quite recently been recognized as a risk factor. It has also been argued that pulse pressure is an even more powerful predictor than traditional blood pressure indices. Study IV shows that in a typical population with type 2 diabetes, the associations between blood pressure and mortality are quite complex and that one cannot generally say that either systolic, diastolic, or pulse pressure is superior to the other indices. The results emphasize that the clinician should always take age and concomitant disease into consideration when the risk associated with any blood pressure level is estimated in patients with type 2 diabetes.
Medical science has in the recent years recognized many factors that are associated with increased arterial stiffness. Unfortunately, factors that improve arterial elasticity have been quite scarce. In that sense, the findings of study V are very encouraging. Not only does the study provide new means of reducing functional arterial stiffness, but the method to do so is non-pharmacological and does not seem to cause any unwanted side-effects. Further studies will undoubtedly be required in order to examine whether intake of milk-derived bioactive tripeptides could have an effect on cardiovascular morbidity and mortality.
10. Acknowledgements

This work was carried out during the years 2002 and 2007 at the Folkhälsan Research Center at the University of Helsinki. I am deeply grateful for excellent facilities I have been provided with.

I owe my most sincere gratitude to my supervisor Per-Henrik Groop. He has had a wonderful way of inspiring my research work. It has been a privilege to be taught and guided by such a supportive and patient supervisor. Although a busy man, Per-Henrik has always found the time to discuss my work, and discussions that were meant to last for five minutes have on repeated occasions lasted for hours.

I was particularly fortunate to have had Ilkka Pörsti and Kaj Metsärinne as reviewers of this thesis. The valuable advice and intelligent comments during the review of the manuscript is highly appreciated. Their comments and suggestions truly improved this thesis.

My introduction to medical science was done by Johan Fagerudd. I sincerely appreciate the brilliant guidance and encouragement I have received. Many times have I had the opportunity to appreciate Johan’s unique ability to find a solution to even the most complex problems.

I also want to express my deepest gratitude to Carol Forsblom for all his help. He supported me and gave me valuable advice on science and statistics whenever I needed it.
During many of my years at Biomedicum, I had the great luck of sitting next to Kim Pettersson-Fernholm, who never failed to entertain me with opera music and funny e-mails. I also owe Kim a great thank for his effort with gathering the FinnDiane material.

I owe my genuine thankfulness to all of my following co-authors.

To Miika Hernelahti for fantastic companionship in the muscle fibre study.

To Esa Hämäläinen and Heikki Tikkanen for providing me with excellent patient material.

To Katja Lampinen for being such a reliable and ambitious co-operator at the Women’s hospital.

To Risto Kaaja for all the good advice and enthusiasm he has shown me.

To Antti Reunanen for kindly providing me with a fantastic control group.

To Bo Isomaa, Tiinamaija Tuomi, and Leif Groop for giving me the opportunity to work with the superb Botnia material.

To Tiina Jauhiainen for her enormous effort in organizing the peptide study.

To Riitta Korpela, Heikki Vapaatalo, and Hannu Kautiainen for your help and insights.

Without supreme technical assistance by Maija Kopo, Minna Riitamaa, Eija Kortelainen, Sinikka Lindh, Anna Sandelin, Maikki Parkkonen, and Minna Hietala the work on this thesis would not have been possible.
I warmly thank all the members of the FinnDiane group for being there: Lena Thorn, Milla Rosengård-Bärlund, Johan Waden, Markku Saraheimo, Daniel Gordin, Outi Heikkilä, Ville Mäkinen, Maija Wessman, Sara Fröjdö, Lisa Sjölin, and all the rest of you.

Furthermore, I am happy to have such great business companions. Work has never been as fun as with you.

This work was financially supported by Finska Läkaresällskapet, Wilhelm och Else Stockmanns Stiftelse, Nylands Nation, Waldemar von Frenckells stiftelse, the Ministry of Education, Samfundet Folkhälsan, K. Albin Johanssons stiftelse, Stiftelsen Dorothea, Olivia, Karl Walter och Jarl Walter Perklens minne, Stiftelsen för Hjärtforskning, and Aarne Koskelon säätiö all of which are gratefully acknowledged.

I am truly grateful to my parents Ulrika and Bengt Rönnback. You cannot ask for a better a great start in life than I had.

I also want to express my gratitude to my brother Jan Rönnback for every now and then reminding me that that there are other things than work in life.

I am also fortunate to have great parents-in-law Eira and Martti Koskenranta. Your care of my children is highly appreciated.

Finally, I wish to dedicate this thesis to my beloved family Ansku, Alvin, and Ebbe. You mean everything to me.
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