DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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Risk markers for cardiovascular disease and low bone mineral density in children with type 1 diabetes

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To my parents

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LIST OF ORIGINAL PUBLICATIONS

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

I Heilman K, Zilmer M, Zilmer K, Kool P, Tillmann V. Elevated plasma adiponectin and decreased plasma homocysteine and asymmetric dimethylarginine in children with type 1 diabetes. Scand J Clin Lab Invest 2009;69:85–91.

II Heilman K, Zilmer M, Zilmer K, Lintrop M, Kampus P, Kals J, Tillmann V. Arterial stiffness, carotid artery intima-media thickness and plasma myeloperoxidase level in children with type 1 diabetes. Diabetes Res Clin Pract 2009;84:168–73.

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

Applicant's contribution to these publications:

Paper I: Study design, identifying and recruiting patients, collecting clinical data, data analysis, writing the paper

Paper II: Study design, identifying and recruiting patients, collecting clinical data, measuring arterial stiffness, data analysis, writing the paper.

Paper III: Study design, identifying and recruiting patients, collecting clinical data, data analysis, writing the paper

ABBREVIATIONS

ACE	angiotensin-converting enzyme
ADMA	asymmetric dimethylarginine
AGE	advanced glycation end product
AIx	augmentation index
AIx@75	augmentation index corrected for a heart rate of 75 beats *per* minute
BA	bone area
BMC	bone mineral content
BMCadj	BMC adjusted for age and height
BMD	bone mineral density
BMDvol	apparent volumetric lumbar BMD
BMI	body mass index
BP	blood pressure
CAD	coronary artery disease
CCA	common carotid artery
CV	cardiovascular
CVD	cardiovascular disease
DM1	diabetes mellitus type 1
DM2	diabetes mellitus type 2
DXA	dual-energy x-ray absorptiometry
F_2-IsoPs	8-iso-prostaglandin F_{2a}
HbA1c	glycosylated haemoglobin
HDL	high-density lipoprotein
hsCRP	high sensitivity C-reactive protein
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IMT	intima media thickness
LDL	low-density lipoprotein
MET	metabolic equivalents
MPO	myeloperoxidase
NO	nitric oxide
Ox LDL	oxidized LDL
OxS	oxidative stress
PKCβ	protein kinase C-beta
PP	pulse pressure
PWA	pressure wave analysis
PWV	pulse wave velocity
ROS	reactive oxygen species
SDS	standard deviation score
tHcy	total homocysteine
TNF-α	tumor necrosis factor-α
Tr	timing of the reflected waveform
WBC	white blood cell

1. INTRODUCTION

Cardiovascular disease (CVD) is currently the leading cause of death in the worldwide and is predicted to remain so for many years, placing a huge financial burden on the world's health resources (Lopez *et al.* 2001; Mathers *et al.* 2006). The two main causes of death are coronary artery disease (CAD) and cerebrovascular disease, which together accounted for 27.2% of all deaths in high-income countries and 21.3% of all deaths in low- or middle-income countries in 2001. The majority of these deaths are caused by underlying atherosclerosis, where disruption of arterial plaques within the coronary or carotid arteries severely reduces blood flow to the target organ.

Regarding type 1 diabetes (DM1), as much as 10% of premature CAD morbidity and mortality in the general population is due to DM1 (Libby *et al*. 2005). One of the main aims in the management of diabetes is the prevention of long-term vascular complications, which can be microvascular (including retinopathy, nephropathy, and neuropathy) or macrovascular (affecting the coronary, cerebral, and peripheral arteries). Clinical practice usually focuses on microvascular risk in DM1 and macrovascular risk in type 2 diabetes (DM2). In fact, microvascular and macrovascular complications are highly relevant to both types of diabetes. Furthermore, the age-adjusted relative risk for CVD in DM1 far exceeds that of DM2 (Libby *et al.* 2005). It has been suggested that atherosclerosis begins at an earlier age in DM1, and the progression of CVD is more aggressive compared with the general population (Valsania *et al.* 1991; Laing *et al*. 2003).

Traditionally, the prevention and treatment of CVD has focused on favourably modifying risk factors, such as hypertension, smoking, hyperglycaemia and dyslipidaemia (Chobanian *et al.* 2003; Graham *et al.* 2007). Although most patients with CAD have at least one identifiable risk factor (Wilson 1994), many ischaemic events occur in the absence of any classical associations (Futterman *et al.* 1998). Furthermore, of the excess CAD risk in people with diabetes, only 25% can be accounted for by established risk factors (Pyorala *et al.* 1987). Much research has therefore focused on accurately identifying subgroups of the general population at the highest risk for CVD. Several new possible risk markers have been suggested, such as markers of inflammation and oxidative stress (OxS) (Ross 1999; Harrison *et al.* 2003), plasma adiponectin, asymmetric dimethylarginine (ADMA) and homocysteine (tHcy) (Wald *et al*. 2002; Maas *et al.* 2007; Dekker *et al.* 2008). These CVD risk markers have been little studied in patients with DM1 and few conclusions have been replicated.

Considering the long latent phase of atherosclerotic pathogenesis before the appearance of symptoms, the ability to evaluate arterial function before the development of angiographically measurable atherosclerotic plaque is an important aspect of early detection and risk classification (O'Rourke *et al.* 2005; Järvisalo *et al.* 2004). Several noninvasive and thus, easy to obtain measures of arterial structure and function have been shown to be clinically useful, including

carotid artery intima-media thickness (IMT) and arterial stiffness (Bots *et al.* 1999; Weber *et al*. 2004). Carotid IMT, a subclinical marker of atherosclerosis, is a strong marker of the burden of CVD and is thus related to CAD, stroke, and cardiovascular (CV) mortality in adults (Lorenz *et al.* 2007). Similarly, arterial stiffness has been associated with adverse CV outcomes (Laurent *et al.* 2006).

An association has been reported between CVD and osteoporosis, perhaps attributable to the presence of common risk factors (Magnus *et al.* 2005; Shaffer *et al.* 2007). Osteoporosis is, by definition, a systemic skeletal disease characterized by low bone mass and microarcitectural deterioration of bone tissue, resulting in an increase in bone fragility and susceptibility to fracture. While primary osteoporosis is a condition of reduced bone mass appearing in postmenopausal women and in elderly individuals, secondary osteoporosis is a condition of reduced bone mass resulting from a variety of specific causes, such as rheumatoid arthritis, long-term immobilization or glucocorticoid treatment. A recent meta-analysis of 80 studies by Vestergaard (2007) showed that patients with DM1 are also at risk of decreased bone mineral density (BMD) and bone fractures. However, data about the influence of gender, age, and metabolic control on bone loss is inconclusive and the exact mechanisms contributing to DM1 related bone loss remain unknown.

The aim of the study was to investigate early risk markers for CVD in children with DM1. We looked at a biochemical panel composed of traditional and new markers, as well as atherosclerosis-related structural and functional changes of the arterial wall, measured by IMT and arterial stiffness. The early detection and prevention of CVD in childhood could reduce CV mortality and morbidity in adulthood. The second part of the study focused on the assessment of BMD in children with DM1 and determining possible associations between BMD and hyperglycaemia, inflammation- and OxS-related markers.

2. REVIEW OF THE LITERATURE

2.1. Definition and epidemiology of type 1 diabetes

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycaemia caused by defects in insulin secretion, insulin action, or both. The abnormalities in carbohydrate, fat, and protein metabolism found in diabetes are due to deficient effects of insulin on the target tissues. The diagnostic criteria for diabetes are based on blood glucose measurements and the presence or absence of symptoms (Craig *et al.* 2006).

An actual etiological classification suggested by the International Society for Pediatric and Adolescent Diabetes (ISPAD) is presented in Table 1 (Craig *et al.* 2006). DM1 only accounts for 5–10% of those with diabetes, but is the most predominant form of diabetes in childhood. In most western countries, DM1 accounts for more than 90% of childhood and adolescent diabetes. DM2 is becoming more common and accounts for a significant proportion of youth-onset diabetes in certain at-risk populations (Pinhas-Hamiel *et al.* 2005). This study focuses on DM1 diagnosed during childhood.

Table 1. Etiological classification of diabetes mellitus

I Type 1 β-cell destruction, usually leading to absolute insulin deficiency
A. Autoimmune
B. Idiopathic
II Type 2 May range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance
III Other specific types
A. Genetic defects of β-cell function
B. Genetic defects in insulin action
C. Diseases of the exocrine pancreas
D. Endocrinopathies
E. Drug or chemical induced
F. Infections
G. Uncommon forms of immune-mediated diabetes
H. Other genetic syndromes sometimes associated with diabetes
IV Gestational diabetes

Individuals with DM1 have an absolute deficiency of insulin secretion. A combination of genetic susceptibility and environmental factors is proposed to lead to humoral and cell-mediated autoimmune destruction of the β-cells in the pancreas. Most cases are primarily T-cell mediated, occurring at a variable rate and becoming clinically symptomatic when approximately 90% of pancreatic β-cells are destroyed (Gepts 1965). Serological markers of a pathologic auto-

immune process, including islet cell, glutamic acid decarboxylase, islet antigen-2 or insulin autoantibodies are present in 85–90% of individuals with fasting hyperglycaemia (Verge *et al.* 1998; Sabbah *et al.* 2000). Susceptibility to auto-immune DM1 is determined by the interaction of multiple genes. Human leucocyte antigen (HLA) genes have the strongest known association, whereby linkage to DQA and DQB genes can be either predisposing or protective (Noble *et al.* 1996; Pugliese *et al.* 1999; Lambert *et al.* 2004). The environmental triggers (chemical and/or viral) remain largely unknown, but the process usually begins months to years before the manifestation of clinical symptoms (Craig *et al.* 2006).

At the start of the 20th century, childhood DM1 was rare and rapidly fatal, but by the end of the century a steady increase in incidence had been reported in many parts of the world. Incidence of DM1 in children aged <15 years is increasing in Europe, particularly among young children and in countries with a low incidence (Patterson *et al.* 2009). The incidence rate of DM1 in children under 15 years of age in Europe varied from 3.6 per 100 000 persons at risk per year in former Yugoslav Republic (FYR) of Macedonia to 43.9 per 100 000 per year in Finland in 1989–1998. The overall annual rate of increase was 3.2% in 1989–1998, with the most pronounced velocity in the 0–4.9 age group (Green *et al.* 2001). A similar pattern was observed over this time in Estonia (Teeäär *et al.* 2009). The prediction is that between 2005 and 2020, new cases of DM1 in European children younger than 5 years will double and that the prevalence in those younger than 15 years will increase by 70% (Patterson *et al.* 2009). Notably, the incidence of DM1 in young adults older than 15 years shows little evidence of rising (Weets *et al.* 2002; Pundziute-Lyckå *et al.* 2002).

One suggestion is that need for genetic susceptibility has lessened over time because of heightened environmental pressure, which results in a raised disease progression rate – especially in individuals with protective HLA genotypes. Several hypotheses based on analytical epidemiological studies have pointed to modern lifestyle habits as possible environmental factors, such as increased weight and height, caesarean section deliveries, or reduced frequency of early infections. Faster rates of increase in countries with low incidence rate – in particular eastern European countries – might be an expression of effects of the lifestyle factors, which are changing rapidly in these countries; therefore, convergence of incidence rates might reflect harmonisation of lifestyle-related risk factors in Europe (Patterson *et al.* 2009).

2.2. Cardiovascular disease in type 1 diabetes

2.2.1. Epidemiology of cardiovascular disease in type 1 diabetes

Patients with DM1 have more than ten times the CVD risk of the general population (Dorman *et al.* 1984; Krolewski *et al.* 1987; Orchard *et al.* 2006). The incidence of CAD is approximately 1–2% per year among asymptomatic adults with DM1 (Orchard *et al.* 2003; Soedamah-Muthu *et al.* 2004) and it is the leading cause of death in people with DM1 (Krolewski *et al.* 1987; Libby *et al.* 2005; Soedamah-Muthu *et al*. 2006). By their mid-40s, about 70% of men and 50% of women with DM1 develop coronary artery calcification (Dabelea *et al.* 2003), a marker of atherosclerotic plaque formation. About 35% of DM1 patients die of CAD by age of 55, in contrast to only 8% of nondiabetic men and 4% of women (Krolewski *et al.* 1987). Women with DM1 are affected as often as men and are 9- to 29-times more likely to die of CAD than nondiabetic women, while the risk for men increases 4- to 9-fold (Krolewski *et al.* 1987; Laing *et al.* 2003) (Figure 1). Although women experience relative protection from CVD compared with men in the general population, diabetes blunts the benefit of female sex (Laing *et al.* 2003; Soedamah-Muthu *et al*. 2006). Recent advances have been successful in decreasing morbidity and mortality from retinopathy, nephropathy and neuropathy of diabetes, but mortality due to CAD in patients with diabetes has not had a similar decrease (Pambianco *et al.* 2006).

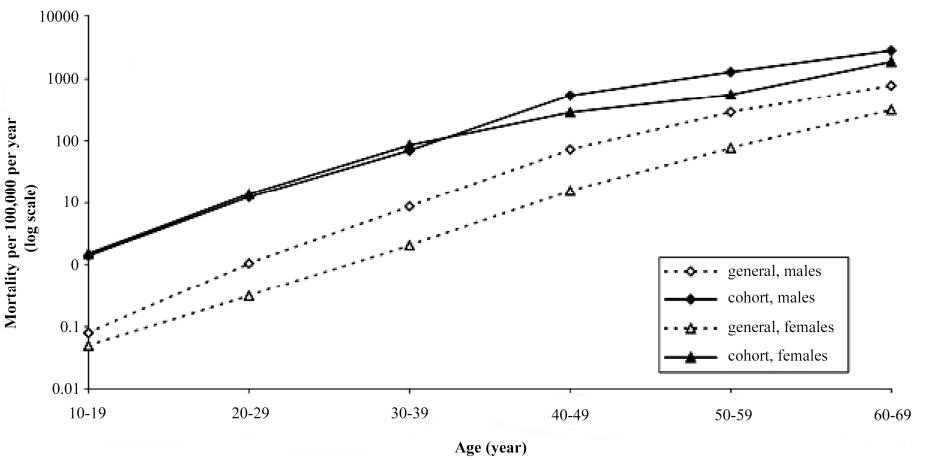

Figure 1. Mortality from ischaemic heart disease in the Diabetes UK cohort (adapted from Laing *et al.* 2003).

The atherosclerotic process begins at an earlier age in patients with DM1 compared to the general population (Laing *et al.* 2003). The Oslo study

demonstrated a high prevalence of stealthy coronary artery atherosclerosis in adult patients with childhood-onset DM1. The mean age at the time of the study was 43 years, the mean diabetes duration was 30 years, and none had symptoms of CAD. Direct intravascular ultrasound examination of the coronary arteries revealed that all had clinically significant atherosclerosis (Larsen *et al.* 2002). Coronary angiography revealed that 34% had >50% vessel stenosis, although only 15% had a pathological exercise ECG (Larsen *et al.* 2002). Carotid IMT was also increased in patients enrolled in the Oslo study (Larsen *et al.* 2005), and resembled that of non-diabetic individuals 20 years older. Atherosclerotic changes in the vessel wall begin long before symptoms appear (Berenson *et al.* 1992). Even in children with a mean age of 11 years and mean diabetes duration of only 4 years, endothelial dysfunction, measured by flow mediated dilatation, is a common manifestation and is associated with increased carotid artery IMT (Järvisalo *et al.* 2004). A recent study by Haller *et al.* (2004) found increased arterial stiffness in children with DM1. These data suggest that children with DM1 are predisposed to the development of early atherosclerosis.

In addition to the tendency for atherosclerotic disease to develop asymptomatically, the disease process seems to be more severe in DM1. Patients with DM1 are more likely to have severe stenosis of the coronary vessels and involvement of all three major coronary arteries (Valsania *et al.* 1991). Since the atherosclerotic process starts at an early age and can be clinically asymptomatic in young adulthood, advanced disease is frequently not detected. As a consequence, the first clinical presentation can be a major CV event with poor outcome (Chun *et al.* 1997; Miettinen *et al.* 1998). Correspondingly, the 5-year mortality rate following myocardial infarction may be as high as 50% for patients with diabetes – more than double that of nondiabetic patients (Herlitz *et al.* 1998).

2.2.2. Pathophysiology of atherosclerosis in type 1 diabetes

Accelerated atherosclerosis is a hallmark of macrovascular disease in DM1. Although the pathophysiology of atherosclerosis in DM1 has not been fully elucidated, current concept supports a model in which circulating factors associated with the perturbed metabolic milieu of DM1 (eg, hyperglycaemia, abnormal levels of glycation and oxidation products) cause endothelial dysfunction, which in turn leads to vasoconstrictive, pro-inflammatory, and prothrombotic changes that contribute to atherosclerotic plaque development and an enhanced potential for thrombosis after plaque rupture (Beckman *et al.* 2002; Libby *et al.* 2005).

The role of glucose in this pathophysiology has received considerable attention. Endothelial cells, because of their strategic anatomic position between the circulating blood and the media layer of arterial wall, regulate vascular function and structure. In normal endothelial cells, biologically active substances are synthesized and released to maintain vascular homeostasis, ensuring adequate blood flow and nutrient delivery while preventing thrombosis and

leukocyte diapedesis (Kinlay *et al.* 2001). In a hyperglycaemic setting, vascular endothelial cells are at particular risk of developing intracellular hyperglycaemia because, unlike many other cell types, they are unable to downregulate glucose uptake (Kaiser *et al.* 1993). The intracellular hyperglycaemia causes overproduction of reactive species by the mitochondrial electron transport chain, thereby creating non-physiological (high-grade) OxS that leads to DNA damage and activation of the reparative enzyme poly (ADP-ribose) polymerase. In addition to its DNA reparative function, this enzyme also mediates the ribosylation and inhibition of glyceraldehyde phosphate dehydrogenase. This promotes glucose diversion away from glycolysis into alternative biochemical pathways that seem to cause hyperglycaemia-induced cellular injury. These proposed alternative pathways include increased formation of advanced glycation end products (AGEs), the activation of protein kinase C-beta (PKCβ), and increased flux through the polyol and hexosamine pathways. AGEs are proteins that have undergone irreversible, non-enzymatic glycation (a physiological process that is upregulated in chronic hyperglycaemia) and interact with specific receptors on target cells, leading to the activation of pathological signalling pathways that increase OxS and promote inflammation and procoagulant activity. Similarly, hyperglycaemia-induced activation of PKCβ signalling increases the generation of reactive oxygen species (ROS), promotes vasoconstriction, upregulates inflammatory factors, and leads to pro-thrombotic changes (Brownlee 2001; Beckman *et al.* 2002; Du *et al.* 2003).

Overall, according to recent paper by Rentakaran *et al.* (2008), these pathways enable hyperglycaemia to induce profound changes in the endothelium, including: downregulation of the endogenous vasodilator nitric oxide (NO) which, coupled with increased endothelin-1 and angiotensin II, causes vasoconstriction; activation of nuclear factor kappa B and inflammatory gene expression, leading to the increased expression of leukocyte-attracting chemokines, inflammatory cytokines, and cellular adhesion molecules; and enhanced production of tissue factor and plasminogen activator inhibitor-1, thereby promoting coagulation. Altered endothelium favours the adhesion and subsequent penetration of circulating monocytes into the arterial intima, where they are activated and converted into macrophages. The macrophages engulf modified lipoproteins (oxidized LDLs) after which they turn into foam cells (Zilmer *et al.* 1999). The localised accumulation of foam cells generates a fatty streak, the hallmark lesion of early atherosclerosis. On formation of the fatty streak, smooth muscle cells migrate from the arterial media to the intima, where they proliferate under the influence of growth factors and lay down a complex extracellular matrix that contributes to the progression of the fatty streak into advanced atherosclerotic plaque (Rentakaran *et al.* 2008).

2.2.3. Conventional risk factors for cardiovascular disease in type 1 diabetes

Family history

While the identification of modifiable risk factors at any age is important, there are certainly genetic factors involved that should be considered. A family history of early CVD (before 55 years of age) and lipid disturbances are considered risk factors for atherosclerosis in the general population (Grundy *et al.* 1998). In the case of a child with DM1, a family history of hypertension, known CAD, dyslipidemia or DM2 will increases the child's risk of developing CVD as an adult (Donaghue *et al.* 2007). A full family history should thus be taken in every child with diabetes, and updated regularly, since parents and grandparents are at an age where CV events are more likely (Dahl-Jørgensen *et al.* 2005).

Impaired blood glucose control

Although controversy exists regarding the direct influence of blood glucose control on the development of atherosclerosis in diabetes, there is increasing evidence for such an effect.

The most important clinical evidence supporting this concept comes from the Diabetes Control and Complications Trial (DCCT). The DCCT was a randomized controlled clinical trial initiated in 1983 in which 1441 patients with DM1 (aged 13–39 years) in the USA and Canada were randomly assigned to receive either intensive diabetes therapy (either three or more insulin injections or continuous subcutaneous insulin infusion by an external pump, with frequent blood glucose monitoring) or the conventional diabetes therapy of the day (one or two insulin injections per day). After mean follow-up of 6.5 years, mean HbA1c in the intensive therapy group was 7.2% and 9.0% in the conventional treatment group. Intensive diabetes therapy was associated with a significant reduction in the incidence and progression of microvascular complications. However, the observed 41% relative risk reduction for macrovascular disease (95% CI –10 to 68) did not achieve statistical significance, mainly because of the limited number of CV events in the young study population (The Diabetes Control and Complications Trial Research Group 1993). In the Epidemiology of Diabetes Interventions and Complications (EDIC) Study, a 6-year non-randomized follow-up of the DCCT, patients with diabetes had increased IMT of their carotid arteries when compared to control subjects, and IMT was related to the HbA1c levels obtained during the DCCT. In patients who had received intensive insulin treatment 6 years previously, thickening had progressed to a lesser extent than in the conventionally treated patients. All participants were instructed on intensive diabetes therapy and over the course of the EDIC study, the mean HbA1c concentrations of the former intensive and conventional therapy groups converged (HbA1c about 8%), such that they were no longer significantly different 5 years after the end of DCCT (Nathan *et al.* 2003).

Furthermore, in 2005, after a mean follow-up of 17 years, it emerged that intensive therapy during the DCCT had reduced the subsequent risk of any CVD event by 42% (95% CI 9–63) and the risk of combined endpoint non-fatal myocardial infarction, stroke, or CV death by 57% (95% CI 12–79) (Nathan *et al.* 2005). Thus, intensive diabetes therapy, with associated reduced glycaemic exposure, initially reduced a surrogate measure of atherosclerotic disease (carotid IMT) and later reduced clinical CV outcomes over the course of the EDIC study, suggesting that intensive treatment should be initiated as early as possible in the management of DM1.

Larsen *et al.* (2002) demonstrated that long-term blood glucose control predicts coronary atherosclerosis as detected by intravascular ultrasound in young childhood-onset DM1 patients with no symptoms of CVD. A 1% increase in mean HbA1c over 18 years implied a 6.4% rise in vessel area stenosis. A meta-analysis of randomized, controlled comparison studies including 1800 DM1 and 4472 DM2 adults associated a reduction in macrovascular events with improvements in glycaemic control for both DM1 and DM2 patients (Stettler *et al.* 2006). The report suggested that improved glycaemic control has a larger reduction in macrovascular risk for DM1 and a smaller reduction of risk for DM2.

According to the standards of care set by the International Society for Paediatric and Adolescent Diabetes (ISPAD), children's HbA1c levels should be kept at <7.5% (Donaghue *et al.* 2007). Intensive diabetes therapy, with a goal of near-normal glycaemic control without excessive hypoglycaemia, should be initiated whenever safely possible in the clinical management of DM1. Cardiovascular benefits of improved glycaemic control might only be realized if sufficiently low levels of glycaemia are achieved and the effect may be greater in the absence of other risk factors (Rentakaran *et al.* 2008). The effect of glucose variability on the long-term risk of macrovascular complications in DM1 needs further investigations.

Lipids and lipoproteins disturbances

Lipids (triglycerides), lipoproteins (LDLs etc.) and lipid-like molecules (cholesterol) are related to the pathogenesis of atherosclerosis (Zilmer *et al.* 1999). Well-controlled DM1 is not associated with gross blood lipid disturbances, but with qualitative abnormalities in lipoproteins, including a preponderance of small dense, atherogenic LDL particles and decreased levels of large, cardioprotective HDL particles. In the DCCT study, poor glycaemic control was associated with these potentially atherogenic changes in lipoprotein particles (Jenkins *et al.* 2003). Furthermore, changes in HDL composition can independently predict incident CVD in patients with DM1 (Groop *et al.* 2007). Additionally, oxLDL has been shown to predict incident CAD in DM1 (Orchard *et al.* 1999). Several recently published articles suggest that oxLDL is a very promising risk marker of CVD (Holvoet *et al.* 2008; De Faire *et al.* 2009). High-

grade OxS, a main reason behind excessive oxLDL, has such a strong impact considering its wide role in the pathogenesis of atherosclerosis.

ISPAD recommends (Donaghue *et al.* 2007) to start screening for fasting blood lipids soon after diagnosis (when diabetes has been stabilized) in all children with DM1 older than 12 years. If normal results are obtained, this should be repeated every 5 years. If there is a family history of hypercholesterolemia, early CVD, or if the family history is unknown, screening should start at 2 years of age. The target LDL cholesterol level should be lower than 2.6 mmol/L. If interventions to improve metabolic control and dietary changes cannot meet this target, statins should be considered, although their long-term safety in children has yet to be established.

Hypertension

Hypertension has a greater impact on CVD in patients with diabetes than in non-diabetic individuals (Stamler *et al.* 1993). Screening for hypertension in young people with diabetes is essential in decreasing risks of both microvascular and macrovascular disease. Blood pressure (BP) should be measured at least annually in children with DM1 (Donaghue *et al.* 2007). Hypertension in children is defined as BP levels at or above the 95th percentile, confirmed by measurements taken on three different days. Blood pressure values between the 90th and 95th percentiles are defined as prehypertension (National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents 2004). Accordantly to the ISPAD guidelines, BP values should be compared with age-appropriate centile charts and maintained at less than the 95th centile in children with DM1 as in all children with hypertension. Angiotensin-converting enzyme (ACE) inhibitors are recommended in children with hypertension. They have been effective and safe in children in short-term studies, but are not safe during pregnancy (Donaghue *et al.* 2007).

Increased urinary albumin excretion

Compared to the general population, the risk of CVD mortality is nearly 100 times higher in patients with diabetic nephropathy (Koivisto *et al.* 1996), but most DM1 patients who develop CVD do not have nephropathy. During the 18-year follow-up of the Oslo study, all patients had preclinical CVD, but only about 15% had microalbuminuria (Larsen *et al.* 2002). Recent evidence suggests that CVD and renal disease share risk factors and develop in parallel (Lloyd *et al.* 1996; Watts *et al.* 1996; Libby *et al.* 2005). Although differences in mean HbA1c during the DCCT accounted for most of the CV benefit associated with intensive therapy, multivariate analyses also suggested that part of the treatment effect on CVD risk was mediated by reduction in the incidence of microalbuminuria or albuminuria. However, the intensive treatment effect on reducing CVD risk persisted after adjustment for micro- and macroalbuminuria,

suggesting that the non-renal effects of intensive diabetes therapy were nonetheless more important (Nathan *et al.* 2005).

Patients with DM1 should be screened annually for microalbuminuria. Guidelines recommend that confirmed persistent microalbuminuria should be treated with ACE inhibitors or angiotensin II receptor antagonists even in the absence of hypertension, and regardless of the age of the patient (Donaghue *et al.* 2007).

Lifestyle factors

Lifestyle modification is the cornerstone of therapy to reduce the risk of CVD in young people with diabetes. Interventions include dietary modification to reduce sodium or fat intake, exercise, and when applicable, weight loss and smoking cessation.

Sedentary men with diabetes were three times more likely to die than active ones (Moy *et al.* 1993). Exercise for young people with or without diabetes is known to have benefits for physical fitness, CV fitness and a sense of well-being that may have further benefits. In young people with DM1, physical fitness is associated with increased insulin sensitivity, improved BP and a better lipid profile (Austin *et al.* 1993; Ridell *et al.* 2006). Obesity and the metabolic syndrome are risk factors for CVD. In the Bogalusa cohort of nearly 10000 healthy children aged between 5 and 17, obese children (weight >95th percentile) had significantly higher risk ratios for CV risk factors than children of normal weight (<85th percentile) (Freedman *et al.* 1999). As observed in the DCCT, intensive insulin therapy can result in excessive weight gain in a subset of patients with DM1 (Purnell *et al.* 1998). This weight gain can be accompanied by components of the metabolic syndrome, including increased visceral adiposity, higher BP and adverse lipoprotein changes (Sibley *et al.* 2003). Importantly, however, a recent analysis showed that, although intensive diabetes therapy was associated with an increased prevalence of the metabolic syndrome over the course of the DCCT (driven by weight gain), baseline metabolic syndrome did not predict subsequent macrovascular disease (Kilpatrick *et al.* 2007). Thus, in the context of the significant reduction in CVD risk associated with intensive therapy in the DCCT, it seems that the CV benefits of improved glycaemic control outweighs the risks associated with the development of the metabolic syndrome in DM1 (Kilpatrick *et al.* 2007).

The diet for young people with diabetes should follow evidence-based nutritional recommendations for all children and adolescents (American Heart Association Nutrition Committee 2006) with the goals of achieving optimal glycaemic control without excessive hypoglycemia, as well as meeting BP and lipid goals.

Smoking is an independent risk factor for atherosclerosis, and DM1 and smoking interact to produce excess CV morbidity and mortality (Gay *et al.* 1992; Zieske *et al.* 2005).

It is important to include these lifestyle factors within an integrated plan of treatment in all children and adolescents from the onset of diabetes onwards.

Lifestyle interventions at an early stage, together with optimal blood glucose control, may be the most promising areas to focus on for improved long-term prognosis (Dahl-Jørgensen *et al.* 2005).

2.3. Markers of inflammation and oxidative stress in cardiovascular diseases and type 1 diabetes

Atherosclerosis is a generalized, chronic, inflammatory vascular disorder leading to CVD (Ross 1999; Libby 2002). This suggestion is supported by the presence of mononuclear cells in arterial lesions and by the ability of various blood markers related to inflammation to predict major coronary events (Danesh *et al.* 1998; Ross 1999; Danesh *et al.* 2000). Previous meta-analyses of long-term prospective studies have reported that the risk of CAD is about 40% greater in people with raised blood white cell counts (WBC), and about 90% greater in those with raised circulating concentrations of CRP, then comparing people in the top third of these factors and those in the bottom third of baseline measurements (Danesh *et al.* 1998; Danesh *et al.* 2000).

Intercellular cell adhesion molecule (ICAM-1) is a marker of WBC interaction with the vascular endothelium, and it facilitates the transendothelial migration of WBCs into the subendothelial space and transformation into macrophages (Smith *et al.* 1989). After LDL particles penetrate into subendothelial space of arteries, they become oxidized and accumulated by macrophages, which are thereafter transformed into foam cells. This process leads to higher levels of macrophage produced inflammatory cytokines that subsequently make the liver produce CRP (Ross 1999). CRP plays a key role in innate immune response and constitutes a stable plasma marker of systemic inflammation, with a half life of 19 hrs. During the acute phase response, its levels may rapidly rise up to 1000-fold above the reference values. The main source of plasma CRP is the liver, which produces most of our CRP. Its production appears to be regulated by several cytokines, mainly interleukin 6 (IL-6) but also tumor necrosis factor (TNF)-alpha and IL-1. Recently, it has been supposed that CRP is produced also in other sites, such as respiratory tract epithelium, macrophages, kidney, neuronal cells, adipocytes, and smooth muscle cells (Calabrò *et al.* 2009).

A "high-sensitivity" CRP (hsCRP) test was developed in the 1990s. It enables the detection of serum CRP levels at lower concentrations than was possible with previous methods. The detection limit is less than 0.1 mg/L and can therefore be used for the evaluation of subclinical inflammation. Increased plasma concentrations of ICAM-1 and hsCRP are found to independently predict future CV events (Hwang *et al.* 1997; Ridker *et al.* 1997; Ridker *et al.* 1998; Ridker *et al.* 2000; Ridker *et al.* 2002; Pai *et al.* 2004; Koenig 2004).

OxS-driven excessive free radical-mediated damage also plays an important role in the pathogenesis of atherosclerosis (Harrison *et al.* 2003). It is now accepted that 8-iso-prostaglandin F_{2a} (F_2-IsoPs) is a very good marker for

quantifying systemic OxS, since they are the end-products of free radical attacks on cell membrane phospholipids (Morrow *et al.* 1990). Urinary F_2-IsoPs levels have also been shown to be an independent predictor for both intima-media thickening and angiographic CAD (Basarici *et al.* 2007).

Myeloperoxidase (MPO) is an enzyme linked to both inflammation and OxS. MPO is a heme protein produced by activated neutrophils, monocytes and tissue macrophages. MPO is released in a state of inflammation and catalyzes the formation of several reactive species, including hypochlorous acid, and thus has a role in host defence against microorganisms (Klebanoff 2005). Elevated levels of MPO have been implicated in initiation and propagation of atherosclerosis. Emerging evidence from recent epidemiological studies has shown that higher concentrations of MPO are associated with an increased CVD risk, independent of classical CVD risk factors (Schindhelm *et al.* 2009). The link between MPO and CVD can be explained by MPO-dependent oxidation of LDL and HDL, which are both atherogenic. MPO activity also diminishes NO bioavailability, resulting in endothelial dysfunction. Finally, MPO may play a role in the transition to unstable atherosclerotic plaques (Schindhelm *et al.* 2009).

Markers of inflammation and oxidative stress in diabetes

DM1 is a subclinical, chronic inflammatory state which is, in part, independent of clinically manifest macro- and microvascular complications, but this subclinical inflammation is strongly correlated to the severity and duration of hyperglycaemia (Targher *et al.* 2005). Recent studies have shown that patients with DM1 have increased plasma levels of ICAM-1 and hsCRP (Schalkwijk *et al.* 1999; Hayaishi-Okano *et al.* 2002; Schram *et al.* 2003; Saraheimo *et al.* 2003; Targher *et al.* 2005; Seckin *et al.* 2006; Jenkins *et al.* 2008). Although the mechanisms that trigger the activation of subclinical inflammation are not fully understood, it is likely that chronic exposure to glucose and high levels of AGEs activates the monocyte-macrophage system and stimulates the production and secretion of cytokines and acute-phase proteins (Baumgartner-Parzer *et al.* 1995; Vlassara *et al.* 1995). Recent studies suggest that a chronic inflammatory state might, at least partially, contribute to the pathogenesis and development of microvascular and macrovascular complications. Subjects developing microalbuminuria showed a progressive rise in hsCRP, with levels significantly higher in the years after the onset of microalbuminuria when compared to levels before onset (Marcovecchio *et al.* 2008). The Diabetes Control and Complications Trial (DCCT) showed that higher plasma ICAM-1 levels predict an increased risk of progressive nephropathy in DM1 and may represent an early risk marker that reflects the important role of vascular endothelial dysfunction in this long-term complication (Lin *et al.* 2008). The same study has previously shown that intensive diabetes control is associated with a clear and sustained reduction in HbA1c levels and of microvascular complications in DM1. However, those assigned to intensive diabetes therapy do have an increased risk of weight gain and obesity (Purnell *et al.* 1998). The intensive glycemic control

was associated with a significant reduction in levels of ICAM-1 but with no change in hsCRP. Futher analyses indicated a significant rise in hsCRP among the intensively treated subjects who gained most weight (Schaumberg *et al.* 2005). This demonstrates that the effect of intensive therapy on inflammation is complex and depends on the presence of other CVD risk factors.

DM1 is also consistently associated with OxS. High-grade OxS may be related to many factors, such as increased ROS production via glucose auto-oxidation, nonenzymatic protein glycation, decreased antioxidant status, and ineffective scavenging of ROS (Baynes *et al.* 1999; West 2000). Previous studies have shown that urinary F_2-IsoPs levels are already increased at DM1 onset. However, F_2-IsoPs levels can decrease significantly with improved metabolic control, although never to control group levels (Davi *et al.* 2003; Flores *et al.* 2004).

MPO levels are higher in patients with DM2 compared to healthy controls (Moldoveanu *et al*. 2006), but there is no data about MPO levels in DM1.

2.4. Adiponectin in cardiovascular diseases and type 1 diabetes

Adiponectin is a 244 amino acid collagen-like protein encoded by the gene APM1, which is mapped to chromosome 3q27 (Vasseur *et al*. 2003). Adiponectin is synthesized mainly by adipocytes, but it is also expressed by skeletal muscle cells, cardiac myocytes and endothelial cells (Piñeiro *et al*. 2005; Delaigle *et al.* 2004; Wolf *et al.* 2006).

Adiponectin exist as a full-length protein, as well as a proteolytic cleavage fragment, which is known as globular adiponectin. Full-length adiponectin can exist as: a trimer (known as low-molecular-weight adiponectin); a hexamer (known as middle-molecular-weight adiponectin); and a high-molecular-weight 12- to 18-mer. The high-molecular weight adiponectin is the most active form (Pajvani *et al.* 2004).

Adiponectin expression is regulated by distinct signalling pathways, involving different transcription factors. Experimental studies have shown that OxS (Kamigaki *et al*. 2006), sympathetic nervous system activity (Fasshauer *et al.* 2001) and pro-inflammatory cytokines such as TNF-α and IL-6 (Kim *et al.* 2005), suppress adiponectin expression. Lower adiponectin levels have been associated with age, male gender (possibly due to androgens) (Nishizawa *et al.* 2002) and smoking status, while a Mediterranean diet and exercise increase its circulating levels (Gable *et al.* 2006; Mantzoros *et al.* 2006; Tsukinoki *et al.* 2005). Moreover, genetic determinants seem to affect adiponectin levels (Gable *et al*. 2006).

Two receptors for adiponectin have been identified (ADIPOR1 and ADIPOR2). ADIPOR 1 is abundantly expressed in skeletal muscle and is activated mainly by globular adiponectin, whereas ADIPOR2 is predominantly expressed in the liver and activated mainly by the full-length variant of

adiponectin (Yamauchi *et al.* 2003). Adiponectin receptor activation has been shown to stimulate AMP-activated protein kinase and peroxisome proliferator-activated receptor-γ ligand activity, fatty-acid oxidation and glucose uptake (Yamauchi *et al.* 2002; Yamauchi *et al.* 2003).

Adiponectin in atherosclerosis

Recent studies have established the principal role of inflammation in mediating all stages of atherosclerosis (Libby *et al.* 2002). Adiponectin modulates the inflammatory responses by inhibiting the proliferation of myelomonocytic cells probably by inducing their apoptosis (Yokota *et al.* 2000), attenuating TNF-α mediated production of ADMA (Eid *et al.* 2007) and adhesion molecule expression (Ouchi *et al.* 2000). Adiponectin has also been shown to inhibit the phagocytic activity of macrophages (Yokota *et al.* 2000), suppress macrophage-to-foam cell transformation (Ouchi *et al.* 2001) and proliferation of vascular smooth muscle cells (Matsuzawa *et al.* 1999). Adiponectin accumulates in the injured vascular walls but not in intact vessels (Okamoto *et al.* 2000). Clinical studies have shown that low adiponectin concentrations are associated with endothelial dysfunction (Ouchi *et al.* 2003), increased carotid IMT (Kojima *et al.* 2005; Iglseder *et al*. 2005), but not with the presence of atherosclerotic plaques (Iglseder *et al.* 2005). Low concentrations of plasma adiponectin have been associated with obesity, metabolic syndrome, DM2 (Weyer *et al.* 2001), essential hypertension (Adamczak *et al.* 2003; Iwashima *et al.* 2004), dyslipidemia (Kazumi *et al.* 2004), stroke (Chen *et al.* 2005; Efstathiou *et al.* 2005), CAD (Kumada *et al.* 2003; Rothenbacher *et al.* 2005) and peripheral artery disease (Iwashima *et al.* 2006). Prospective studies have found inconsistent results. While some studies reported that adiponectin is not independently associated with future CVD (Lawlor *et al.* 2005; Lindsay *et al.* 2005; Sattar *et al.* 2006), others have found that low adiponectin concentration to be a significant risk factor (Efstathiou *et al.* 2005; Frystyk *et al.* 2007; Kojima *et al.* 2007). However an increasing number of studies have indicated that high plasma adiponectin concentrations, not hypoadiponectinemia, independently predict CV mortality, particular in patients with already prevalent CVD (Dekker *et al.* 2008; Cavusoglu *et al.* 2006).

Adiponektin and type 1 diabetes

Hyperadiponectinemia is associated with autoimmune diseases, as DM1, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus (Fantuzzi *et al.* 2008) and Graves disease (Sieminska *et al.* 2008). Plasma adiponectin levels during DM1 onset are comparable to those in healthy children, but begin to increase during the first six month of disease (Galler *et al.* 2007). This increase is mainly due to an increase in the high molecular weight subform

of adiponectin (Leth *et al.* 2008). Reports have shown that patients with DM1 who have higher adiponectin concentrations have an increased risk of microvascular complications (Frystyk *et al.* 2005; Hadjadj *et al.* 2005).

It is likely that glycosylation is one of the major posttranslational modifications of adiponectin (Wang *et al*. 2002). With constant hyperglycaemia, the adiponectin glycosylation process is probably altered, and this could lead to an altered function. The altered adiponectin molecule could lead to a diminished negative feedback and thus to increased adiponectin concentrations in diabetes (Saraheimo *et al.* 2005). It has also been suggested that elevated adiponectin levels may just reflect the hyper-catabolic state (Behre 2008), since adiponectin is a hormone which helps provide fuel in catabolic states. Delaigle *et al.* (2004) found that adiponectin is up-regulated in vivo and in vitro in response to inflammatory cytokines, which may be viewed as a protective mechanism against excessive inflammatory reactions.

2.5. Asymmetric dimethylarginine in cardiovascular diseases and type 1 diabetes

ADMA is a naturally occurring derivative of the amino acid arginine that circulates in the plasma, is excreted in urine, and is found in tissues and cells. Endogenous ADMA acts as a competitive inhibitor of NO synthase leading to reduced NO synthesis (Vallance *et al.* 1992, Leiper *et al.* 1999). ADMA is synthesized when L-arginine residues in proteins are methylated by the action of protein arginine methyltransferases (Clarke 1993; McBridge 2001). Proteolysis of proteins containing methylated arginine residues releases free ADMA into the cytosol, plasma and tissues. Thus the amount of ADMA generated within a cell is dependent on the extent of L-arginine methylation in proteins and the rates of protein turnover (Vallance 2004).

Methylarginines are eliminated in part by renal excretion, but over 90% of ADMA may be metabolized by the enzyme dimethylarginine dimethylaminohydrolases (DDAHs) into L-citrulline and dimethylamine (Leiper *et al.* 1999). NO directly inhibits DDAH activity (Leiper *et al.* 1999). Thus, high-output NO production nitrosates DDAH, resulting in the accumulation of ADMA and inhibition of NO synthase. This provides a potentially important mechanism of NO homeostasis.

When administered to healthy volunteers, pathophysiologically relevant ADMA blood concentrations decrease cardiac output, increase systemic vascular resistance and BP, and decrease effective renal plasma flow (Kielstein *et al.* 2004). Inhibition of NO production results in vasoconstriction, platelet activation and aggregation, proliferation of smooth muscle cells and adhesion of monocytes to the endothelial cells. All these events contribute to a pro-inflammatory state of the vascular wall and thereby promote atherosclerosis (Böger 2006).

Increased plasma concentrations of ADMA are associated with endothelial dysfunction and subclinical atherosclerosis (Böger *et al.* 1998*;* Böger *et al.* 2006; Juonala *et al.* 2007; Maas *et al*. 2009; Ayer *et al.* 2009), which are key precursors of overt CVD (Hansson 2005).

ADMA accumulation has been reported in a wide range of CV risk states, including hypertension (Goonasekera *et al.* 1997), diabetes (Xiong *et al.* 2003), hyperhomocysteinaemia (Böger *et al.* 2001), and in individuals with overt atherosclerotic disease (Miyazaki *et al.* 1999). Evidence for ADMA being causally related to CVD comes from both case-control (Schulze *et al.* 2006) and prospective cohort (Valkonen *et al.* 2001*;* Maas *et al.* 2007*;* Schnabel *et al.* 2005; Meinitzer *et al.* 2007) studies. These associations between ADMA and CVD risk have also been observed in those in the community who have no history of prior CVD (Valkonen *et al.* 2001; Maas *et al.* 2007*)*, as well as in people with pre-existing overt CVD (Schnabel *et al.* 2005; Meinitzer *et al.* 2007).

ADMA and diabetes

Elevated ADMA concentrations have been described in patients with DM2 (Abbasi *et al.* 2001). Lin *et al.* (2002) demonstrated in an animal model that hyperglycaemia elevates ADMA by impairing DDAH activity in vascular smooth muscle and endothelium. The study by Päiva *et al.* (2003) found that the only significant predictor of plasma ADMA levels is glomerular filtration rate in patients with DM2. Few studies have been carried out on ADMA plasma levels in patients with DM1, and they have had conflicting results. A recent study by Altinova *et al.* (2007) found increased ADMA plasma levels in adult patients with uncomplicated DM1, but Jehlicka *et al.* (2009) did not find a statistically significant elevation of ADMA levels in children with DM1. The most recent study by Sibal *et al.* (2009) found a decreased ADMA level in adolescents and young adults with uncomplicated DM1. Lajer *et al.* (2008) showed that ADMA plasma levels are related to the progression of renal disease and predict CV morbidity and mortality in DM1 patients with diabetic nephropathy.

2.6. Homocysteine in cardiovascular diseases and type 1 diabetes

Hcy, a sulphur-containing amino acid, is an intermediate product in the normal metabolism of methionine. Hcy is predominantly metabolised by the enzyme N^{10}-methylenetetrahydrofolate reductase (MTHFR) back to methionine and by cystathionine β-synthase (CβS) to cysteine. The activity of MTHFR is strongly dependent on the presence of the folate and cobalamine (vitamin B_{12}), and the activity CβS of pyridoxine (vitamin B_6) as cofactors (Audelin *et al.* 2001).

Elevated plasma level of Hcy was first suspected to be associated with atherogenic and thrombogenic tendencies in patients with classic homocysteinuria (caused by a defect in the gene encoding for CβS) (McCully 1969). Strategies that reduced Hcy levels in these children also decreased vascular event rates (Wilcken *et al.* 1976; Mudd *et al.* 1985). Experimental studies indicate that Hcy may have a harmful effect on endothelial cells, increase coagulability and have a proliferative effect on smooth muscle cells (Fryer *et al.* 1993; Brown *et al.* 1998; Demuth *et al.* 1999; Zhang *et al.* 2000; Su *et al.* 2005).

Meta-analysis of cohort studies found a positive association between serum Hcy contcentrations and ischaemic heart disease events and stroke. A 3 μmol/l decrease in serum Hcy lowers the risk of myocardial infarction by 11–16% and stroke by 19–24% (The Homocysteine Studies Collaboration, 2002; Wald *et al*. 2002). However, a recent large meta-analysis confirmed the lack of statistically significant associations between the MTHFR gene polymorphism and CAD in subjects living in Europe, Asia, North America and Australia. Patients with the MTHFR C677T genotype have moderately raised Hcy concentrations (Lewis *et al*. 2005). Furthermore, randomized controlled studies have not consistently shown that folate supplementation, which decreases Hcy serum concentrations, reduces CVD risk among persons with established vascular disease (Joseph *et al.* 2009). The debate remains over whether raised serum Hcy concentrations cause CAD and stroke or the increased Hcy is merely due to the presence of pre-existing atherosclerotic processes (Faeh *et al.* 2006).

Homocysteine and diabetes

Mean plasma Hcy concentrations are normal or low in patients with uncomplicated DM1 (Cotellessa *et al.* 2001; Wiltshire *et al.* 2001; Meloni *et al.* 2005). The cause of low plasma Hcy relative to the high prevalence of future CVD is not conclusively known. However a direct relation between micro- and macroalbuminuria (Chico *et al.* 1998; Robinson *et al.* 1998), decreased glomerular filtration rate (Wollesen *et al.* 1999), diabetic nephropathy (Buysschaert *et al.* 2000), CVD (Parving *et al.* 1996) and increased plasma Hcy levels have been reported.

2.7. Arterial stiffness

The role of arterial stiffness in the pathophysiology of cardiovascular events

The function of large arteries such as the aorta is to deliver continuous and steady blood flow into the arterioles and capillaries. The conduit function of larger arteries is dependent on mean BP, blood flow and the relationship between them. Ventricular ejection generates a primary pressure wave that moves away from the

heart at a finite speed, measured as pulse wave velocity (PWV). Propagation of the pulse wave is inversely related to the distensibility of the arterial tube. Mechanical properties of arterial walls are important determinants of the propagation and reflection of pressure waves along the arteries. The pressure wave is reflected at any point of structural or geometric discontinuity of the arterial tree, generating a reflected wave that travels backward toward the ascending aorta. This reflected wave returns to the central aorta during diastole, enabling the heart to receive adequate blood flow through the coronary arteries to meet its metabolic requirements. The higher is the arterial stiffness, the higher is the speed of travel of the forward and retrograde waves. This can cause a premature return of the reflected waves during late systole, increasing central pulse pressure (PP), thus systolic BP. Systolic BP increases the load on the left ventricle, increasing myocardial oxygen demand. The increase in central PP and the decrease in diastolic BP may directly cause subendocardial ischaemia. In addition, arterial stiffness is associated with left ventricle hypertrophy, a known risk factor for coronary events (Laurent *et al.* 2006).

The aortic stiffening which accompanies aging and CV risk factors is caused by various phenomena, including breaks in elastin fibers, accumulation of collagen, fibrosis, inflammation, medial smooth muscle necrosis, calcifications, and the diffusion of macromolecules within the arterial wall (Laurent *et al.* 2005).

Assessment of arterial stiffness and wave reflections

Increased central arterial stiffening is a hallmark of the aging process and the consequence of many disease states such atherosclerosis, diabetes and chronic renal damage. The measurement of arterial stiffness is thus assuming an increasing role in the clinical assessment of patients. Three groups of non-invasive methods for the assessment of arterial stiffness are used: 1) measuring PWV, 2) assessing arterial pressure waveforms, and 3) relating change in the diameter of an artery to the distending pressure measured by ultrasound or magnet resonance imaging.

Aortic PWV has emerged as the "gold-standard" measurement of regional arterial stiffness, since it is the simplest noninvasive and reproducible method, and is supported by the greatest number of epidemiological studies for its predictive value for CV events (Laurent *et al.* 2006). PWV measured along the aortic and aortoiliac pathway is the most clinically relevant since the aorta and its first branches, which are elastic arteries in young subjects, are responsible for most of the pathophysiological effects of arterial stiffness. The waveforms are obtained transcutaneously over the common carotid artery (CCA) and the right femoral artery. Arterial pulse waves can be detected by using pressure-sensitive transducers, Doppler ultrasound, or applanation tonometry. The distance (D) covered by the waves is assimilated to the distance measured between the two recording sites. The time delay (t) from the proximal to the peripheral artery is divided by the distance between the measurement sites to calculate the velocity (PWV= D (meters) /t (seconds)).

Pressure wave analysis (PWA) gives additional information about arterial stiffness. According to a recent consensus document on arterial stiffness (Laurent *et al.* 2006), arterial pressure waveforms should be analysed at the central level (at the ascending aorta) since it represents the true load imposed on the left ventricle and central large artery walls. The aortic pressure waveforms are estimated from the radial artery waveform, using a transfer function, derived from invasive pressure and flow data obtained by cardiac catheterization (Pauca *et al.* 2001). The pressure waveform can be recorded noninvasively with applanation tonometry. The integral software generates an averaged peripheral and corresponding aortic waveform that is used for the determination of the augmentation index (AIx) and timing of the reflected waveform (Tr). The arterial pressure waveform is a composite of the forward pressure wave created by ventricular contraction and the reflected wave. In the case of stiffer arteries, PWV rises and the reflected wave arrives back at the central arteries earlier, adding to the forward wave and augmenting the systolic pressure. This phenomenon can be quantified through the AIx, defined as the difference between the second and first systolic peaks expressed as a percentage of PP (Figure 2). The first peak of the pulse wave is caused by left ventricular ejection, while the second peak is the result of wave reflection. The AIx, a predominant determinant of wave reflections, depends also on several factors, including gender, ventricular ejection, height, heart rate, mean arterial BP and aortic PWV, and provides an indirect measure of arterial stiffness. The Tr represents the composite travel time of the pulse wave to the periphery, the main reflectance site (aortic bifurcation) and its return to the ascending aorta, thus providing the surrogate measure of aortic PWV. Pulse waves should be analysed through three major parameters: central PP, central systolic BP and the AIx (Laurent *et al.* 2006). PP is the difference between systolic and diastolic BP, and is determined by cardiac output and arterial stiffness (Dart *et al.* 2001).

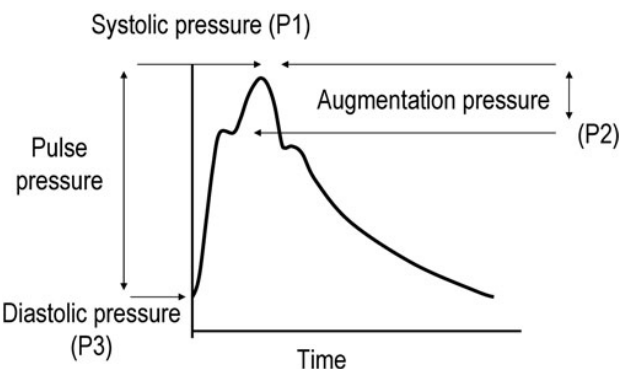

Figure 2. Carotid pressure waveform is recorded by applanation tonometry. The height of the late systolic peak (P1) above the inflection (P2) defines the augmentation pressure, and the ratio of augmentation pressure to PP defines the AIx (in percent) (adapted from Laurent *et al.* 2006).

McEniery *et al.* (2005) studied normal vascular aging and found that, although both the AIx and aortic PWV increased significantly with age, these changes were nonlinear. Thus AIx increased more in individuals younger than 50 years and PWV in subjects of age greater than 50 years.

Echotracking systems provide optimal conditions for a precise determination of local arterial stiffness, which is directly measured and requires no assumption from models of the circulation. But this method requires a high degree of technical expertise, and takes longer time than measuring PWV, therefore it is indicated for mechanistic analysis in pathophysiology, pharmacology, and therapeutics, rather than for epidemiological studies (Laurent *et al.* 2006).

Predictive value of arterial stiffness and wave reflections for CV events

Aortic stiffness has independent predictive value for all-cause and CV mortality, fatal and non-fatal coronary events and fatal strokes in patients with uncomplicated essential hypertension (Boutouyrie *et al.* 2002; Laurent *et al.* 2001; Laurent *et al.* 2003), DM2 (Cruickshank *et al.* 2002) and end-stage renal disease (Blacher *et al.* 1999; Shoji *et al.* 2001), as well as in elderly subjects (Meaume *et al.* 2001; Sutton-Tyrrell *et al.* 2005). The Rotterdam Study group (Mattace-Raso *et al.* 2006) and the Danish participants in the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) health survey (Willum Hansen *et al*. 2006) reported that aortic PWV provides prognostic information beyond that from traditional risk factors, including age, gender, BP, cholesterol, diabetes, and smoking. One reason may be that aortic stiffness integrates the damage of CV risk factors on the aortic wall over a long period of time, whereas BP, lipids and blood glucose concentration can fluctuate over time and their values, recorded at the time of risk assessment, may not reflect the true values damaging the arterial wall (Laurent *et al.* 2006).

Central AIx and PP is found to be an independent predictor of all-cause mortality in end-stage renal disease (London *et al.* 2001), and CV events in patients undergoing percutaneus coronary intervention (Weber *et al.* 2005) and in the hypertensive patients of the CAFÉ study (Williams *et al.* 2006).

A recent consensus document (Laurent *et al.* 2006) stated that arterial stiffness and central pressure measurements should be considered as recommended tests for the evaluation of CV risk, particularly in patients in whom target organ damage is not discovered by routine investigations.

Treatment of arterial stiffness

A large number of publications and several reviews have reported changes in arterial stiffness and wave reflections after various interventions. The consensus document on arterial stiffness (Laurent *et al.* 2006) summarizes them. Non-pharmacological treatments which are able to reduce arterial stiffness include

exercise training, dietary changes (including weight loss, low salt diet, moderate alcohol consumption, garlic powder and fish oil). Pharmacological treatments which are able to reduce arterial stiffness include antihypertensive treatment (such as diuretics, beta-blockers, ACE-inhibitors and calcium-channel inhibitors); alpha-linoleic acid; treatments of congestive heart failure; hypolipidaemic agents such as statins; antidiabetic agents, such as thiazolidinediones; estrogen replacement therapy; sildenafil, and AGE-breakers such as alagebrium (ALT-711).

The AGE-breakers break down established AGE crosslinks between proteins within the arterial wall, thereby reducing arterial stiffness. The results of clinical trials so far have shown some beneficial CV effects of alagebrium in elderly patients with increased systolic pressure, diastolic heart failure or increased aortic stiffness. However, the effects were far less than expected from the results of animal studies. Alagebrium breaks only one type of cross-links (a-diketone), but does not affect the more abundant glucosepane cross-links (Susic 2007).

Long-term, large-scale therapeutic trials are needed to determine whether a reduction in arterial stiffness proves to be more effective in preventing CV events than usual care and is a desirable goal in terms of hard clinical endpoints such as morbidity and mortality.

Arterial stiffness in childhood

Both aortic and carotid stiffness increase throughout childhood and adolescence, with the fastest increase observed during the first few years of life (Fernhall *et al.* 2008). The aortic capacitance (flow at a given pressure) increases throughout childhood, primarily as a function of increased arterial size, whereas the distensibility of the arterial wall actually decreases (Ahimastos *et al.* 2003; Senzaki *et al.* 2002). The increase in arterial capacitance suggests that arterial buffering capacity (the ability of the aorta to facilitate increased blood flow with minimal changes in BP) increases independently from changes in arterial wall elasticity. Thus the increase in arterial size appears to offset the increase in wall stiffness, preventing an increase in afterload that could adversely affect ventricular performance (Fernhall *et al.* 2008). This is different from the effect of agining in adults, where arterial capacitance decreases with a corresponding increase in arterial wall stiffness.

The age-associated increase in arterial wall stiffness in children is also supported by several studies showing increases in both aortic and peripheral PWV from childhood through puberty (Avolio *et al.* 1983; Cheung *et al.* 2002; Niboshi *et al.* 2006). However, CV risk factors such as higher BP and body mass index (BMI), physical inactivity and high dietary fat intake are related to increased arterial stiffness in healthy children (Jourdan *et al.* 2005; Schack-Nielsen *et al.* 2005). It has been suggested that the degeneration of the arterial wall begins in childhood, causing decreases in elastin, increases in collagen, and ultimately the beginning of the atherosclerotic process (Newman *et al.* 1991;

Berenson *et al.* 2002; Niboshi *et al.* 2006). Cheung *et al.* (2004) showed that preterm children with intrauterine growth retardation have increased systemic arterial stiffness and mean BP later in childhood, indicating the importance of prenatal influences on vascular function.

Arterial stiffness and diabetes

There are only a few previous studies using PWA to measure arterial stiffness in adults with DM1 (Brooks *et al.* 1999; Wilkinson *et al.* 2000; Tryfonopoulos *et al.* 2005; Sommerfield *et al.* 2007; Gordin *et al.* 2007; Gordin *et al.* 2008) and only one study in children with DM1 (Haller *et al.* 2004). Most of the studies support the concept of increased arterial stiffness in DM1. Altered pressure wave reflections are even apparent in children with DM1 as young as 10 when compared to matched controls (Haller *et al.* 2004). The effects of large and small artery changes in diabetes combine to alter arterial stiffness and wave reflection characteristics. Whether aortic stiffness is more important than changes in central pressure wave shape in the pathophysiology of CVD in DM1 is not known and further studies are needed.

There is a poor correlation between arterial stiffness to the traditional CVD risk factors (HbA1c, LDL-cholesterol and family history) and even to the novel serum CVD risk factors (IL-6, tumor necrosis factor, hsCRP, monocyte chemoattractant protein-1 and NO) in DM1 (Haller *et al.* 2004; Haller *et al.* 2006; Zineh *et al.* 2009). Notably, a genetic association between arterial stiffness and a NO3 gene polymorphism has been seen (Zineh *et al.* 2007). A recent report from EURODIAB investigators showed that the magnitude of PP, an estimate of arterial stiffness, was strongly and independently associated with increased formation of AGEs (Schram *et al.* 2005) and with the incidence of CVD (Schram *et al.* 2003) in young DM1 individuals. Acute hyperglycaemia during the hyperglycaemic clamp is found to influence wave reflections (Gordin *et al.* 2007) and the higher mean daily glucose concentration increases aortic PWV (Gordin *et al.* 2008), emphasizing the importance of strict daily glycaemic control. The potential reduction of arterial stiffness following atorvastatin therapy is publicized by the Pediatric Atorvastatin in Diabetes Trial (PADIT) (Haller *et al.* 2009).

2.8. Carotid artery intima media thickness

Arteriosclerosis is a term that usually describes diffuse thickening and stiffening of mainly large- and medium-sized arteries. The clinical significance of arteriosclerosis is related to the progressive stiffening of arterial trunks, progressive narrowing at some particular sites, and the risk of atherothrombosis. Decades of silent arterial wall alterations precede vascular clinical events, which then reflect advanced atherosclerotic disease. The first morphological abnormalities

of arterial walls can be visualized by B-mode ultrasonography. This high-resolution, non-invasive technique is one of the best methods for the detection of early stages of atherosclerotic disease, because it is rapidly applicable, readily available and demonstrates the wall structure (Touboul *et al.* 2007). Ultrasound measurement of the two internal layers of the carotid artery is a validated technique (Pignoli *et al.* 1986) and correlates well with histological findings of the same region of the vessel wall (Persson *et al.* 1994).

The recent consensus document of carotid IMT measurements (Touboul *et al.* 2007) definite IMT as a double-line pattern visualized by echotomography on both walls of the CCA in a longitudinal image. It is formed by two parallel lines, which consist of the leading edges of two anatomical boundaries: the lumen-intima and media-adventitia interfaces. According to this consensus document, the standard sites of IMT measurement are the CCA, carotid bulb, and internal carotid artery. The arterial wall segments should be assessed in a longitudinal view and IMT should be measured preferably on the far wall, along a minimum of 10 mm length of an arterial segment. Measurement of IMT should be performed in a region free of plaque where the double-line pattern is observed and mean IMT values are averaged across the segment. It is accepted that IMT values from the left and right side could be averaged. Adventitia-to-adventitia diameter and intraluminal diameter of CCA must also be measured as IMT is significantly correlated with the arterial diameter. The standard equipment includes a high-resolution B-mode system operating with preferentially linear ultrasound transducers at frequencies >7 MHz. An appropriate depth of focus (e.g. 30-40 mm) should be used and the optimal frame rate is 25 Hz (>15 Hz).

Several large population-based studies have shown that IMT can predict future clinical events such as myocardial infarction or stroke, and IMT contains information beyond the classic CV risk factors. A recent meta-analysis with data of 200 000 person-years in 8 studies found that carotid IMT is a strong predictor of future vascular events. For an absolute carotid IMT difference of 0.1 mm, the future risk of MI increased by 10% to 15%, and the stroke risk increased by 13% to 18% (Lorenz *et al.* 2007).

The *Paroi Art´erielle et Risque Cardio-vasculaire* (PARC) study found a significant correlation between CCA-IMT and all components of the Framingham CVD risk score. When the analysis was performed according to decades, younger subjects had a steeper relationship. However variations in CCA IMT only explained a modest part of the Framingham score and vice versa- the variances explained by each other were 19% in men and 28% in women (Touboul *et al.* 2007). This variation in disease is probably due do genetic susceptibility combinations of different risk factors and interactions between genetic and environmental factors. Since the classical CVD risk factors provides only a limited part of the real prediction, additional IMT measurement could help in selecting and targeting subjects at intermediate risk in primary prevention. In some countries recommendations already suggest that carefully performed IMT measurement can add incremental information to traditional risk factor assessment (Graham *et al.* 2007).

The associations between IMT and novel CVD risk markers have also been studied. Lorenz *et al.* (2007) did not find a correlation between hsCRP and carotid intima-media progression. There is also data that plasma Hcy level is not associated with IMT (Durga *et al.* 2004 and 2005). Maas *et al.* (2009) observed that higher plasma ADMA concentrations were independently associated with greater internal carotid artery/bulb IMT, but not with CCA-IMT in the large community-based sample of the Framingham Heart Study.

Pooled analysis of published randomized controlled trials by Bots *et al.* (2003) showed an overall annual rate of change in mean CCA-IMT of 0.0147 mm/y (95% CI 0.0122–0.0173) and for subjects with previous coronary heart disease, estimates were 0.0170 mm/y (95% CI 0.0114–0.0227).

There are also an increasing number of paediatric publications evaluating IMT in children. The normative values for IMT have been described in relation to age, height and gender (Jourdan *et al.* 2005; Böhm *et al.* 2009). A mean IMT increase of 0.02 mm in 2 years was found for girls until an age of 14/15 years. In boys, the mean IMT increased 0.04 mm from age 8/9 to 10/11. IMT values were significantly higher in boys than girls. Predictors for age-adjusted IMT were systolic BP in boys and weight in girls (Böhm *et al.* 2009). The Cardiovascular Risk in Young Finns Study showed that some childhood risk factors were independently associated with increased adult IMT, including elevated systolic BP, high LDL-cholesterol and smoking, but not CRP (Juonala *et al.* 2006).

IMT in patients with type 1 diabetes

Although clinically evident macroangiopathic complications of DM1 infrequently occur in children, increased CCA-IMT has been found as early as 4 years after diagnosis (Järvisalo *et al.* 2004). The prospective study by Pozza *et al.* (2009) found that mean IMT progression during a 2-year period did not exceed the physiological IMT increase in children with DM1, in whom an increased IMT had been found at the beginnig of the study. Only children with a higher HbA1c and a higher systolic BP showed progressive IMT. The DCCT/EDIC showed that intensive therapy during the study, presumably mediated through improved glycaemic control, resulted in decreased progression of IMT six years after the end of the DCCT trial. The differences in the HbA1c values during the DCCT explained 96 percent of the long-term differences between IMT groups at year 6 (Nathan *et al.* 2003). The best predictor for IMT progression in regard to markers of endothelial dysfunction and inflammation (hsCRP and fibrinogen, soluble vascular cell adhesion molecule-1, ICAM-1, and E-selectin and fibrinolytic markers) was plasma fibrinogen (Lopes-Virella *et al.* 2008).

Sander *et al.* (2009) found HbA1c and hsCRP to be independent predictors of IMT progression in adult patients with DM1. Subjects with HbA1c and hsCRP in the upper 2 quartiles had a four times increased risk for new vascular events.

2.9. Bone mineral density in type 1 diabetes

Osteoporosis is a systemic disease characterized by low bone mass and micro-architectural deterioration of bone tissue, resulting in an increase in bone fragility and susceptibility to fracture. Osteoporosis in childhood is defined as low bone mineral content (BMC) or BMD in the presence of a clinically significant fracture history (either a long bone fracture of the lower extremities, a vertebral compression fracture, or two or more long-bone fractures of the upper extremities). Low BMC or BMD are defined as Z-score values equal or less than −2.0, adjusted for age, gender, and body size as appropriate (Bianchi *et al.* 2009). Peak BMC, defined as maximum bone mass gain, is mostly maintained until the end of the second decade of life and it is an important factor determining bone mass in adult life (Matkovic *et al.* 1994).

DM1 is the most frequent endocrine disorder which may have negative effects on bone turnover and mineralization. Recent meta-analysis by Vestergaard (2007) showed that patients with DM1 are at increased risk of bone fractures and reduced BMD. A survey of a prospective cohort of 32,089 postmenopausal women in the Iowa Women's Health Study revealed that women with DM1 were 12 times more likely to report hip fractures than women without DM1 (Nicodemus *et al.* 2001).

The risk of hip fractures and loss of bone density are evident in both male and female patients with DM1 (Miao *et al.* 2005). However, data about the gender influence on bone loss is inconsistent, with reports indicating decreased BMD in only males (Hamilton *et al.* 2009) or in only females patients with DM1 (Auwerx *et al.* 1988). Patients with diabetes complications, i.e. retinopathy, neuropathy, nephropathy and macroangiopathy have increased risk of fracture and lower BMD (Miao *et al.* 2005). The increased fracture risk could also be related to nonskeletal risk factors, such as a propensity for falls (Leidig-Bruckner *et al.* 2001), conceivably mediated through impaired proprioception, balance, and gait due to neuropathy, visual impairment from diabetic retinopathy and cataracts (Ivers *et al.* 2001), or frequent nocturia (Nelson *et al.* 2001).

Bone loss can begin at the onset of diabetes in children (Gunczler *et al.* 2001; Heap *et al*. 2004; Salvatoni *et al.* 2004; Moyer-Mileur *et al*. 2004; Léger *et al.* 2006), but there are reports of children with DM1 who do not exhibit bone loss (Pascual *et al.*1998; Karagüzel *et al.* 2006; Brandao *et al.* 2007). Moreover, it has recently been showed that patients who manifest DM1 at an early age may have only transiently impaired bone development (Bechtold *et al.* 2007). Insufficient skeletal mineralization during puberty has been implicated as a mechanism that might explain the lower BMD in patients with DM1.

Mechanisms contributing to DM1 related bone loss are unknown, but there are many theories. The decrease in osteoblast number and/or osteoblast differentiation is suggested to contribute to the reduced bone formation (McCabe 2007). Insulin deficiency and the resultant deficiency of insulin-like growth factor have been proposed as an important factor in impaired bone formation (Jehle *et al.* 1998). Increased Ca-excretion due to hyperglycaemia (McNair *et*

al. 1979), functional hyperparathyroidism and disturbances in vitamin D metabolism, which are particularly prominent in patients with nephropathy (McNair *et al.* 1981; Bouillon 1991), are other plausible factors. Mechanisms that lead to a decreased bone biomechanical competence besides decreases in BMD are alterations in the glycosylation of collagen. The AGE-s and their receptors play an important role in bone metabolism and bone strenght (Yamagishi *et al.* 2005).

Studies of bone mineralization in children and adults with DM1 have using various techniques and there is a great variability in auxologic data and metabolic control of the patients, making definitive conclusions difficult. Moreover, the interpretation of densitometric data in young people is difficult because the "normal" BMD values to be used for comparison are continuously changing with age, and also depend on other variables such as gender, body size, pubertal stage, skeletal maturation and ethnicity. Routine screening or initiation of preventative medications for osteoporosis is not currently recommended in DM1. The prevalence of DM1 is growing, and many more patients will survive long enough to develop hip fractures. Hip fractures are associated with considerable morbidity and long-term mortality (Clague *et al.* 2002), posing a major and growing burden on health care. Further studies are needed to clarify the mechanisms contributing to diabetic bone loss and patient selection for osteoporosis screening and prevention.

3. AIMS OF THE STUDY

1. To compare the biochemical cardiovascular risk markers between children with type 1 diabetes against age- and sex-matched controls, using an array of traditional and novel markers.
2. To compare arterial stiffness and carotid intima-media thickness, as markers of subclinical atherosclerosis, between children with type 1 diabetes against matched controls.
3. To compare bone mineral density parameters between children with type 1 diabetes against matched controls.
4. To determine the relationships between bone mineral density, physical activity, glycaemic control and markers of oxidative stress and inflammation, in children with type 1 diabetes.

4. SUBJECTS AND METHODS

4.1. Study population

The study included 30 Caucasian children with DM1 (mean age 13.1±3.6 [range 4.7–18.6] years, 19 boys) attending the Diabetes Clinic at the Tartu University Children's Hospital, Estonia and 30 healthy Caucasian control subjects, matched by age (±2 years), sex and body mass index (BMI) (±3 kg/m^2). The clinical characteristics of the study groups are shown in Table 2. By Tanner pubertal stage, patients with DM1 were divided: 6 boys and 1 girl in stage 1, 4 boys and 4 girls in stages 2 or 3, 9 boys and 6 girls in stages 4 or 5. Control subjects were divided by pubertal stage: 7 boys and 1 girl in stage 1, 3 boys and 3 girls in stage 2 or 3, 9 boys and 7 girls in stage 4 or 5. All patients with diabetes were without clinical evidence of vascular complications. The mean duration of diabetes was 5.4±3.4 [range 1.0–14.6] years. The patients were treated with a multiple insulin injection regimen (n=21) or two daily injections of a mixture of short- and long-acting insulin (n=9). The mean daily insulin dose was 0.88±0.2 IU/kg/day. Inclusion criteria both for children with diabetes and control subjects were: age between 3 and 20 years, without acute infection and no history of using antihypertensive or lipid-lowering medications. Children with diabetes were included only if at least 1 year had passed from the diagnosis. The diagnosis of DM1 was based on the American Diabetes Association's criteria. Two children with diabetes also had celiac disease.

Table 2. Clinical characteristics of the study groups.

Variables	Diabetes group (n=30)	Control group (n=30)	*P*-value
Age (years)	13.1±3.6	13.2±3.9	0.9
Gender (M/F)	19/11	19/11	
Tanner stage 1 (n)	7	8	
Tanner stage 2 (n)	5	2	
Tanner stage 3 (n)	3	4	
Tanner stage 4 (n)	9	9	
Tanner stage 5 (n)	6	7	
Height (cm)	155.9±20.8	160±19.6	0.2
Height SDS	0.3±1.4	0.6±0.9	0.3
Weight (kg)	52.2±17.7	54.5±20.3	0.4
Body mass index (kg/m^2)	20.2±3.5	20.4±4.1	0.8
Duration of diabetes (years)	5.4±3.4	–	
HbA1c (%)	9.8±1.5	NM	
Glucose (mmol/l)	17.5 (10.7;19.7)	5.2 (4.9;5.4)	<0.0001
Insulin dosage (IU/kg/day)	0.88±0.24	–	
Creatinine (μmol/l)	60.4±14.2	62.0±16.0	0.6

Variables	Diabetes group (n=30)	Control group (n=30)	*P*-value
Creatinine clearance (ml/min/1.73m^2)	124.8±25.9	127.7±22.9	0.6
Systolic blood pressure (mmHg)	115.0±8.1	108.4±7.8	0.001
Diastolic blood pressure (mmHg)	62.6±5.7	57.0±5.2	0.003

The means with ±SD or median with inter-quartile range are shown.
NM, not measured; HbA1c, glycosylated haemoglobin

4.2. Methods

4.2.1. Study protocol

The study was planned as cross-sectional study.

Blood and urine samples were taken and PWA was performed after overnight fasting between 8:00 and 09:00 AM and in subjects with diabetes before insulin administration. Height was measured on a wall-mounted stadiometer and weight with a digital scale. The body mass index was calculated as weight (kilogram)/ height (meter)^2. Pubertal stage was assessed according to Tanner.

Children over 12 years of age and parents of those under 12 years completed a questionnaire about the child's milk and dairy product consumption, and about exercise level approximately 1 week before their clinic appointment. We also asked data about children's possible smoking habits and about family history of CVD.

IMT and DXA examination were made at the same visit with blood sample drawing or with an interval of a one week. After the first visit, subjects were asked to wear the accelerometer on 3 consecutive days.

Daily insulin doses and mean HbA1c over the 12 months before the study were obtained from the registry of the Outpatient Diabetes Clinic.

Informed consent was obtained from each subject and/ or their parents. The local ethical committee approved the protocol. The investigations were carried out in accordance with the principles of the Declaration of Helsinki.

4.2.2. Measurement of cardiovascular risk markers

4.2.2.1. Biochemical markers

The following biochemical markers were measured according to the manufacturer's instructions:

1. Creatinine, glucose, lipids (total cholesterol, LDL- and HDL-cholesterol and triglycerides) were analyzed by standard laboratory methods using certified assays in the local clinical laboratory. Glomerular filtration rate

was calculated using the paediatric Schwartz formula (Schwartz *et al.* 1976) (Paper I, II).

2. MPO plasma concentration was determined using an enzyme-linked immunosorbent assay kit (BIOXYTECH® MPO-EIA, OXIS Research, USA) (Paper II).
3. ADMA plasma concentration was determined using an validated ELISA kit (DLD Gesellschaft für Diagnostika und Medizi-nische Geräte mbH; Germany) (Paper I)
4. ICAM-1 plasma concentration was determined using human soluble ICAM-1 CD54 immunoassay (R&D Systems Europe; United Kingdom) (Paper I, III)
5. tHcy plasma concentration was determined using an Axis Homocysteine EIA kit (Axis-Shield Diagnostics Ltd; United Kingdom) (Paper I)
6. Adiponectin plasma concentration was determined using Human Adiponectin/Acrp30 Immunoassay (R&D Systems Europe Ltd; United Kingdom) (Paper I)
7. hsCRP was determined in serum using a chemiluminescence enzyme immunometric assay on an Immulite immunoassay analyser (DPC; USA) (Paper I, III)
8. The urinary content of F_2-IsoPs was determined using a competitive ELISA assay (BIOXYTECH® 8-Isoprostane Assay, OxisResearch®, USA) (Paper III). The urinary concentration of F_2-IsoPs was corrected by urinary creatinine concentration to account for the differences in renal function.
9. HbA1c was measured by latex immunoagglutination inhibition test using a DCA 2000+ Analyzer (Bayer Diagnostics Europe Ltd; Ireland).

The subjects above the upper limit of the normal range of hsCRP (>5 mg/l) were excluded in the comparison of hsCRP between the diabetes and the control group (n=3 in diabetes group and n=1 in control group), as having apparently some infection or inflammatory disorder (Paper I and III).

4.2.2.2. Blood pressure measurement

Periferal arterial BP was measured three times after at least 5 minutes rest using a validated oscillometric technique and a cuff of appropriate size on the patient in supine position (OMRON M4-I; Omron Healthcare Europe BV®, Netherlands). The mean of the triplicate measurements was used in analysis.

Central arterial BP, PP and mean arterial BP were calculated from the integration of the radial pressure waveform using the Sphygmocor software (SphygmoCor Px, Version 7.0; AtCor Medical, Australia) (Paper II). Peripheral PP was calculated as the difference between peripheral systolic and diastolic BP (Paper II).

4.2.2.3. Assessment of arterial stiffness using systolic pulse wave analysis

Indices of arterial stiffness were measured using the SphygmoCor apparatus (SphygmoCor Px, Version 7.0; AtCor Medical, Australia). A high-fidelity micromanometer (SPT-301B; Millar Instruments, USA) was placed on the radial artery, and gentle pressure was applied until 15–20 sequential unvaried waveforms were produced. The integral software was used to generate an averaged peripheral and corresponding aortic waveform that was used for the determination of the AIx and Tr. AIx represents the difference between the second and first systolic peaks of the central arterial waveform, expressed as the percentage of central PP. AIx have been used as a measure of wave reflection. Because AIx depends on heart rate (Wilkinson *et al.* 2000), AIx corrected to a heart rate of 75 (AIx@75) was used for the analysis. The Tr represents the composite travel time of the pulse wave to the periphery, the main reflectance site (aortic bifurcation) and its return to the ascending aorta. Aortic PWV was measured using the same device by sequentially recording ECG-gated carotid and femoral artery waveforms. The difference in carotid to femoral path length was estimated from the distance from the sternal notch to the femoral and carotid palpable pulse.

Because age-adjusted normative values for children are missing, we used a case-control comparison for evaluating arterial stiffness in the diabetes group. PWA was performed only in children older than 8 years (n=52). Measurements with a quality index >74 were used in analysis of Tr and AIx@75 (n=48). A quality index is calculated by the software and represents reproducibility of waveform. A value of <74 is considered to demonstrate insufficient waveform consistency, according to the manufacturer's instructions. The four children whose PWA quality index was <74 were not different from the others in any of the clinical characteristics shown in Table 2. Therefore the data of PWA in 24 case-control pairs were used to compare AIx@75 and Tr, in 26 case-control pairs to compare PWV.

4.2.2.4. Ultrasound examination of carotid artery intima-media thickness

Ultrasound examinations were performed using Acuson Sequoia 512 (Siemens Medical Solutions, Mountain View, USA) ultrasound equipment with a 14.0 MHz linear-array transducer. Both CCA were scanned by a single radiologist using standardized protocol (Iglesias del Sol *et al.* 2002). Using the zoom function magnified images 2 cm high and 3 cm wide in an optimal longitudinal view were obtained and recorded in a moving scan and sent to the Picture Archiving and Communication System. At least three scans were performed on both CCA with continuously recorded electrocardiograms. The images were recorded from an angle that showed the greatest distance between the CCA lumen-intima-media thickness. The actual measurements of IMT were performed off-line. Digitally stored images were manually analyzed by a single reader. The best-quality end-diastolic frame was selected from each stored clip.

From this image at least three measurement of the CCA far wall were taken approximately 10 mm proximal to the bifurcation. The average of the IMT of each of the three selected images was calculated. For each individual, the final common carotid IMT was determined as the average of far-wall measurements of both the left and right arteries. The performer-reader of the ultrasound images was unaware of the case status of the subject. Six subjects were studied twice within two days and the between-study coefficient of variation in IMT measurements was 1.2%.

For the calculation of the SDS of IMT, the age and height-specific normative values by Jourdan *et al*. (2005) were used. As the normative values were available only for children older than 10 years and a height of more than 139 cm, 24 case-control pairs out of 30 were composed. Because IMT is found to be more closely related to height rather than age (Jourdan *et al*. 2005), we used height-specific IMT SDS for correlation analysis.

4.2.2.5. Heredity

We obtained information about the family history of hypertension and early heart disease (before 55 years of age) in the first and second-degree relatives of a child.

4.2.3. Measurement of bone mineral density

Total body and lumbar (L1–L4) BMC and BA were measured by DXA (GE Lunar DPX-IQ; USA). BMD was calculated by BMC/BA. The BMD data obtained from DXA is expressed as grams per centimeter squared, not as true volumetric density and therefore, is influenced greatly by the bone size. To minimize the effect of bone size on BMD values, a total BMC (g) were adjusted for age and height (BMCadj; %) by Warner *et al*. (1998) and we calculated the apparent volumetric lumbar BMD (BMDvol; g/cm^3) using the Kröger formula (Kröger *et al.* 1995).

4.2.4. Measurement of physical activity

4.2.4.1. Questionnaire

Children over 12 years of age and parents of those less than 12 years completed a validated modified questionnaire (Godin *et al.* 1985) about the child's exercise level approximately 1 week before their clinic appointment. Subjects were asked to indicate how many times they participate in different forms of exercise for more than 15 minutes during their free time in an average week. Exercise was classified into three categories, strenuous exercise (heart beats rapidly), moderate (not exhausting) and mild exercise (minimal effort). A total score was

generated (exercise in an average week) by summing the reported frequencies for each of the three categories after multiplying each category with the corresponding MET score (9, 5, or 3, respectively).

4.2.4.2. Accelerometer

Physical activity was measured by a uniaxial accelerometer (ActiGraph, USA). Subjects were asked to wear the accelerometer through the day (not during the night) on 3 consecutive days. Each 3-day period included 2 days in school and 1 day at weekend. Data was analyzed by a specific software program provided by the manufacturer (ActiSoft Analysis Software version 3.2; USA). The mean activity count per hour over the 3-day period was used in the analysis.

4.2.5. Evaluation of milk and dairy products consumption

A questionnaire was used to assess daily milk (ml) and weekly dairy product consumption (milk, yogurt, cheese and ice cream). Patients were divided subsequently into three groups: low – none or only one dairy product once (or less) per week; moderate – at least two dairy products two to three times a weeks or daily milk consumption 125–250 ml; and high – two dairy products six to seven times a week or four to five times a week and daily milk consumption >250 ml, or daily milk consumption >500 ml.

4.2.6. Statistical analysis

The statistical calculations were performed using the statistical SAS Version 8.02 package (SAS Institute Inc., USA) (Paper I and II) and software R, version 2.4.1 for Windows (Paper III).

All data were tested for normality using the Kolmogorov-Smirnov test (Papers I–III). Continuous variables are presented as mean values with 95% confidence intervals (95% CI) in Paper I, while in Paper II and III variables with normal distribution are presented as mean values with SD. The variables with non-normal distribution are presented as median with the inter-quartile range. Qualitative variables are presented as absolute and relative frequencies.

Comparisons between the groups were performed using the Student's two-tailed t-test (Paper III) or non-parametric Mann-Whitney U test (Paper I). When analyzing the matched case-control pairs, comparisons between the groups were performed using the paired t-test or the non-parametric sign test (Paper II). To compare proportions (qualitative variables), the Fisher's Exact Test for counting data was used. Odds ratios (OR) and 95% CI were used to estimate relative risk (Paper I and II).

Relationships between two variables were evaluated by linear regression analysis, including partial correlation analysis (Paper II and III) or the Spearman Rank Correlation Test (Paper I).

Stepwise multiple linear regression analysis was used to determine the best set of predictors of BMD (Paper III) and blood pressure (Paper I). Since the distribution of the hsCRP values was skewed to the left, logarithmic transformation and a natural logarithm was employed to achieve approximate normality before the analysis.

All *P*-values were two-sided and differences were considered statistically significant if $P < 0.05$.

5. RESULTS

5.1. Heredity and smoking habits (Paper I, II)

The diabetes group had an increased family history of arterial hypertension (*15 cases vs. 9, OR =2.3 [95%CI: 0.7–7.7], P=0.19*) and smoking (*3 cases vs. 1, OR =3.2 [95%CI: 0.2-174.7], P=0.6*) compared to the control group, but this was not statistically significant.

5.2. The biochemical risk markers for CVD (Paper I, II, III)

5.2.1. The biochemical risk markers for CVD in children with type 1 diabetes and in healthy controls

Lipids profile

The two groups did not differ regarding serum total cholesterol, LDL- and HDL-cholesterol and triglycerides levels (Table 3).

hsCRP and ICAM-1

The levels of hsCRP and ICAM-1 were significantly higher in the diabetes group compared to the control group (Table 3). There were no significant associations between serum hsCRP concentrations and other measured CVD risk markers.

Myeloperoxidase and F2-Isoprostane

Median plasma MPO level was significantly higher in the diabetes group compared to the control group (Table 3). Children in the diabetes group with a positive family history of arterial hypertension had higher plasma MPO levels than children with a negative family history (*144.8±72.7 vs. 92.0±43.6 ng/ml, P=0.04*). Urinary F_2-IsoPs levels were not statistically different between the groups (Table 3).

Adiponectin

The mean plasma adiponectin concentration was significantly higher in the DM1 group compared with the control group (Table 3). In the diabetes group adiponectin correlated inversely with BMI (*r=–0.36*), but it was statistically not significant (*P=0.06*). In the control group adiponectin correlated positively with ICAM-1 concentration and negatively with pubertal stage and creatinine level (Table 4).

ADMA

The mean plasma ADMA concentration was significantly lower in the diabetes group compared with the control group (Table 3). Plasma ADMA concentration correlated inversely with age (Figure 3), pubertal stage and BMI in both groups and in addition with serum creatinine level in the diabetes group (Table 4).

Homocysteine

The mean plasma tHcy concentration was significantly lower in the diabetes group compared to the control group (Table 3). Plasma tHcy concentration correlated positively with pubertal stage, serum creatinine level and systolic BP and inversely with plasma ICAM-1 concentration in both groups. It also correlated positively with age and BMI in the diabetes group (Table 4).

Table 3. Biochemical markers of the study groups.

Variables	Type 1 diabetes group (n=30)	Control group (n=30)	*P*-value
Homocysteine (μmol/l)	5.9 (5.3–6.5)	7.8 (6.9–8.8)	0.0008
ADMA (μmol/l)	0.55 (0.5–0.6)	0.67 (0.6–0.8)	0.03
Adiponectin (μg/ml)	14.45 (11.8–17.1)	10.67 (8.5–12.8)	0.03
hsCRP (mg/l)	0.9 (0.7–1.2)	0.7 (0.3–1.2)	0.02
ICAM-1 (ng/ml)	293 (276–310)	251 (227–276)	0.002
Myeloperoxidase (ng/ml)	88.7 (63.0;168.0)∗	61.4 (48.0;72.3)∗	0.006
F_2-IsoPs (pg/mg creatinine)	1773±814∗	1595±774∗	0.5
Cholesterol (mmol/l)	4.6 (4.2–5.0)	4.2 (3.9–4.5)	0.2
LDL-cholesterol (mmol/l)	2.8 (2.5–3.2)	2.5 (2.3–2.8)	0.2
HDL-cholesterol (mmol/l)	1.7 (1.6–1.8)	1.7 (1.5–1.8)	0.8
Triglycerides (mmol/l)	1.0 (0.7–1.2)	0.8 (0.6–0.9)	0.6

The means with 95% confidence intervals are shown.
∗The mean with ±SD or median with inter-quartile range are shown.
ADMA, Asymmetric dimethylarginine; hsCRP, high sensitivity C-reactive protein; ICAM-1, Intercellular adhesion molecule-1; F_2-IsoPs, 8-iso-prostaglandin F_{2a}; LDL, low-density lipoprotein; HDL, high-density lipoprotein

5.2.2. Associations between biochemical markers of atherosclerosis and glycaemic control and blood pressure

The duration of diabetes, HbA1c and blood glucose level did not demonstrate any significant correlations with serum hsCRP, urinary F_2-IsoPs levels or plasma ADMA, tHcy, adiponectin and MPO concentrations. Children with diabetes had significantly higher systolic and diastolic BP than the controls

(Table 2). In the multivariate regression analysis diabetes group, BMI and tHcy were the most important determinants of systolic BP, explaining 38% of its variability ($R^2adj=0.38;\ P<0001$). Triglycerides and the diabetes group were the most important predictors of diastolic BP, explaining 27% of its variability ($R^2adj=0.27;\ P<0.0001$). However, the multiple regression analysis was based only in markers used in Paper I.

Table 4. Spearman correlation coefficients in type 1 diabetes and control group

	Type 1 diabetes group (n=30)			Control group (n=30)		
	Adiponectin	tHcy	ADMA	Adiponectin	tHcy	ADMA
Age	n.s	0.50 **	–0.59 **	n.s	n.s	–0.51 **
Tanner stage	n.s	0.54 **	–0.55 **	–0.38 *	0.37 *	–0.50 **
BMI	n.s	0.36 *	–0.52 **	n.s	n.s	–0.46 *
Creatinine	n.s	0.48 **	–0.46 *	–0.42 *	0.40 *	n.s
ICAM-1	n.s	–0.45 *	n.s	0.44 *	–0.40 *	n.s
Systolic BP	n.s	0.38 *	n.s	n.s	0.37 *	n.s
Diastolic BP	n.s	n.s	n.s	n.s	n.s	n.s

BMI, body mass index; ADMA, Asymmetric dimethylarginine; tHcy, total Homocysteine; ICAM-1, Intercellular adhesion molecule-1; BP, Blood pressure
n.s, not significant (*P>0.05)*
* *P<0.05*
** *P<0.01*

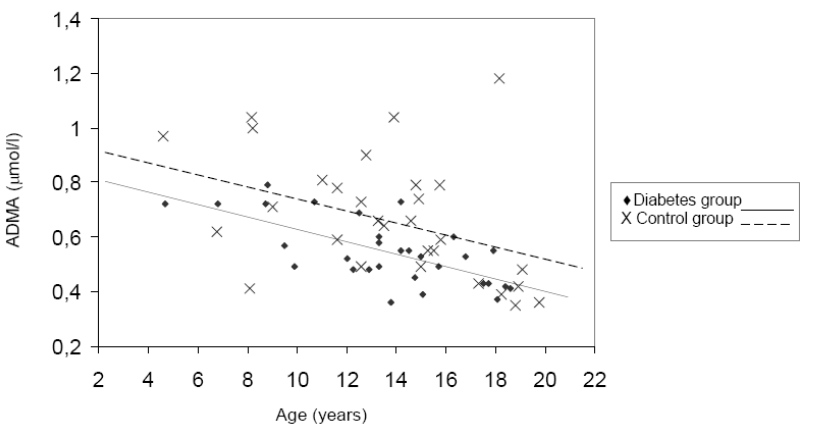

Figure 3. Correlation between plasma asymmetric dimethylarginine (ADMA) level and age in patients with diabetes (*diamonds, solid line*) (*r=–0.59; P=0.001*) and in the control group (*crosses, dashed line*) (*r=–0.51; P=0.004*).

5.3. Arterial stiffness and carotid artery intima-media thickness in children with type 1 diabetes and in healthy children (Paper II)

5.3.1. Arterial stiffness

Children with diabetes had increased wave reflection, measured by higher AIx@75 (Table 5). The two groups did not differ regarding either PWV or Tr. Central and peripheral PP were not significantly different between the groups. In the diabetes group PWV was partially correlated with diabetes duration after adjusting for mean arterial BP and age, which are both well-known determinants of PWV (*r=0.49, P=0.02*). Blood glucose concentration and HbA1c value did not correlate with any of the characteristics obtained by PWA.

5.3.2. Carotid artery intima-media thickness

The absolute IMT and IMT SDS were significantly higher in children with diabetes (Table 5). IMT SDS correlated positively with HbA1c in the diabetes group (*r=0.39, P=0.05*) (Figure 4).

Table 5. PWA, PWV and IMT characteristics of the study groups.

Variables	Type 1 diabetes group (n=30)	Control group (n=30)	*P*-value
Central systolic BP (mmHg)	97.0±5.2	90.6±6.1	<0.0001
Central diastolic BP (mmHg)	64.7±5.3	58.7±5.2	0.001
Mean arterial BP (mmHg)	78.1±4.9	71.34±5.0	<0.0001
Central PP (mmHg)	53.8±7.7	52.9±5.8	0.7
Peripheral PP (mmHg)	32.3±4.8	31.9±4.0	0.8
IMT (mm)	0.453±0.06	0.419±0.05	0.005
IMT SDS height-specific (n=24)	1.44±1.2	0.68±0.8	0.02
IMT SDS age-specific (n=24)	1.45±1.2	0.85±0.9	0.05
AIx (%) (n=24)	2.85±11.6	3.66±8.85	0.8
HR (beats/min) (n=24)	74.02±14.5	63.19±9.9	0.01
AIx@75 (%) (n=24)	4.787±13.0	–2.22±10.6	0.02
Tr (ms) (n=24)	145.8 (137.8;151.8)	148.0 (136.7;180.0)	0.1
PWV (m/s) (n=26)	5.42±0.7	5.16±0.6	0.1

The mean with ±SD or median with inter-quartile range are shown.
BP, blood pressure; PP, pulse pressure; IMT, Intima media thickness; IMT SDS, IMT standard deviation score; HR, heart rate; AIx, Augmentation index; AIx@75, Augmentation index corrected for heart rate 75; Tr, Timing of the reflected waveform; PWV, pulse wave velocity; PWA, pressure wave analysis

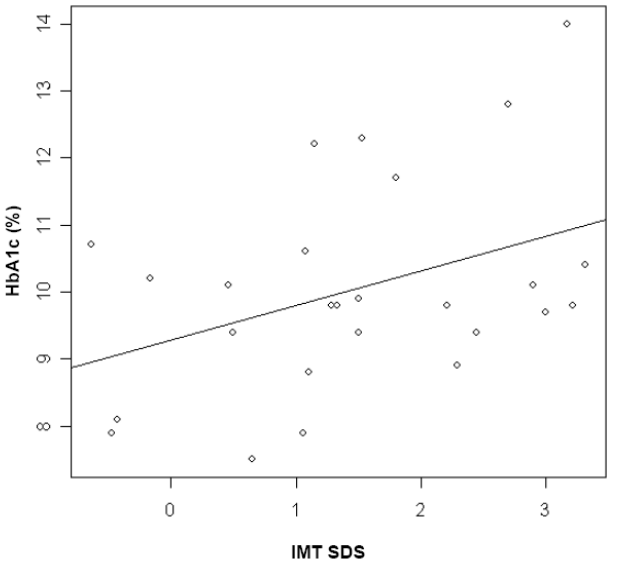

Figure 4. Correlation between mean HbA1c (%) and IMT standard deviation score (IMT SDS) in patients with diabetes (*r=0.39, P=0.05*).

5.4. Bone mineral density in children with type 1 diabetes, associations with inflammation- and oxidative stress-related markers, physical activity and glycaemic control (Paper III)

5.4.1. Characteristics of bone mineral density

BMC, BA and areal BMD of total body and lumbar region were not different between the diabetes and the control group. The mean lumbar BMDvol and total BMCadj were significantly lower in the diabetes group than in their sex- and age-matched controls (Table 6). Differences remained significant even after the two children with celiac disease were excluded from analysis. The differences in these two parameters were mostly caused by the differences in boys (total BMCadj *99.7±8.0 vs. 105.8±6.2, P=0.01*; lumbar BMDvol *0.29±0.06 vs. 0.33±0.07, P=0.07*), whereas in girls total BMCadj and lumbar BMDvol did not differ significantly. According to stepwise regression analysis where all 60 subjects were included, the most significant determinants of total BMCadj were the presence of diabetes and male gender (both negatively) ($R^2adj=0.19$, *P=0.001*); whereas the most important determinants of lumbar BMDvol were age (positively), male gender (negatively) and the presence of diabetes (negatively) ($R^2adj=0.58$, *P<0.0001*). There was no significant difference in BMD characteristics in either group regarding their Ca intake.

Table 6. Comparison of values of bone and physical activity characteristics.

Variables	Type 1 diabetes group (n=30)	Control group (n=30)	*P*-value
Total BMC (g)	2001.8±800	2268±865	0.2
Total BA (cm^2)	1931.6±549	2046.3±549	0.4
Total BMD (g/cm^2)	1.0028±0.14	1.067±0.16	0.1
Total BMCadj (%)	101.8±7.7	107.0±5.7	0.005
Lumbar BMC (g)	39.4±17.6	45.6±21.7	0.2
Lumbar BA (cm^2)	42.2±9.5	43±10.6	0.7
Lumbar BMD (g/cm^2)	0.88±0.24	1.007±0.29	0.07
Lumbar BMDvol (g/cm^3)	0.32±0.08	0.36±0.09	0.05
Physical activity (count/h) Entire group	18151±7962	21295±7519	0.1
Physical activity (count/h) Boys	18231±6613	24145±7449	0.04
Physical activity (count/h) Girls	18014±10252	16372±4689	0.8
Activity score by questionnaire	81.0±42.7	71.9±40.6	0.8

The means with ±SD are shown.
BMC, bone mineral content; BA, bone area; BMD, bone mineral density; Total BMCadj, predicted total bone mineral content; Lumbar BMDvol, apparent volumetric lumbar bone mineral density

5.4.2. BMD associations with physical activity

Weekly activity score and physical activity measured by accelerometer were not different between the diabetes and the control group. However, boys with diabetes were less active than the control boys measured by accelerometer (Table 6). We did not find any correlations between physical activity and BMD parameters.

5.4.3. BMD associations with inflammation- and oxidative stress-related markers

In the diabetes group lumbar BMDvol, but not total BMCadj, was negatively correlated with plasma ICAM-1 levels (*r=–0.4; P=0.02*) (Figure 5) and urinary F_2-IsoPs levels (*r=–0.5; P=0.005*) (Figure 6). We did not find any correlations between hsCRP and BMD parameters.

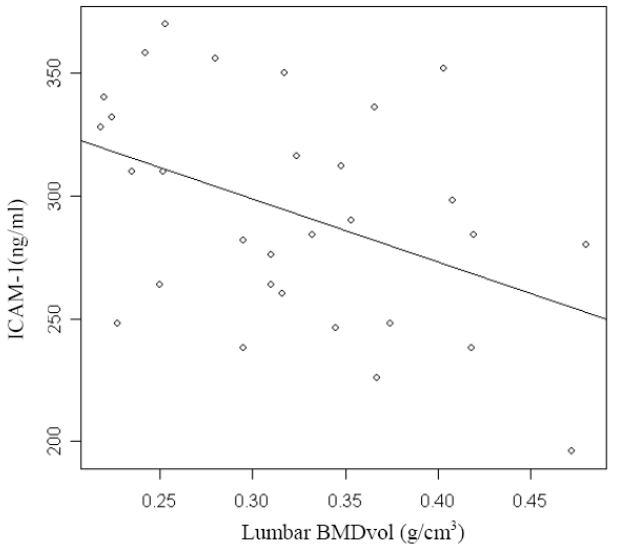

Figure 5. Correlation between mean plasma intercellular adhesion molecule-1 (ICAM-1) levels and Lumbar BMDvol in patients with diabetes (*r=–0.4; P=0.02*).

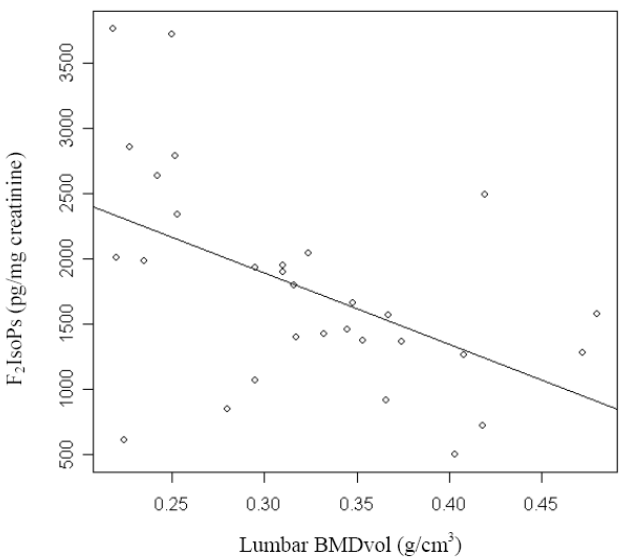

Figure 6. Correlation between mean urinary 8-iso-prostaglandin F_{2a} (F_2IsoPs) levels and Lumbar BMDvol in patients with diabetes (*r=–0.5; P=0.005*).

5.4.4. BMD associations with the glycaemic control

There was a negative correlation between total BMCadj and HbA1c level (*r= –0.4; P=0.02*) (Figure 7). The correlation remained significant after adjustment for gender in partial correlation analysis. Children with HbA1c level more than 10.6% had statistically significantly lower total BMCadj than those with HbA1c levels below this cut-off value (*103.28±7.6 vs 96.75±6.3; P=0.05*). Lumbar BMDvol is highly age dependent and therefore has to be adjusted for age. Using partial correlation analysis lumbar BMDvol was significantly correlated with HbA1c when adjusted for age (*r=–0.45; P<0.05*), as well as when adjusted for age and gender (*r=–0.39; P<0.05*). In multivariate regression analysis HbA1c, age and gender were the most important determinants of lumbar BMDvol in the diabetes group explaining 58% of its variability ($R^2adj=0.58$, *P<0.0001*).

We did not find any significant correlation between BMD parameters and physical activity, ICAM-1, F_2-IsoPs, HbA1c or hsCRP levels, if examined separately in boys or girls with diabetes.

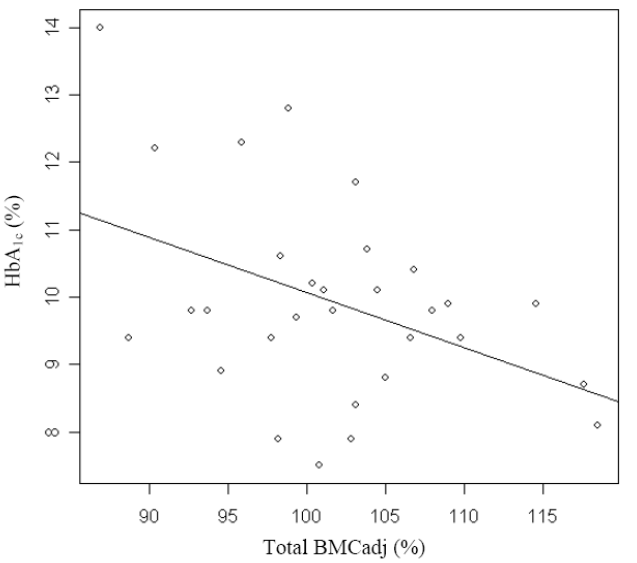

Figure 7. Correlation between mean glycosylated haemoglobin (HbA1c) levels over the last year and Total BMCadj in patients with diabetes (*r=–0.4; P=0.02*).

6. DISCUSSION

6.1. Importance of testing vascular function and bone mineral density in patients with type 1 diabetes

DM1 is an important risk factor for CVD (Laing *et al*. 2003). The cumulative incidence of CVD increases significantly with the presence of diabetic nephropathy (Tuomilehto *et al.* 1998) and when conventional CV risk factors such as hyperlipidemia, smoking and hypertension are present (Stamler *et al.* 1993).

Endothelial dysfunction has been demonstrated to occur early in the course of diabetic vascular complications, starting in childhood. With DM1 hyperglycaemia, increased circulating fatty acid levels and altered lipoproteins provoke molecular mechanisms that contribute to vascular dysfunction: decreased bioavailability of NO, profound OxS and prothrombotic activity, disturbances of intercellular signal transduction, and activation of receptors for AGE-s (Creager *et al.* 2003).

Over the last decade, several new possible risk markers have been suggested as contributors for CVD. An increase in ICAM-1 (Ridker *et al.* 1998), tHcy (Nygard *et al.* 1997) and ADMA (Schulze *et al.* 2006) levels or a decrease in plasma adiponectin concentrations (Cesari *et al.* 2006) are in focus. These CVD risk markers are not sufficiently studied in patients with DM1 and the previous studies reveal conflicting results. Therefore we studied a panel of potential biochemical CVD risk markers (comprising traditional and new markers) as well as atherosclerosis-related structural and functional changes of the arterial wall, measured by IMT and PWA in children with DM1 (Paper I and II). The early detection of CVD risk factors and prevention of CVD in childhood could be a potentially valuable tool in reducing CV mortality and morbidity in adulthood.

Patients with DM1 are also at risk of decreased BMD (Vestergaard 2007). The mechanisms behind impaired bone metabolism in DM1 are not clear. Puberty is known to be an important time period in terms of peak bone mass gain, in which bone formation occurs at a higher rate compared to other stages of life (Matkovic *et al.* 1994). Insufficient skeletal mineralization during puberty has been implicated as a mechanism that might explain the lower BMD in adult patients with DM1. Therefore we decided to investigate BMD in children and adolescents with DM1 and to determine the relationships between BMD, physical activity, glycaemic control and markers of oxidative stress and inflammation (Paper III).

6.2. The biochemical risk markers for cardiovascular disease in children with type 1 diabetes (Paper I, II and III)

It is generally accepted that HbA1c levels have a central role in the prediction of micro- and macrovascular complications in DM1 (Donaghue *et al.* 2007). However, the recommendations in current guidelines and the level of metabolic control actually achieved during routine care, differ widely. In a multicenter study which took place in Austria and Germany only 45% of children and young adults had a good glycaemic control (as defined HbA1c values <7.5%) (Gerstl *et al.* 2008). Our study group had a rather poor metabolic control with a mean HbA1c level of 9.8%. In our study about two thirds (n=23) of diabetic children were in puberty. It has been shown that in puberty the average HbA1c has been significantly higher than in childhood (Mortensen *et al.* 1997; Danne *et al.* 2001), probably due to poor compliance and decreased insulin sensitivity. Thus, achieving good metabolic control in patients with DM1 still remains a challenge for paediatric endocrinologists.

Dyslipidemia is a traditional risk factor for atherosclerosis and CVD. Patients with DM1 usually exhibit a normal conventional lipid profile. However, poor glycaemic control is associated with alterations in lipoprotein subclass distribution (Jenkins *et al.* 2003) and qualitative abnormalities of lipoproteins, such as glycation and oxidation (Orchard *et al.* 1999). These changes make lipoproteins more atherogenic. The serum triglyceride, total cholesterol, LDL and HDL levels did not differ between the diabetes and control groups in our study. Nevertheless, traditional risk factors of CVD such as diabetes itself, higher serum triglycerides level and BMI were significant predictors for raised BP. Data from the Pittsburgh Epidemiology of Complications Study, a 10-year follow-up of patients with childhood onset DM1, showed that high BP and increased LDL were independent risk factors for microvascular disease, macrovascular disease, and mortality (Orchard *et al.* 2001). Although nobody met the criteria of arterial hypertension in our study, children with DM1 had higher BP compared to the control group. It has been suggested that hypertension has a greater impact on CVD in patients with diabetes than in non-diabetic individuals (Stamler *et al.* 1993).

Hyperglycaemia is known to modulate expression of cell adhesion molecules and cytokines which through monocyte-endothelium interactions leads to the initiation and progression of atherosclerosis. It has been recently shown in the Diabetes Control and Complications Trial that higher plasma ICAM-1 levels predict an increased risk of progressive nephropathy in DM1 and may represent an early risk marker that reflects the important role of vascular endothelial dysfunction in this long-term complication (Lin *et al.* 2008). Children with DM1 had the elevated level of markers of endothelial activation and inflammation, as ICAM-1 and hsCRP, compared to the healthy children in our study. Several inflammatory biomarkers, particularly hsCRP, have been identified as likely predictors for the development of CVD (Ridker 2003). According to a

statement of the American Heart Association and the Centers for Disease Control and Prevention (2003), an hsCRP value more than 1 mg/l is considered to be a risk factor for CVD in the adult population (Pearson *et al*. 2003). Our children with diabetes had a mean hsCRP 0.9 mg/l, which is very close to this limit.

Hyperglycaemia in diabetes is also closely associated with both glucose autooxidation and protein glycation. This increases the production of reactive species and OxS-driven consiquences (Brownlee 2000). We measured F_2-IsoPs as a marker of systemic OxS. F_2-IsoPs are formed by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid and can be reliably measured in both plasma and urine (Awad *et al.* 1993; Wang *et al.* 1995). F_2-IsoPs can nowadays be considered as one of the most reliable markers of OxS (Morrow *et al.* 1996). Recent studies have shown that the formation and urinary excretion of F_2-IsoPs is elevated in children with DM1 (Flores *et al.* 2004; Davì *et al.* 2003). We found slightly elevated F_2-IsoPs levels in children with DM1 compared to controls, but this difference was not statistically significant. The small sample size may be one reason why we were not able to show significant differences.

Modification of LDL by OxS (production of oxLDL) and extensive leukocyte activation are both important mechanisms of atherogenesis. MPO is a haemoprotein that is expressed in polymorphonuclear leukocytes and is secreted during their activation. In recent years, the activated MPO has been suggested as a trigger in the atherogenic modification of LDL as well as HDL *in vivo* (Heineke *et al.* 1999; McMillan *et al.* 2005, Schindhelm *et al.* 2009). High plasma MPO level is seen in patients with CAD (Zhang *et al.* 2001) and is an independent predictor for future CV events (Baldus *et al.* 2003; Brennan *et al.* 2003). The vascular-bound MPO can use high-glucose-stimulated hydrogen peroxide to amplify ROS species-induced vascular damage and the impairment of endothelium-dependent relaxation in animals with acute hyperglycaemia (Zhang *et al.* 2004).

We found significantly higher MPO plasma levels in children with DM1 than in the controls. According to our knowledge there have been no studies looking at the MPO levels in children with DM1. Children with a positive family history of arterial hypertension had higher plasma MPO levels than children without such a history. In the study by Vita *et al.* (2004) a significant inverse correlation was found between serum MPO level and NO-dependent flow-mediated dilatation of the brachial artery. An important consequence of MPO activity is consumption of NO and thereby induction of endothelial dysfunction. The enzyme MPO can convert NO into nitrating oxidants (Eiserich *et al.* 1998), which have been found to be potential mediators in CVD (Shishehbor *et al.* 2003). Therefore we can speculate that the oxidative stress-affected MPO pathway could be one link between systemic inflammation and exacerbation of diabetic vascular disease.

This study evaluated the plasma ADMA levels, a powerful endogenous inhibitor of NO-synthase (Segarra *et al.* 2001), in children with DM1. We found reduced ADMA concentration in children with diabetes. Most recent study by

Sibal *et al.* (2009) confirms our results. Nonetheless, normal or increased plasma ADMA levels have been found in patients with uncomplicated DM1 (Jehlicka *et al.* 2009; Altinova *et al*. 2007). Recently markedly elevated ADMA levels have been demonstrated in adult patients with diabetic nephropathy (Tarnow *et al.* 2004). In the study by Layer *et al.* (2008) ADMA levels above the median in patients with DM1 nephropathy significantly predicted fatal and nonfatal CV events and the development of end-stage renal disease. One conclusion might be that ADMA level is low in young patients with diabetes, whereas this increases in later stages with renal impairment.

There may be a number of reasons why children with DM1 have low plasma ADMA levels. One of the possible explanations is as follows: ADMA is formed during proteolysis of methylated proteins. Protein methylation is a mechanism of post-translational modification of proteins resulting in a modification of the tertiary structure and the biofunction of proteins. This process is catalysed by a group of enzymes named S-adenosylmethionine protein N-methyltransferases. Thus, formation of ADMA is a very complicated process and a persistent elevated level of glucose may result in the non-enzymatic glycation (modification) of enzymes/proteins involved in the formation of ADMA.

The decreased ADMA level may be one of the possible explanations why patients with diabetes have a higher level of NO and nitrosative stress. Recent studies on experimental animals have indicated that in hyperglycaemia NO production is increased, and when NO reacts with a superoxide anion, it forms peroxynitrite, a powerful damaging factor to endothelium (Coppey *et al.* 2001). Increased NO levels have also been found in human diabetes (Seckin *et al.* 2006). Furthermore low ADMA is found to be a significant determinant for a high LDL oxidation rate (Päiva *et al.* 2006). However, previous studies have shown that high, but not low plasma ADMA concentration is associated with increased risk of CVD in general population (Böger *et al.* 2009). Although the large community based Framingham Offspring Study observed significant association between plasma ADMA levels and all-cause mortality in individuals without diabetes, there was no association in those with diabetes (Böger *et al.* 2009). Therefore further studies evaluating the role of ADMA in patients with DM1 are necessary.

Plasma tHcy level depends on the kinetic properties of enzymes which participate simultaneously in tHcy and ADMA synthesis. A link between plasma hyperhomocysteinemia and nephropathy in patients with both DM1 and DM2 has been reported (Hofmann *et al.* 1998; Buysschaert *et al.* 2000). However, reduced tHcy concentrations have been found in children and adults without diabetic nephropathy (Cotellessa *et al.* 2001; Wiltshire *et al.* 2001; Meloni *et al.* 2005). As there are conflicting results regarding whether hyperhomocysteinemia is a cause or a result of CVD (The Homocysteine Studies Collaboration 2002), we also included tHcy in our panel to clarify its relation with early courses of atherosclerosis.

In our study children with diabetes had significantly lower tHcy levels than the controls. However the exact tHcy values remained within the normal reference values published by Vilaseca *et al.* (1997). Increased glomerular

filtration has been proposed as the mechanism which explains the lower than normal tHcy in patients without overt nephropathy (Wollesen *et al.* 1999). Plasma tHcy level correlated positively with creatinine level also in our study. But mean serum creatinine concentration and creatinine clearance did not differ between the groups. As we used calculated and not measured glomerular filtration rate, it is possible that in this way we were not able to detect mild changes in renal function. The decreased tHcy concentrations have also been partially explained by higher B_{12} vitamin and folate status in children with diabetes (Wiltshire *et al.* 2001) and by insulin deprivation (Abu-Lebdeh *et al.* 2006). We did not measure serum folate and vitamin B12 levels in these subjects and therefore can not rule out their protective effect on serum tHcy levels. Blood glucose level, HbA1c or insulin daily dose did not demonstrate any significant correlation with tHcy in our study. Although patients with diabetes had a lower plasma concentration of tHcy, in multivariate analysis tHcy was a significant predictor of higher systolic BP indicating its value as a risk marker for CVD.

The present study demonstrated that children with DM1 had elevated adiponectin levels, in agreement with the results of Celi *et al.* (2006), Barnes *et al.* (2008) and Galler *et al.* (2007). Adiponectin, a polypeptide synthesised mainly by adipocytes, has a wide range of biological activities, including an anti-inflammatory and anti-atherogenic effect (Matsuzawa 2005). Plasma adiponectin levels are reduced in patients with DM2 (Lindsay *et al.* 2002), obesity (Okamoto *et al.* 2006) and CAD (Cesari *et al.* 2006.). Conversely, increased plasma levels of adiponectin have been observed in patients with DM1 (Imagawa *et al.* 2002; Heliövaara *et al.* 2006). Patients with microvascular complications have even higher serum adiponectin levels than patients without complications (Frystyk *et al.* 2005). The mechanism responsible for the elevated adiponectin levels is not clear. Adiponectin levels have also been reported to be higher in dialysis patients (Zoccali *et al.* 2002). While adiponectin is excreted through kidneys, it has been suggested that decreased renal function may lead to an elevated adiponectin concentration (Looker *et al.* 2004). But this will not explain why children with a normal or increased glomerular filtration rate have elevated adiponectin levels. The previous studies have found an independent positive correlation between plasma adiponectin and markers of endothelial activation/injury in patients with DM1 (Schalkwijk *et al.* 2006), chronic renal failure and healthy individuals (Malyszko *et al.* 2004). The adiponectin expression is shown to be induced by inflammatory cytokines in vivo and in vitro (Delaigle *et al.* 2004). Therefore, increased adiponectin concentration could represent a beneficial compensatory mechanism to existing vascular damage. The increased plasma adiponectin levels have been found in patients with heart failure (Wannamethee *et al.* 2007) and high adiponectin levels were predictive for mortality in patients already afflicted with CVD (Dekker *et al.* 2008).

The limitation of the study was that the different isoforms of adiponectin were not measured. Adiponectin circulates as various isoforms and polymers, which may differ in receptor affinities as well as metabolic effects. Recent data

indicate that it is the plasma fraction of high molecular-weight polymers rather than the total concentration of adiponectin that is associated with its vasculo-protective effects (Aso *et al.* 2006). However, this information remains to be elucidated in the future.

In summary we found that children with DM1 with relatively poor glycaemic control have increased levels of plasma inflammatory markers, OxS and endothelial activation. We found lower levels of new possible CVD risk factors such as tHcy and ADMA, and higher levels of protective adiponectin. These three results are somewhat inexplicable, since children with diabetes had a higher risk of future CVD, which is normally associated with the opposite biochemical picture. Hcy and ADMA are intermediate products in normal metabolism of aminoacids. Lower tHcy and ADMA plasma level in DM1 is probably a reflection of altered protein metabolism due to high grade OxS and glucosylation or early renal dysfunction. These changes are most likely secondary to disturbed metabolic milieu in DM1.

6.3. Structural and functional changes of arteries in children with type 1 diabetes (Paper II)

PWA is a simple, non-invasive, reproducible method for assessing the indices of arterial stiffness (Laurent *et al.* 2006). Increased arterial stiffness is recognized as an independent CV risk factor and may also have a role in the process of atherosclerosis itself (Arnett *et al.* 1994). Arterial stiffening increases PWV and wave reflection, which augments central systolic pressure.

There are only a few previous studies using PWA to measure arterial stiffness in DM1 (Brooks *et al.* 1999; Wilkinson *et al.* 2000; Haller *et al.* 2004; Tryfonopoulos *et al.* 2005; Sommerfield *et al.* 2007; Gordin *et al.* 2007; Gordin *et al.* 2008). The study by Haller *et al.* (2004) has demonstrated increased AIx@75 in children with DM1. We found that children with diabetes have increased wave reflection, expressed as AIx@75, but normal PWV. This indicates that in children with DM1 the early atherosclerotic changes are more likely to occur in increased pulse wave reflection from periphery rather than aortic stiffness. Stiffening of the small arteries, as a consequence of either vasoconstriction or structural change in diabetes, can alter the magnitude and timing of reflected waves. Structural changes in the aorta are probably a later manifestation of atherosclerotic disease. We found that longer diabetes duration was related to stiffer aorta, expressed as PWV. Thus, we may speculate that adults with early onset DM1 are more likely to have increased stiffness of the aorta than those whose diabetes has started later.

The increased carotid IMT, measured by ultrasound, is an early marker of atherosclerotic changes of the arterial wall. Our children with diabetes had increased IMT compared to the control group as well as to the previously published normative data by Jourdan *et al.* (2005). Thus, children with DM1 have subclinical atherosclerosis as early as 5 years after diagnosis, similar to the

studies by Järvisalo *et al.* (2004) and Dalla Pozza *et al.* (2007), where the average diabetes duration was 4.4 and 6.2 years, respectively. Although controversy exists regarding the direct influence of blood glucose control on the development of atherosclerosis in diabetes, the relationship between glycaemic control and microvascular and macrovascular complication is well documented (The diabetes control and complication trial research group 1993; Nathan *et al.* 2005). The cornerstone of diabetes management is optimal control of hyperglycaemia. Our study group had relatively poorly compensated diabetes with a large variety of HbA1c. We found a positive correlation between mean HbA1c value over the year and IMT. The Diabetes Control and Complications Trial showed that progression of IMT was associated with the mean HbA1c value over the study period and that progression is slower using intensive insulin therapy (Nathan *et al.* 2003). Our study was not powered enough to study the impact of the insulin treatment regimen on IMT. We used recently published height and age-specific carotid IMT normative values published by Jourdan *et al.* (2005). Our control group had slightly increased IMT SDS (mean +0.68) compared to the normative values by Jourdan *et al.* (2005). This result is comparable to the study of Dalla Pozza *et al.* (2007), who used the same normative values and also found slightly increased IMT SDS in healthy children. They concluded that the different result might in part depend on the different techniques used for IMT measurement in studies or metabolic characteristics of the study population (Dalla Pozza *et al.* 2007). We used the same methodology for IMT measurement as Jourdan *et al.* (2005) with one exemption: we did not exclude children with a positive family history of CVD or arterial hypertension, as they did. This may explain the higher mean IMT in the control group.

The limitation of our study is that we did not measure diameter of CCA. According to recent consensus document (Touboul *et al.* 2007) adventitia-to-adventitia diameter and intraluminal diameter of CCA must also be measured as IMT is significantly correlated with the arterial diameter.

The study demonstrates that children with diabetes have atherosclerosis-related structural and functional changes of the arterial wall as early as five years after the diagnosis. Increased IMT is associated with poor glycaemic control. Therefore, the mainstay of DM1 management must be the optimal control of hyperglycaemia.

6.4. Bone mineral density in children with type 1 diabetes (Paper III)

Patients with DM1 have increased risk of osteopenia and fractures (Vestergaard 2007). Bone loss can begin already in childhood (Gunczler *et al.* 2001; Salvatoni *et al.* 2004; Moyer-Mileur *et al.* 2004; Heap *et al.* 2004; Léger *et al.* 2006), but there are reports of children with DM1 who do not exhibit bone loss (Pascual *et al.* 1998; De Schepper *et al.* 1998; Liu *et al.* 2003). BMD and BMC have been measured in children using different methods. Especially in children,

the DXA method has limitations because of the two-dimensional measurement and therefore dependence on height. To minimize the effect of bone size on BMD values, we adjusted total BMC for age and height and calculated the apparent volumetric lumbar BMD, which corrects the areal BMD value with the width of the vertebra.

The total BMCadj and lumbar BMDvol were significantly lower in children with DM1 than in controls. Some data indicates that a decrease in osteoblast number and/ or osteoblast differentiation contributes to reduced bone formation in DM1 (Krakauer *et al.* 1995). We found that the male gender was associated with lower BMD in children with DM1, similar to the study by Heap *et al.* (2004). In children with diabetes, a larger bone mass deficit has been described in girls compared to boys (Léger *et al.* 2006), whereas some other studies did not find sex-specific effect on BMD (Pascual *et al.* 1998; De Schepper *et al.* 1998). The study by Liu *et al.* (2003) found that BMD values were significantly lower, compared to the controls, only in women with DM1 who were older than 20 years and not in the younger group.

Our patients and controls were quite well matched by pubertal stage, but there were more prepubertal boys than girls. Puberty is known to be an important time interval in terms of peak bone mass gain, in which bone formation occurs at a higher rate compared to other stages of life (Matkovic *et al.* 1994). Although the mean age of boys at pubertal stages 2 or 3 in our study did not differ between the patients (n=4) and the controls (n=3) delayed onset of puberty can be still one reason why the boys with diabetes had lower BMD. More than a decade ago in much bigger study, Estonian boys with DM1 had a tendency to have delayed pubertal development with slightly reduced testicular volume in relation to age (Tillmann *et al.* 1996). Recent study by Rohrer *et al.* (2007) in a large German cohort of more than 2400 paediatric DM1 patients found that male, and not female gender was a significant determinant of the delayed pubertal onset. The same study found that elevated HbA1c level was associated with significantly delayed pubertal onset. In our study male patients had slightly higher mean HbA1c value than females (10.1% vs. 9.5%), but the difference was not statistically significant. However, our girls with diabetes had also lower BMD parameters compared to healthy girls, but these differences were statistically not significant. The small sample size (n=11) may be also one reason why we were not able to show significant differences between the girls regard to BMD characteristics.

Higher urine F_2-IsoPs and serum ICAM-1 level in children with DM1 correlated inversely with lumbar BMDvol, which represents more trabecular bone and not to total BMCadj, which represents more cortical bone. Trabecular bone where bone turnover is higher than in cortical bone is therefore potentially more susceptible to damage by inflammation and OxS. Cellular interactions through adhesion molecules and their impact together with cytokines represent an important concept in bone metabolism, particularly in inflammatory diseases. It has been suggested that elevated cytokine levels can activate osteoclast bone resorption and suppress osteoblast differentiation and bone formation (McCabe 2007). However, the exact mechanism of how ICAM-1 affects bone formation

is not entirely understood. In vitro studies have shown that OxS inhibits osteoblastic differentiation (Mody *et al.* 2001; Bai *et al.* 2004) and induces osteoblast insults and apoptosis (Chen *et al.* 2005). Hamada *et al.* demonstrated that streptozotocin-induced DM1 mice exhibited low-turnover osteopenia associated with increased OxS (Hamada *et al.* 2007). A link between the increased level of urinary F_2-IsoPs and reduced lumbar spine BMD has also been shown in population based human studies (Basu *et al.* 2001). The fact that ICAM-1 and F2-IsoPs in our study were correlated to lumbar BMDvol and not to total BMCadj suggests that lower spine BMD in patients with DM1 is associated with increased oxidative stress and inflammation.

In summary, the results of Paper III demonstrate that children with DM1 have lower BMD, which is related to poor glycaemic control, elevated markers of inflammation and OxS. These results suggest that children with diabetes who have a higher risk for vascular complications have also a higher risk for osteopenia. Therefore it is important to pay early attention also to BMD in those patients in addition to classical micro- and macrovascular complications.

7. CONCLUSIONS

1. Compared to healthy controls, children with type 1 diabetes had elevated markers of systemic inflammation (hsCRP), endothelial activation (ICAM-1) and cellular inflammation and oxidative stress (MPO). Children with diabetes had higher levels of adiponectin and lower levels of novel atherosclerotic metabolite risk markers (ADMA, tHcy). Systemic oxidative stress (measured by urinary F_2-IsoPs levels) did not differ between children with diabetes and healthy controls.

   The levels of traditional markers of dyslipidemia did not differ between the diabetes and control groups.

2. Children with type 1 diabetes had increased carotid intima-media thickness and pulse wave reflections as early as five years after diagnosis, indicating atherogenesis and an increased risk of CVD in those patients.

3. Compared to healthy children, children with type 1 diabetes had lower bone mineral density, which was particularly seen in the boys.

4. Poor glycaemic control, elevated markers of inflammation (ICAM-1) and oxidative stress (F_2-IsoPs), but not physical activity, were associated with lower bone mineral density in patients with DM1.

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SUMMARY IN ESTONIAN

Südame-veresoonkonna haiguste riskimarkerid ja luu mineraalne tihedus 1. tüüpi suhkurtõvega lastel

1. tüüpi suhkurtõbi (DM1) on autoimmuunhaigus, mis läbi kõhunäärme β-rakkude hävingu viib insuliini tootmise lakkamisele ja hüperglükeemiale. Suhkruhaiguse esinemissagedus suureneb üle maailma ning haigestuvad üha nooremad lapsed (Patterson *jt.* 2009). Kuigi on tõestatud, et hea glükeemiline kontroll aitab ennetada hilistüsistusi (The Diabetes Control and Complications Trial Research Group 1993), ei ole seda alati kerge saavutada. Tänapäevaste ravimeetoditega on õnnestunud vähendada küll mikroangiopaatiate esinemist, kuid mitte oluliselt suurte veresoonte ateroskleroотiliste kahjustuste teket DM1 patsientidel (Pambianco *jt.* 2006). Südame ja veresoonkonna haigused on täiskasvanueas DM1 patsientide peamine surma põhjus (Libby *jt.* 2005).

Tänapäeval on välja töötatud mitmeid uuringumeetodeid avastamaks veresoonte varaseid kahjustusi enne südame-veresoonkonna haiguste kliinilise pildi väljakujunemist. Arteriaalse süsteemi jäikuse ja unearteri sise- ja keskkesta paksuse hindamine mitteinvasiivsete meetoditega on saanud olulisteks vahenditeks varajase riski hindamisel (Lorenz *jt.* 2007; Laurent *jt.* 2006).

Subkliiniline põletik ja kestev oksüdatiivne stress omavad olulist rolli ateroskleroosi patogeneesis (Ross 1999; Harrison *jt.* 2003). Eelnevate uuringutega on näidatud, et mitmete põletiku- ja oksüdatiivse stressi markerite tase veres aitab ennustada haigestumust südame-veresoonkonna haigustesse (Danesh *jt.* 2000; Basarici *jt.* 2007). 8-isoprostaglandiin F_{2a} (F_2-IsoPs) tekib oksüdatiivse stressi poolt vahendatud rakumembraani fosfolipiidide lõhustumisel ning tema tase uriinis on üks paremaid süsteemse oksüdatiivse stressi markereid. Põletikutaseme ja sellega seotud ateroskleroотiliste haiguste riski hindamiseks on kasutatud kõrgtundlikul meetodil määratavat C-reaktiivset valku (hsCRV), plasma intertsellulaarse adhesioonimolekuli (ICAM-1) ning müeloperoksidaasi (MPO) taset. Ensüüm MPO eritatakse aktiveeritud leukotsüütide poolt ning kõrgenenud plasma MPO tase on seotud nii suurenenud põletikuaktiivsuse kui ka oksüdatiivse stressiga organismis (Klebanoff 2005). Kõrgenenud ICAM-1 tase veres viitab endoteeli seisundi häirele. Adhesioonimolekulid kontrollivad vererakkude kleepumist arterite sisekestale ning nende edasist liikumist läbi endoteeli, mis on oluline etapp ateroskleroosi patogeneesis.

Lisaks on uuringud näidanud, et esineb seos südame-veresoonkonna haiguste esinemise ja homotsüsteiini (tHcy), asümmeetrilise dimetüülarginiini (ADMA) ning adiponektiini plasma taseme vahel (Wald *jt.* 2002; Maas *jt.* 2007; Frystyk *jt.* 2007). tHcy ja ADMA kõrge tase veres on olulised ateroskleroosi tekke riskifaktorid. Hüperhomotsüsteineemia aterogeenset toimet seostatakse peamiselt võimega vallandada oksüdatiivset stressi. ADMA kõrgenened tase pärsib aga lämmastikoksiidi tootmist. Lämmastikoksiid on võimas antiateroskleroотiline molekul, mis osaleb arterite jäikuse regulatsioonis. Adiponektiin omab põletikuvastast toimet, samuti osaleb ta nii rasvade kui ka süsivesikute aine-

vahetuses. Metaboolse sündroomiga, 2. tüüpi suhkurtõbe ja südame-veresoonkonna haigusi põdevatel inimestel on leitud adiponektiini madal tase veres (Weyer *jt.* 2001; Rothenbacher *jt.* 2005).

Veresoonte varase kahjustuse mehhanismide parem tundmine võimaldab välja töötada täpsemaid parameetreid südame-veresoonkonna haiguste riski hindamiseks. Seetõttu on käesoleva uurimustöö eesmärgiks hinnata DM1 põdevatel lastel arterite funktsionaalset ja struktuurset seisundit paralleelselt biokeemiliste südame-veresoonkonna haiguste riskimarkeritega.

Eelnevad uuringud on näidanud, et südame-veresoonkonna haigused esinevad sageli koos osteoporoosiga (Magnus *jt.* 2005; Shaffer *jt.* 2007). Täiskasvanueas on DM1 põdevad inimesed ohustatud osteoporoosist ning seeläbi on neil ka suurenenud luumurdude tekke risk (Vestergaard 2007). Oma uuringus määrasime luutiheduse erinevaid parameetreid DM1 lastel. Kuna osteoporoosi sagedase esinemise põhjus DM1 patsientidel ei ole täpselt teada, siis otsustasime uurida seost luutiheduse ja oksüdatiivse stressi, põletikumarkerite ning kehalise aktiivsuse vahel.

Uurimuse eesmärgid

1. Uurida klassikalisi ja uusi südame-veresoonkonna haiguste biokeemilisi riskimarkereid 1. tüüpi suhkurtõvega lastel võrrelduna tervete lastega.
2. Võrrelda subkliinilise ateroskleroosi markereid – arteriaalse süsteemi jäikust ning unearteri sise- ja keskkesta paksust 1. tüüpi suhkurtõvega ning tervetel lastel.
3. Hinnata luutihedust 1. tüüpi suhkurtõvega lastel võrdlevalt tervete lastega.
4. Uurida kehalise aktiivsuse, glükeemilise kontrolli ning põletiku ja oksüdatiivse stressi markerite seost luutihedusega 1. tüüpi suhkurtõvega lastel.

Patsiendid ja meetodid

Uuritavateks olid 30 (11 tüdrukut) DM1 põdevat last, vanuses 4,7 kuni 18,6 aastat ja 30 sama soo ja sarnase vanuse (±2 aastat) ning kehamassiindeksiga (±3kg/m^2) tervet last. Suhkurtõve keskmine kestus oli 5,3±3,4 aastat ning ühelgi patsiendil ei esinenud suhkurtõve tüsitusi. Kõik DM1 lapsed olid jälgimisel SA TÜK Lastekliinikus.

Uuringud viidi läbi hommikul tühja kõhuga ning sukruhaigetel enne insuliini süsti. Määrati laste pikkus, kaal, kehamassiindeks, vererõhk ja puberteedi staadium.

Vereanalüüsist määrati seerumi hsCRV, veresuhkru, kreatiniini, lipiidide tase, plasma glükosüleeritud hemoglobiini, ICAM-1, adiponektiini, tHcy, MPO ja ADMA tase. Uriinist määrati F_2-IsoPs tase. Vere ja uriini analüüsid teostati Tartu Ülikooli Kliinikumi Ühendlaboris ja Tartu Ülikooli Biokeemia Instituudis.

Arteriaalse süsteemi jäikus määrati süstoolse pulsilaine analüüsi meetodil (SphygmoCor Px, versioon 7.0, AtCor Medical, Australia). Parema käe radiaalarterilt registreeriti perifeerne rõhulaine ja kasutades valideeritud ülekandefunktsiooni (Pauca *jt.* 2001) tuletati tsentraalne rõhulaine aordis, millelt mõõdeti tsentraalne vererõhk ja augmentatsiooni indeks (AIx). AIx on edasiliikuva ja tagasipeegeldunud rõhulaine vahe ülenevas aordis, väljendatuna protsentides pulsirõhust. AIx näitab seega eelkõige rõhulainete tagasipeegeldumise ulatust väikestest arteritest ja arterioolidest, mis on seoses nende jäikusega. Sama aparatuuri kasutades määrati ka pulsilaine levikukiirus aordis, mis võimaldab otseselt hinnata ateroskleroотilistest muutustest tingitud aordi jäikust (Laurent *jt.* 2006).

Unearteri sise- ja keskkesta paksuse mõõtmisel kasutati ultraheliaparaati Acuson Sequoia 512 (Siemens Medical Solutions, Mountain View, USA) ja 14.0 MHz andurit.

Kogu keha ja lumbaalpiirkonna luutihedus määrati densitomeetrial (GE Lunar DPX-IQ; USA). Luutiheduse näitajad kohandati vastavalt lapse pikkusele ning vanusele (Warner *jt.* 1998) või L1–L4 selgroolüli laiusele (Kröger *jt.* 1995).

Laste kehalist aktiivsust hinnati küsimustiku ja aktseleromeetriga (ActiGraph, USA). Küsimustik hõlmas lapse kehalist aktiivsust viimase ühe nädala jooksul (Godin *jt.* 1985). Aktseleromeetrit kanti 3 päeva jooksul (2 tööpäeva ja 1 puhkepäev). Aktseleromeeter ActiGraph on väike, kerge monitor, mis kinnitatuna puusale registreerib keha kiirendusi suuruses 0,05–2,0 G, sagedusega 0,25–2,50 Hz.

Suhkruhaige ambulatoorsest kaardist saadi andmed suhkurtõve kestuse, kompensatsiooni, tüsistuste esinemise ja insuliini raviskeemi kohta.

Tulemused ja järeldused

1. Võrreldes tervete lastega oli 1. tüüpi suhkurtõvega lastel kõrgem süsteemse põletiku näitaja (hsCRV), endoteeli aktivatsiooni markeri (ICAM-1), rakulise põletiku ning oksüdatiivse stressi markeri (MPO) tase veres. Suhkruhaigetel lastel esines kõrgem adiponektiini ning madalam uute biokeemilis-metaboolsete ateroskleroosi riskimarkerite (ADMA, tHcy) tase plasmas. Süsteemse oksüdatiivse stressi markeri (F_2-IsoPs) tase uriinis ei olnud suhkurtõvega lastel erinev tervetest.

   Klassikaliste düslipideemia markerite tase seerumis ei erinenud suhkruhaigetel lastel kontrollgrupi omast.
2. 1. tüüpi suhkurtõvega lastel esines suurenenud unearteri sise- ja keskkesta paksus ning rõhulainete tagasipeegeldumine. Antud tulemus näitab, et veresoonkonna varased ateroskleroотilised muutused esinevad lastel juba viie aasta möödudes suhkurtõve diagnoosimisest ning neil on suurenenud risk haigestuda südame-veresoonkonna haigustesse.

3. 1. tüüpi suhkurtõvega laste luutihedus oli madalam võrreldes tervete lastega. Muutused olid eriti väljendunud poistel.
4. Halb suhkurtõve glükeemiline kontroll, kõrgenenud oksüdatiivse stressi (F_2-IsoPs) ning põletikumarkeri (ICAM-1) tase, kuid mitte kehaline aktiivsus, olid seotud madalama luutihedusega suhkruhaigetel lastel.

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PUBLICATIONS

ELULOOKIRJELDUS

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Teadustegevus

Peamiseks uurimisvaldkonnaks on varased veresoonkonna aterosklerootilised muutused ja luutihedus 1. tüüpi suhkurtõvega lastel. Sellel teemal on ilmunud 3 teaduslikku artiklit, kaks suulist ja kolm posterettekannet rahvusvahelistel konverentsidel.

Teadusartiklite üldarv on kaheksa (neist seitse rahvusvaheliselt eelretsenseeritavates ajakirjades).

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