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NOVEL CARDIOVASCULAR RISK FACTORS IN CHILDHOOD

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2008

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LIST OF PUBLICATIONS

This thesis is based on following papers, which will be referred to by the Roman numerals:

I **Odermarsky M**, Andersson S, Pesonen E, Sjöblad S, Ylä-Herttuala S, Liuba P. Respiratory infection recurrence and passive smoking in early atherosclerosis in children and adolescents with type 1 diabetes. Eur J Clin Invest. 2008;38:381-388.

II **Odermarsky M**, Nilsson A, Lernmark A, Sjöblad S, Liuba P. Atherogenic vascular and lipid phenotypes in young patients with type 1 diabetes are associated with diabetes high-risk HLA genotype. Am J Physiol Heart Circ Physiol. 2007;293:H3175-H3179.

III **Odermarsky M**, Lernmark A, Truedsson L, Liuba P. Cutaneous microvascular dysfunction is associated with human leukocyte antigen-DQ in youths with type 1 diabetes. Pediatr Res. 2008;63:420-422.

IV **Odermarsky M**, Lernmark A, Sjöblad S, Truedsson L, Liuba P. Environmental tobacco smoke, diabetes-risk HLA and heart rate variability in young patients with type 1 diabetes. Submitted to Pediatric Cardiology.

V **Odermarsky M**, Pesonen E, Persson K, Liuba P. Cumulative adverse effects of repeated challenge with Chlamydia pneumoniae on cutaneous microvascular function in apoE-knockout mice. Submitted to American Journal of Physiology - Heart and Circulatory Physiology.

ABBREVIATIONS

Ach	acetylcholine
ANCOVA	analysis of covariance
ANOVA	analysis of variance
apoE-KO	apolipoprotein E-knockout
AUC	area under the curve
BMI	body mass index
C. pneumoniae	Chlamydia pneumoniae
CAC	carotid artery compliance
cGMP	cyclic guanosine monophosphate
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETS	environmental tobacco smoke
FMD	flow-mediated dilatation
GTN	glycerol trinitrate
HbA_{1c}	glycosylated hemoglobin A_{1c}
HDL	high-density lipoprotein
HF	high frequency
HFn	normalized high frequency
HLA	human leukocyte antigen
HRV	heart rate variability
hsCRP	high sensitivity C-reactive protein
ICAM	intracellular adhesion molecule
IMT	intima-media thickness
LDL	low-density lipoprotein
LF	low frequency
LFn	normalized low frequency
NO	nitric oxide
oxLDL	oxidized low-density lipoprotein
PU	perfusion unit
PCR	polymerase chain reaction
RTI	respiratory tract infection
SBP	systolic blood pressure
SDNN	standard deviation of normal-to-normal beats
SI	stiffness index
SNP	sodium nitroprusside
T1D	type 1 diabetes
TC	total cholesterol
TNF-alpha	tumor necrosis factor alpha

INTRODUCTION

Type 1 diabetes and cardiovascular disease

Individuals with type 1 diabetes (T1D) have a 2 to 3-fold increased risk for cardiovascular disease compared to non-diabetic subjects (1). Accelerated atherosclerosis appears to play an important role in the excess cardiovascular morbidity associated with T1D. Evidence suggests that non-invasive measures of early subclinical atherosclerosis in peripheral arteries of young patients with T1D could have prognostic value in cardiovascular risk prediction (2-4).

During the past decades, it has become increasingly apparent that exposure to a broad panel of risk factors in childhood may cause arterial damage with potentially long-term consequences for atherosclerosis-related morbidity. Many of such lesions occurring in early life are likely to become arrested or regress particularly in children that lack cardiovascular risk factors. In those with T1D, which commonly onsets in childhood, the prevalence of cardiovascular complications due to accelerated atherosclerosis remains high in the adult life.

Like in many other disorders with cardiovascular risk, much of the current data support the notion that atherosclerotic vasculopathy in T1D has a multifactorial etiology. A growing concept attributes an equal importance to both intrinsic and extrinsic factors that act in concert in the development of atherosclerosis (5-7).

It has been suggested that T1D increases the risk of cardiovascular disease through inflammatory, oxidative, and glucose-related events that lead to vascular endothelial damage (8) which is a key mechanism in atherosclerosis through all of its stages (9). Increasing evidence points out that a close-to-normal glycemic control in diabetic patients is not sufficient to fully prevent the widespread vasculopathy (10).

In patients with T1D, the atherosclerotic process begins at an earlier age compared with the general population (11). Children with T1D show increased intima-media thickness (3,12,13) and impaired endothelium-dependent vasomotor function (14) of peripheral arteries. Loss of carotid artery elasticity was previously demonstrated in young individuals with cardiovascular risk factors (15), and was suggested to independently predict cardiovascular events (16), supporting the view that arterial stiffening progresses hand in hand with atherosclerosis.

Autonomic neuropathy is a common complication of T1D, affecting several organ systems (17,18). Cardiovascular autonomic neuropathy, caused by damage of the autonomic nerve fibres innervating the heart and blood vessels, results in abnormalities of heart rate control and vascular dynamics (19,20). Decreased heart rate variability (HRV) is significantly related to development of coronary heart disease in individuals with T1D (21-23). In adult diabetic patients, decreased high frequency (HF) has been shown to be linked to cardiovascular morbidity and mortality (24).

Role of infections in atherosclerosis development

Infections have been hypothesized to contribute to atherosclerosis particularly in conjunction with other cardiovascular risk factors (25) but their precise role remains in dispute.

In experimental studies, a broad spectrum of pathogens has been proposed to contribute to atherosclerosis through multiple mechanisms including vascular endothelial damage, dyslipidemia, autoimmunity and endovascular infection (25). In a few prior pediatric studies, detrimental effects on arterial homeostasis were associated with clinically manifest acute infections. Thus, impairment of the brachial artery's endothelial function and thickening of the carotid artery intima-media were detected in otherwise healthy children but also in those with T1D weeks to months after clinical recovery from acute infections in the respiratory or urinary tract (26-28). In these studies, the magnitude of arterial damage observed after recovery from the infectious illness was not related to the severity of the illness, rendering conceivable the assumption that both mild and severe infections are important in triggering vascular changes. Flu-like infections have also been linked to increased incidence of major coronary events in middle-aged people (29).

Many of the previously suggested influences of infection on vasculature could be coupled to atherosclerosis if certain conditions are met. Pathogen burden, infection recurrence and co-existence with cardiovascular risk factors have been proposed as important prerequisites in order for this association to take place. Studies by Törmäkangas et al. showed that repeated rather than single infections in mice worsened atherosclerosis in the aorta through increased lipid accumulation although the blood levels of cholesterol remained unchanged (30). Intimal accumulation of lipids with subsequent lipid peroxidation contributes to stiffening of the arterial wall in part via inflammatory and proliferative changes initiated by oxidized lipoproteins (31). Recently, Mayr et al. showed in adults a rise in oxidized LDL (oxLDL) antibodies with increasing number of microbes known to cause chronic infection (32). Persistent co-infection with multiple pathogens predisposes to frequent infectious relapses, and this mechanism might have

played a role in the observed association between pathogen burden and lipid peroxidation. In Mayr's study, although the magnitude of atherosclerosis in the carotid arteries increased with pathogen burden, the investigators found no independent association between oxLDL antibodies and carotid atherosclerosis, which could imply that the infection-mediated pro-atherosclerotic effects involve multiple pathways.

In view of the multifactorial etiology of atherosclerosis, it is likely that the additional burden imposed by other vascular risk factors is important in the development of vascular changes following infection. This concept might have particular relevance in T1D since diabetic hyperglycemia seems to predispose to increased recurrence of both bacterial and viral infection by lowering the efficacy of the cell-mediated immune response (33).

Environmental tobacco smoke and vascular disease

Environmental tobacco smoke (ETS) exerts adverse vascular effects nearly as much as active smoking (34). Previous prospective study demonstrated an additive interaction between both active and passive smoking and chronic infections on plaque progression (35). In healthy children and young adults, ETS exposure correlated with the magnitude of atherogenic changes in peripheral arteries (36,37). Previous cell culture studies have shown an inhibitory effect of both infection (38) and ETS (39) on the vascular endothelial nitric oxide (NO) pathway. ETS could also affect the immune system, especially in the respiratory tract, via noxious substances in the tobacco smoke (40). This could in part explain the increased susceptibility of smoke-exposed children to RTI (41,42).

Human leukocyte antigen (HLA) system and vascular disease

Although the concept of heritability of cardiovascular disease has gained mounting attention during the past decade (43), there have been very few studies looking at the association between genetic factors predisposing for T1D and early stages of vascular disease in young diabetic patients (44,45).

A genetic susceptibility involving class II HLA genes is recognized in up to 80 % of young patients with T1D (46). The primary loci of genetic susceptibility have been mapped to the HLA-DQ region, which is located on chromosome 6 (47). Two HLA haplotypes, A1*0501-DQB1*0201 (DQ 2) and A1*0301-DQB1*0302 (DQ 8), appear to confer the highest risk for developing T1D (48), especially when both are present in the genotype (i.e., HLA-DQ 2/8).

The precise mechanisms whereby certain HLA increase diabetes susceptibility are not yet thoroughly clarified. There is a general consensus that the HLA-DQ molecules exert their effects in part via presentation of peptides from islet antigens to T cells, which contribute to destruction of insulin-producing cells (47,49). Previous studies (50,51) have shown that the expression of HLA molecules on the endothelial cells of islet microcirculation goes hand in hand with lymphocyte infiltration, thereby suggesting a possible pathogenic role of HLA-mediated endothelial vasculopathy in the development of T1D. Greening and colleagues demonstrated that both pancreatic and aorta endothelial cells expressing HLA class II molecules have the ability to process and present islet autoantigens (52). It is therefore conceivable to assume that endothelial cells of other arterial beds might also owe HLA-mediated antigen-presenting capacity in T1D. Particular HLA-DQ phenotypes, including DQ 2/8, appear to facilitate the presence of potentially pathogenic T cells in the peripheral circulation via thymic selection (53).

Lymphocyte accumulation within the arterial wall is an important mechanistic component of atherosclerosis development (54) and contributes to endothelial injury (55). Endothelial injury promotes, in turn, additional immune events, including release of chemokines and cytokines, with further endothelial transmigration of immune cells and CRP synthesis via liver activation by interleukin-6 (56). The increased inflammatory activity leads to alteration of lipoprotein metabolism characterized, in part, by an increase in LDL and a decrease in HDL cholesterol. Dyslipidemia prevails in T1D (57) and has an important role in endothelial injury and plaque development (58,59). CRP is slightly, yet significantly, elevated in diabetic children (60). Nevertheless, a slight rise in CRP, i.e., over 1 mg/l, appears to be predictive of the relative risk of future cardiovascular events in apparently healthy adults (61). Jarvisalo and colleagues found that even lower levels of CRP (i.e., 0.7 mg/l) were associated with decreased endothelial vasodilatory function of the brachial artery (62).

AIMS

The aims of studies I - IV were to investigate in young patients with T1D whether:

Study I	the frequency of acute infections correlates with atherogenic vascular changes
Study II – III	diabetes-predisposing genotype HLA-DQ 2/8 is associated with vascular phenotypes of atherosclerosis and systemic microvascular dysfunction
Study IV	exposure to ETS affects HRV, and whether this association is influenced by diabetes-risk HLA

The aim of:

Study V	was to investigate in an animal model susceptible to vascular disease whether repeated challenge with a respiratory pathogen poses cumulative adverse effects on microvascular function

MATERIALS

Subjects

All patients with T1D from the pediatric outpatient clinic at Lund University Hospital, fulfilling eligibility criteria, were invited to participate. Of 160 patients, 98 patients consented to participate.

Inclusion criteria were: diabetes duration of at least 6 months and age of at least 6 years. Exclusion criteria were: family history for other major cardiovascular risk factors (primary hypercholesterolemia, hypertension, and premature coronary and cerebrovascular disease), active smoking, systemic hypertension, asthma and allergy.

All patients were on insulin treatment (Levemir® or Lantus®, and Novorapid®). Four patients were on thyroid hormone replacement therapy (Levaxin®).

Patients were investigated up to 3 times, approximately 1 year apart.

The number of patients and the main methods per visit are summarized in Table 1.

Papers included in this thesis are based on data from the following timepoints:

1st visit: papers I and II
2nd visit: paper III
3rd visit: paper IV

Table 1. Overview of methods and number of patients per visit.

	1st visit	2nd visit	3rd visit
Number of patients	98	86	74
Health (including infection) questionnaire	+	+	+
Tobacco smoke exposure questionnaire	+	+	+
Carotid artery ultrasound	+	+	+
Brachial artery ultrasound	+	+	+
Laser Doppler and iontophoresis		+	+
Heart rate variability			+
HLA genotyping	+	+	+
Blood sampling	+	+	+

Data on demographic characteristics, medical history including age at onset of diabetes and diabetes duration, medication, weight, height, systolic and diastolic blood pressure, results of ocular examination, parental education and current occupation were obtained at each visit.

Detailed infection history (type and number of acute infections during the past year, type, length and severity of symptoms, medication if any, and time elapsed since the last infection prior to the visit) was determined by questionnaire.

Exposure to ETS was assessed using a previously standardized questionnaire (63), and defined as occasional or regular cigarette smoking in the presence of study participants in or outside the home (e.g. in or around school, playground or other public places). Household exposure to ETS was categorized in relation to the average number of cigarettes smoked per day in or around the home by the patient's cohabitants. The number of household smokers was also obtained.

Both questionnaires were filled out by patients or their guardians upon the visit, but were reviewed in a blind fashion (i.e., without being aware of the vascular, HRV, HLA, and biochemical data) after all patients completed the visit.

Study I

Ninety-eight children and adolescents (54 male and 44 female) aged between 7 and 22 (mean 15) years with diabetes duration between 1 – 19 (mean 7) years were included.
Patients were grouped as follows in relation to the number of acute infections during the past year: group 1 = 0 – 1 infections (low frequency); group 2 = 2 - 3 infections (moderate frequency), and group 3 = ≥ 4 infections (high frequency). Based on exposure to ETS, patients were divided into three groups: group 1 = no exposure during the past year; group 2 = occasional exposure, i.e. presence in a smoky environment less than once a week; and group 3 = weekly to daily exposure. The parental social status was deduced based on the level (secondary/high school = low, university or professional qualification = high) and number of years of education of the mother and the mother's partner.

Study II

Eighty-six children and adolescents (49 male and 37 female) aged between 7 and 22 (mean 15) years with diabetes duration between 1 and 19 (mean 7) years were included.

Study III

Seventy-five children and adolescents (45 male and 30 female) aged between 9 and 21 (mean 16) years with diabetes duration between 2 and 20 (mean 8) years were included.

Study IV

Seventy-four children and adolescents (38 male and 36 female) aged between 10 and 22 (mean 17) years with diabetes duration between 3 and 20 (mean 9) years were included.

Patients were divided into 2 groups based on exposure to ETS: group 1 = no or occasional exposure and group 2 = frequent (weekly to daily) exposure to ETS.

All studies were approved by the ethical committee for human research at the Lund University. Written informed consent was obtained from all participants over 18 years, or from their guardians for those under 18 years of age.

Animals

Study V

Twenty-one male, 6-week old apolipoprotein E-knockout (apoE-KO) mice, purchased from the Jackson Laboratories (Bar Harbor, Maine, USA), were divided randomly into 3 groups as follows (Figure 1): control group (n=7) - inoculated with saline at 6, 7, and 8 weeks of age; single-infected group (n=7) - inoculated with saline at 6 and 7 weeks, and with Chlamydia pneumoniae (C. pneumoniae, type AR-39, 2 x 10^5 inclusion-forming units per mouse) at 8 weeks of age; and repeatedly infected group (n=7) - inoculated with C. pneumoniae at 6, 7, and 8 weeks of age. All animals were fed standard normocholesterolemic chow and water ad libitum.

One week after the last inoculation, at the age of 9 weeks, cutaneous microvascular function was assessed by laser Doppler iontophoresis of acetylcholine (Ach) and sodium nitroprusside (SNP). In order to obtain a hairless skin area on the back of animals, depilatory cream was used 1 day before laser Doppler assessment. Prior to each inoculation and laser Doppler, mice were sedated by intraperitoneal injection of mixture of ketamine (Ketalar®, Pfizer; 60 mg/kg) and xylazine (Rompun®, Bayer; 10 mg/kg). The study protocol was approved by the ethical committee for animal research at Lund University.

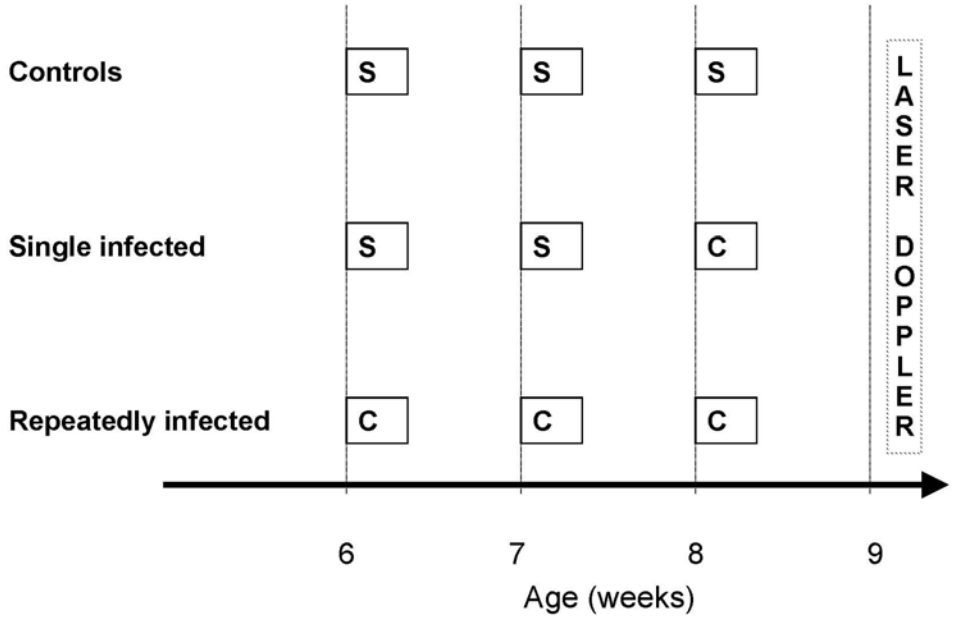

Figure 1: Laser Doppler was performed 1 week after the last inoculation with either saline (S) or C. pneumoniae (C).

METHODS

Assessment of cardiovascular function

Carotid artery ultrasound

A high-resolution ultrasound system (Acuson Sequoia C512, Siemens AG, Germany) equipped with a 15 MHz probe was used. The imaging protocol was described in detail previously (64). In short, longitudinal scans in bi-dimensional mode of the 1-cm-long distal end of the left common carotid artery were imaged so that the lumen-intima and intima-media interfaces were clearly distinguishable. All scans corresponded to the R-wave on the ECG. Four to six scans from each individual were recorded on videotape for off-line analysis of the carotid artery compliance (CAC), stiffness index (SI), and intima-media thickness (IMT). The mean carotid IMT of four measurements along a 1-cm segment was calculated from each scan. Mean IMT values obtained from all scans from the same subject were averaged, and the resulted mean IMT was used for statistical analyses.

CAC and SI were calculated according to the following formulas: CAC = $[(D_s - D_d)/D_d]/(P_s - P_d)$, and SI = $\ln(P_s/P_d)/[(D_s - D_d)/D_d]$, where D_s is systolic diameter, D_d is diastolic diameter, P_s is systolic blood pressure, and P_d is diastolic blood pressure. CAC reflects the ability of arteries to expand in response to the pulse pressure caused by cardiac contraction and relaxation. SI was introduced to reduce the impact of the curvilinear pressure-stiffness relationship on arterial stiffness, and is therefore considered to be relatively independent of blood pressure (65).

Brachial artery ultrasound

Longitudinal scans of the brachial artery (non-dominant arm) were imaged several centimeters above the antecubital fossa via a 15-MHz linear ultrasound transducer of an Acuson Sequoia C512 (Siemens). The ultrasound beam frequency was set at 8 MHz. Once the image was obtained, the transducer was positioned throughout the ultrasound study with the aid of a transducer holder (Great Ormond Street Hospital, London, UK). ECG-gated end-diastolic scans of the artery were recorded at baseline, and a pressure cuff tourniquet placed around the forearm was thereafter inflated to 200 mmHg (minimum 50 mmHg over the systolic blood pressure) for 5 minutes. A new series of frames were taken for 15 s before and 120 s after cuff deflation. Arterial flow velocity was obtained before and during the first 15 s after cuff release by pulsed-Doppler signal at 70 ° to the vessel with the range gate in the centre of the artery. Blood flow volume was calculated by multiplying the velocity-time integral of the Doppler signal by the vessel's cross-sectional area. Reactive hyperemia was calculated as the percent increase in flow after cuff release compared with baseline flow.

Following a 10-minutes recovery period, additional frames were taken before and over a 4-minutes period after sublingual administration of 400 µg glycerol nitrate (GTN) spray. Flow-mediated and GTN-induced dilation of the brachial artery was expressed as maximum percent dilatation following cuff deflation and GTN administration, respectively.

Laser Doppler with iontophoresis

Cutaneous blood flow responses to endothelium-dependent and independent agonists were assessed by using a laser Doppler multifiber probe (481–1, Perimed AB, Stockholm, Sweden) during transdermal iontophoresis of acetylcholine (Ach) and sodium nitroprusside (SNP), respectively, on the volar side of the forearm. The non-dominant upper extremity was chosen in all patients. Anodal iontophoresis was used for Ach, whereas SNP was delivered via cathodal iontophoresis. The current was set at 100 µA for 20 s for both drugs, based on previous work (66). Five

consecutive doses were applied for both drugs to generate dose-response curves. Baseline perfusion and changes in response to Ach and SNP were expressed as area under the curve (AUC).

In mice, cutaneous blood flow was recorded using a laser Doppler multifiber probe (481-1, Perimed AB, Stockholm, Sweden) during transcutaneous iontophoresis applied to 1.2 cm^2 area on the hairless back of the animals. Endothelium-dependent responses were assessed using anodal iontophoresis with Ach (5.5 mmol/l; Sigma-Aldrich, Sweden) with a current application of 100 µA for 10 s. Endothelium-independent responses were assessed using cathodal iontophoresis with SNP (67 mmol/l; Sigma-Aldrich, Sweden) with a current application of 100 µA for 20 s (67,68). For both drugs, three consecutive doses were applied.

Heart rate variability

All measurements were performed after 20 minutes of rest, with patient in supine position. Patients were asked to restrain from caffeine-containing beverages at least 12 hours prior the HRV measurement. Heart rate variability was recorded for 5 minutes using handheld HRV device (DailyCare BioMedical Inc., Taiwan). The 5 minutes length of recording is in keeping with recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology (69). Recordings were transferred to computer and data were automatically analyzed by HRV analysis software (DailyCare BioMedical Inc, Taiwan). Time-domain [standard deviation of normal-to-normal beats (SDNN)] and frequency-domain HRV parameters [high frequency power (HF) and normalized high frequency (HFn), low frequency power (LF) and normalized low frequency (LFn), and low frequency/high frequency ratio (LF/HF)] were obtained. Normalized units represent the relative value of each power component in proportion to the total power minus the very low frequency component.

Blood analyses

High-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol (TC) were analyzed from lithium heparin plasma by enzymatic method (Roche/Hitachi 912, Roche Diagnostics, Mannheim, Germany). Plasma high-sensitivity C-reactive protein (hsCRP) was measured from lithium heparin plasma by ELISA using polyclonal antibodies (DACO Diagnostics, Glostrup, Denmark). Plasma fibrinogen was assessed by automated coagulation analyzer from sodium citrate plasma (Sysmex CA-7000, Sysmex Corporation, Mundelein, IL, USA).

Plasma tumor necrosis factor-alpha (TNF-alpha) was detected using chemiluminescent immunometric assay from serum (Immulite 1000 LKNF1, Siemens Medical Solutions Diagnostics, Llanberis, UK).
Plasma cyclic guanosine monophosphate (cGMP) was measured by an enzyme immunoassay (Amersham Pharmacia Biotech UK, Buckinghamshire, UK) according to the manufacturer's recommendations (70). In brief, 125 µl of EDTA plasma was precipitated with 500 µl of ice cold ethanol. After centrifugation of this suspension (4000 rpm for 5 minutes), the supernatant was removed and the remaining precipitate was washed with 500 µl of ethanol, centrifuged and dried at 56 °C. The dried extract was dissolved in 500 µl of assay buffer for analysis. The detection limit was 0.02 pmol/ml.
For measurement of nitrite-nitrate, 200 µl plasma samples were first deproteinized by adding 400 µl $ZnSO_4$ and 500 µl NaOH. The samples were mixed and after 10 minutes centrifuged at 1000 ×/g at 4 °C. Nitrate (NO_3) in supernatants was reduced to nitrite (NO_2) with copper-coated granules of cadmium. The concentration of nitrite was then determined after reaction with a Greiss reagent using an Elx808 Bio-Tek microtiterplate instrument reader (71). A modified ELISA was used to determine antibodies against oxidized LDL (oxLDL) in serum. The technique is described in details elsewhere (72). The data are expressed as the ratio of binding to oxLDL to binding to native LDL. Plasma levels of soluble intercellular adhesion molecule (sICAM)-1 were measured by an ELISA method (R&D Systems, Minneapolis, MN, USA).

HLA typing

HLA genotypes were determined in dried spots of peripheral blood by polymerase chain reaction followed by DELFIA® hybridization assay (73). Briefly, DNA in the blood was amplified, and presence of particular alleles was determined by a hybridization reaction using allele-specific, short oligonucleotides labeled with lanthanide chelates.

Serology and PCR for Chlamydia pneumoniae (Study V)

Serum anti C. pneumoniae IgG antibodies were measured by microimmunofluorescence. Presence of C. pneumoniae DNA in the lungs and ascending aorta specimens from each group was determined by PCR. Both techniques are described elsewhere (74).

Statistics

Due to skewed distribution, HRV and hsCRP values were log-transformed. Between-group comparisons were done by one-way analysis of variance

(ANOVA). Analysis of covariance (ANCOVA) was used to control between-group differences for possible confounding effects of age, diabetes duration, BMI and HbA_{1c}. ANOVA for repeated measures was used to assess the differences in responses to Ach and SNP between groups. Correlation between two continuous variables was assessed by univariate regression analysis. A multiple regression model was used to identify independent factors affecting variable of interest. Data are presented as mean±SD, mean±SE or as median (interquartile range) when appropriate. A probability (p) level ≤ 0.05 was considered statistically significant. All analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Atherogenic effects of infections and ETS (Study I)

The infection questionnaire was answered by 72 (41 male and 31 female) patients (73 %). Fifteen patients experienced none or 1 episode of RTI during the past year (group 1), 33 patients had 2 to 3 RTI (group 2), while the remaining (24) patients had $\geq$ 4 RTI (group 3). No other types of clinically manifest acute or chronic infections were identified through questionnaire.

The questionnaire enquiring ETS exposure was answered by 93 patients (95 % of study participants). Twenty respondents (22 %) indicated weekly to daily ETS exposure (group 3), while rare exposure (group 2) was indicated by 44 patients (47 %). Data on the number of smokers per home and the average daily cigarette consumption by patients' cohabitants were provided by 93 patients. Non-smoking homes were indicated by 66 respondents (71 %), while the remaining respondents indicated 1 (16 respondents, 17 %) and $\geq$ 2 smokers per home (11 respondents, 12 %).

In univariate analysis, weak but significant inverse correlations were observed between CAC and diabetes duration (p=0·007, r=–0.29), HbA_{1c} (p=0.004, r=–0.31), age (p=0.004, r=–0.30), and frequency of RTI (p=0.01, r=–0.32; Figure 2, left panel). CAC was decreased in patients with a high frequency of RTI compared to those with low and moderate frequency (Figure 2, right panel).

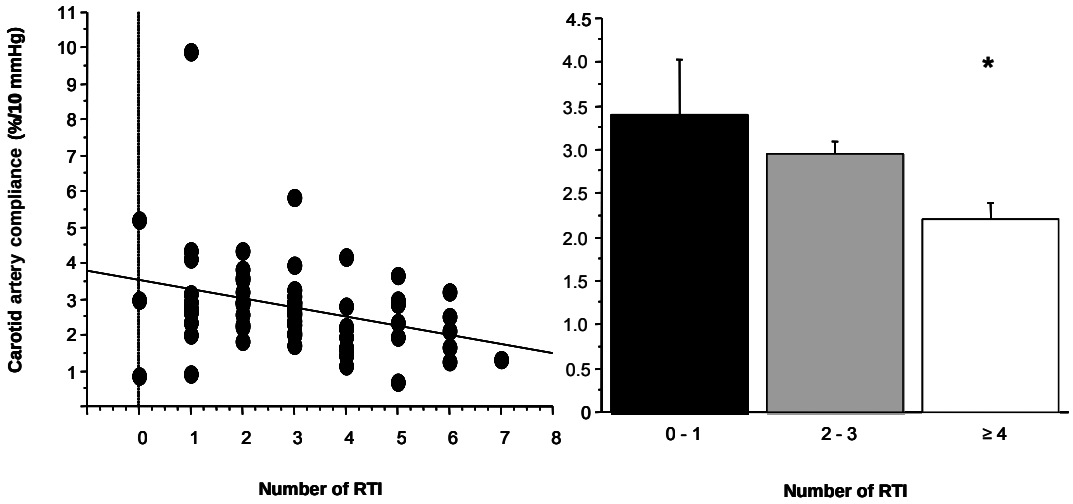

Figure 2: Relationship between carotid artery compliance and frequency of respiratory tract infections (RTI) during the past year. * denotes p<0.01, 1st versus 3rd group.

Although diabetes duration was significantly greater in the third infection group, the relationship between RTI and CAC remained significant when diabetes duration along with other variables (age, BMI, IMT, hsCRP and HbA_{1c}) were entered in a multiple regression analysis. In this model, in addition to RTI, HbA_{1c} was also found to independently predict the decrease in CAC (p=0.01, r=–0.36).

Carotid SI was correlated with RTI frequency (p=0.01, r=0.31; Figure 3, left panel), being greater in the high frequency group than in the remaining two groups (Figure 3, right panel).

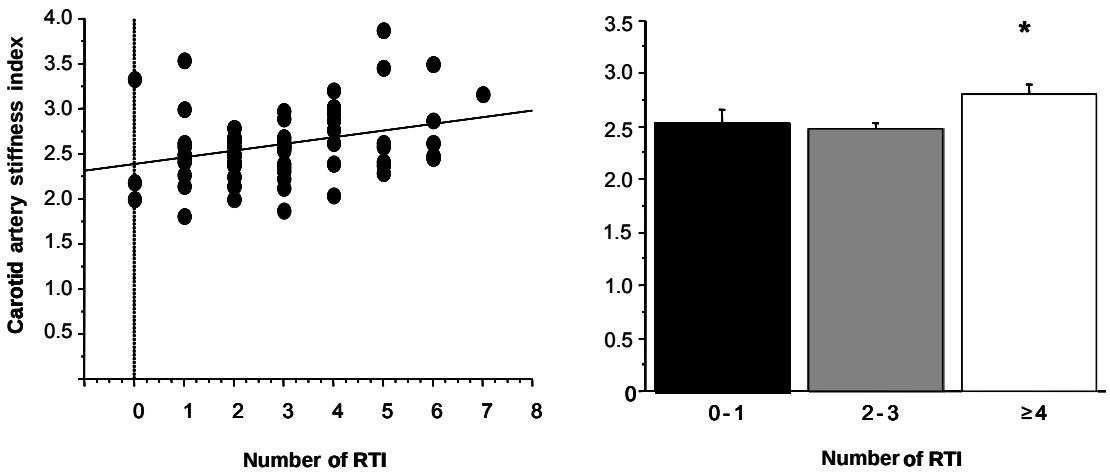

Figure 3: Relationship between carotid artery stiffness and frequency of respiratory tract infections (RTI) during the past year. * denotes p<0.05, 1st versus 3rd group.

Patients with moderate to high prevalence of RTI (≥ 2/year) also frequently exposed to ETS had lower CAC than those presenting either one or none of these factors ($p<0.05$; ANCOVA after adjustment for age, diabetes duration, and HbA_{1C}; Figure 4). Also, the relationship between CAC and RTI recurrence became strongly significant ($p=0.0002$, $r=-0.56$) in patients with ETS exposure (2nd and 3rd group). In patients with frequent ETS exposure (3rd group), but not in the 1st ($p=0.61$), and 2nd ($p=0.13$) group, plasma levels of oxLDL antibodies were correlated with the frequency of RTI ($p=0.04$, $r=0.53$).

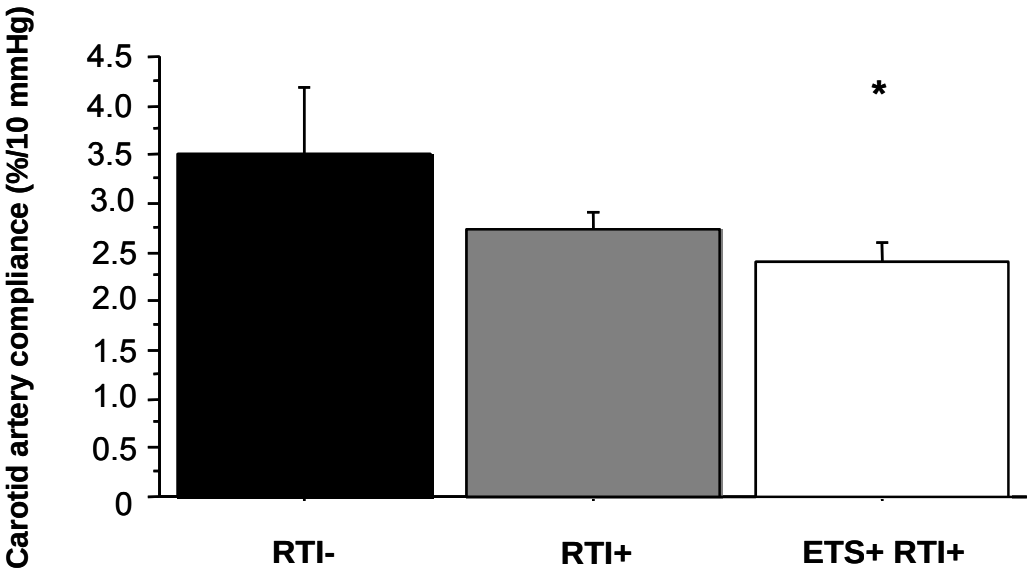

Figure 4: Interaction between frequency of respiratory tract infections (RTI) and exposure to environmental tobacco smoke (ETS) on carotid artery compliance by ANCOVA (adjustment for age, diabetes duration and HbA_{1C}). * denotes $p<0.05$, 1st versus 3rd group. n=12 in the 1st group; n=32 in the 2nd group; n=12 in the 3rd group.

Exposure to ETS expressed as daily cigarette consumption in and around the home was associated with LDL:HDL ratio ($p=0.001$, $r=0.36$; Figure 5).

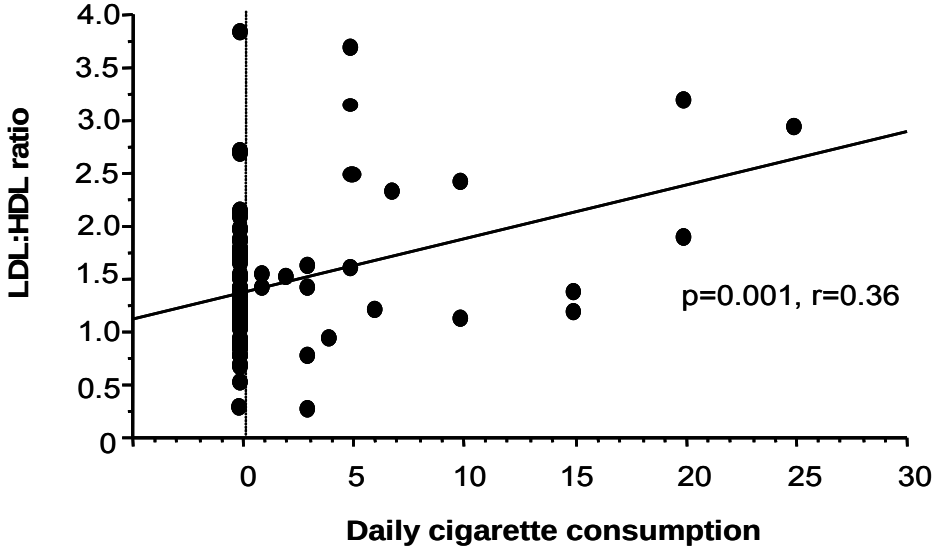

Figure 5: Relationship between LDL:HDL ratio and exposure to ETS expressed as daily cigarette consumption by cohabitants in or around the home.

High-risk HLA and arterial dysfunction (Study II)

Thirty-four patients (39 %, 18 male and 16 female) were positive for the HLA-DQ 2/8 genotype. Among the remaining 52 patients (31 male and 21 female), 15 had one of the two haplotypes (DQ 2 or DQ 8).

LDL:HDL ratio was significantly higher in the DQ 2/8 group (1.8±0.2) than in the non-DQ 2/8 group (1.3±0.2, $p<0.01$; Figure 6, left panel). The difference remained significant after adjustment for age, BMI, diabetes duration, HbA_{1C}, and hsCRP ($p<0.02$ by ANCOVA). When the patients were grouped based on the genotype, the correlation between hsCRP and LDL:HDL ratio became significant among the DQ 2/8 patients ($p<0.001$, $r=0.6$; Figure 6, right panel). In this group, the association between LDL:HDL ratio and hsCRP remained significant after adjustment for age, BMI, diabetes duration, and HbA_{1C} ($p<0.01$).

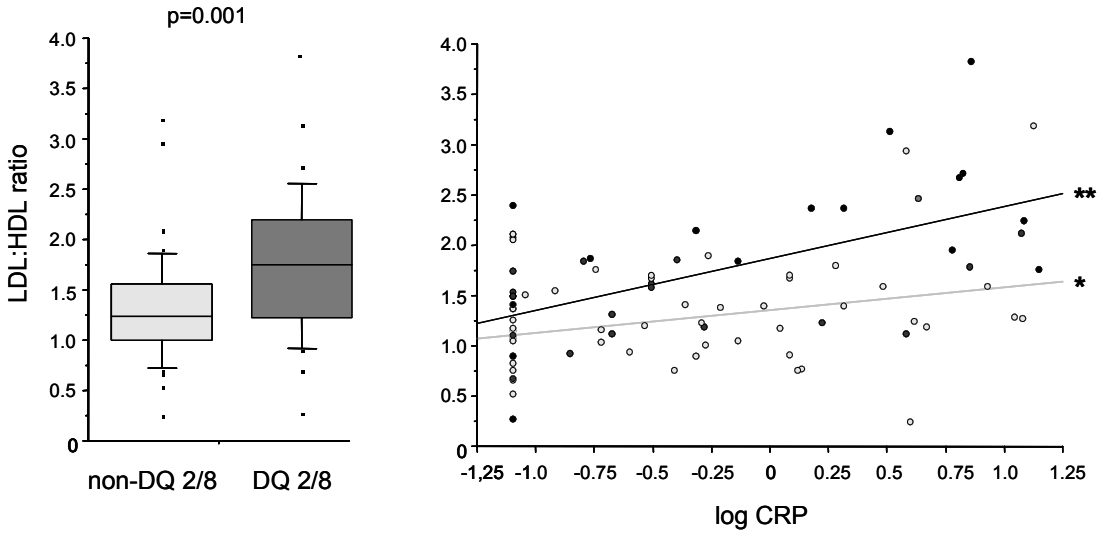

Figure 6: Box plot illustrating the differences in LDL:HDL ratio between the DQ 2/8 and non-DQ 2/8 groups (left panel). The box plot displays the 25th percentile, median, and 75th percentile, as well as the 10th and 90th percentiles as horizontal lines outside the box. Association of LDL:HDL ratio with C-reactive protein (CRP) in DQ 2/8 (*full circles*) and non-DQ 2/8 patients (*empty circles*) (right panel). * denotes p=0.05, r=0.3; ** denotes p=0.0004, r=0.6.

No significant difference was noted between the groups with regard to FMD of brachial artery (5.3±0.7 % in the DQ 2/8 group vs. 6.7±0.5 % in the non-DQ 2/8 group, p=0.08; Figure 7, left panel). However, in the DQ 2/8 group, FMD inversely correlated with CRP (p=0.01, r=-0.5), but no such association was observed in the non-DQ 2/8 group (p=0.9, r=0.02; Figure 7, right panel).

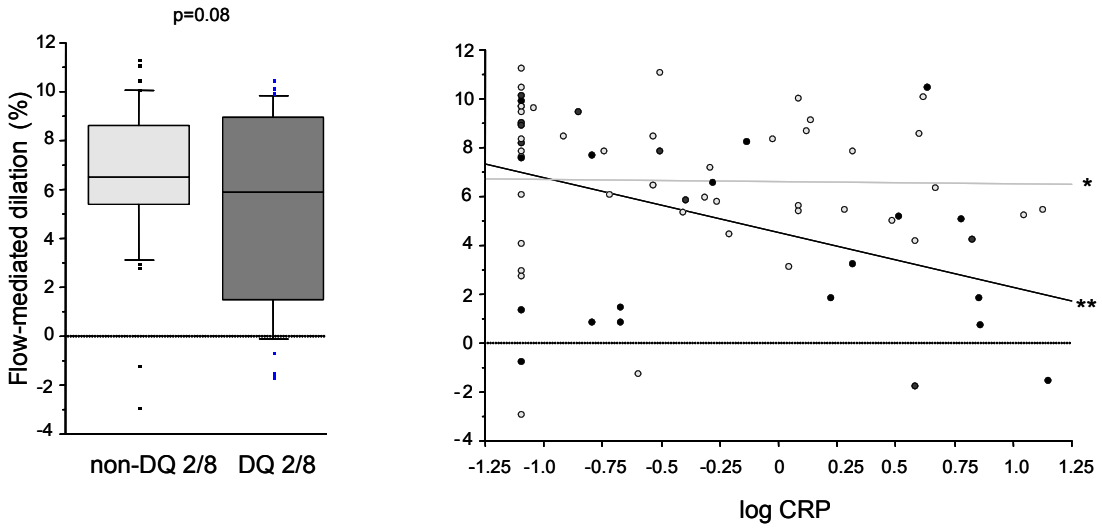

Figure 7: Left Panel: Box plot illustrating the differences in flow-mediated dilatation of brachial artery between the DQ 2/8 and non-DQ 2/8 groups. The box plot displays the 25th percentile, median, and 75th percentile, as well as the 10th and 90th percentiles as horizontal lines outside the box. Right Panel: Association of flow-mediated dilation of brachial artery with CRP in DQ 2/8 (*full circles*) and non-DQ 2/8 patients (*empty circles*). * denotes p=0.9, r=0.02); ** denotes p=0.01, r=-0.5.

When the patients were further categorized according to their CRP levels (cut-off value, 1 mg/l), both LDL:HDL ratio (Figure 8, left panel) and FMD (Figure 8, right panel) showed a shift toward a more atherogenic profile in the DQ 2/8 and CRP ≥ 1 subgroup (2.2±0.2 % and 3±1.1 %, respectively) compared with the remaining subgroups.

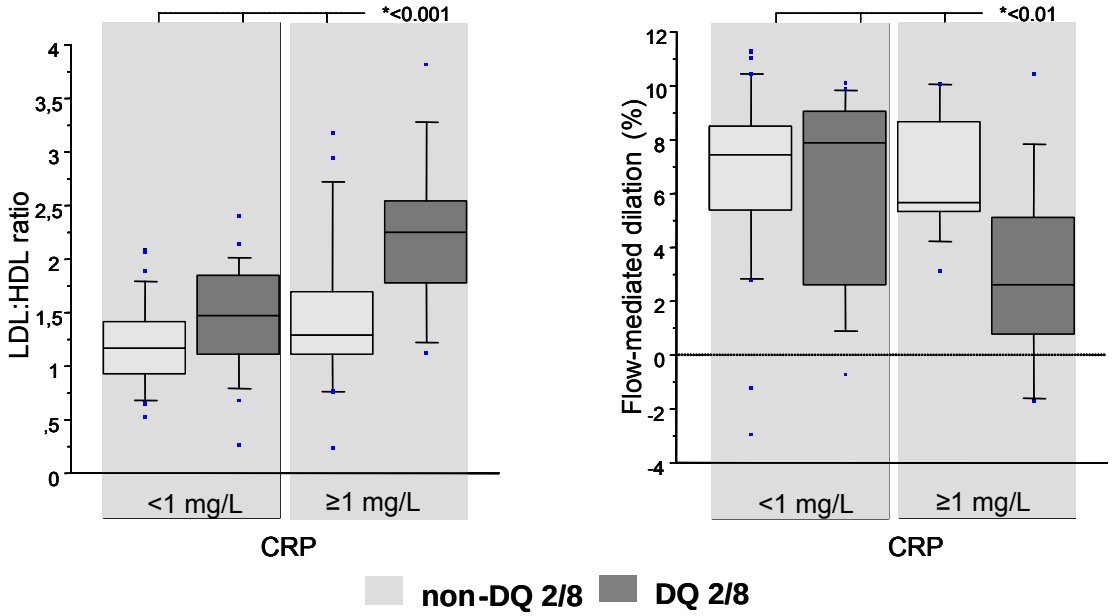

Figure 8: Box plot distribution of LDL:HDL ratio (left panel) and flow-mediated dilation of brachial artery (right panel) in relation to the HLA-DQ 2/8 genotype and CRP (cut-point, 1 mg/l). The box plot displays the 25th percentile, median, and 75th percentile, as well as the 10th and 90th percentiles as horizontal lines outside the box. * denotes p value.

High-risk HLA and microvascular dysfunction (Study III)

HLA-DQ 2/8 was identified in 29 patients whereas the remaining 46 patients were negative for this genotype.

Although the baseline skin perfusion values were similar (p=0.29), the microvascular responses to Ach were decreased in DQ 2/8 group compared with the non-DQ 2/8 group (p=0.01; Figure 9, left panel). The difference remained significant after adjustment for age, diabetes duration, HbA_{1c}, and BMI (ANCOVA, p=0.03). In contrast, no significant difference in the responses to SNP was noted between the groups (p=0.16; data not shown).

Among the DQ 2/8 patients, CRP showed significant correlation with both systolic (p<0.001, r=0.76) and diastolic (p<0.01, r=0.50) blood pressure (Figure 9, left panel). No such association was observed in the non-DQ 2/8 group (p=0.2 and r=0.2 for both).

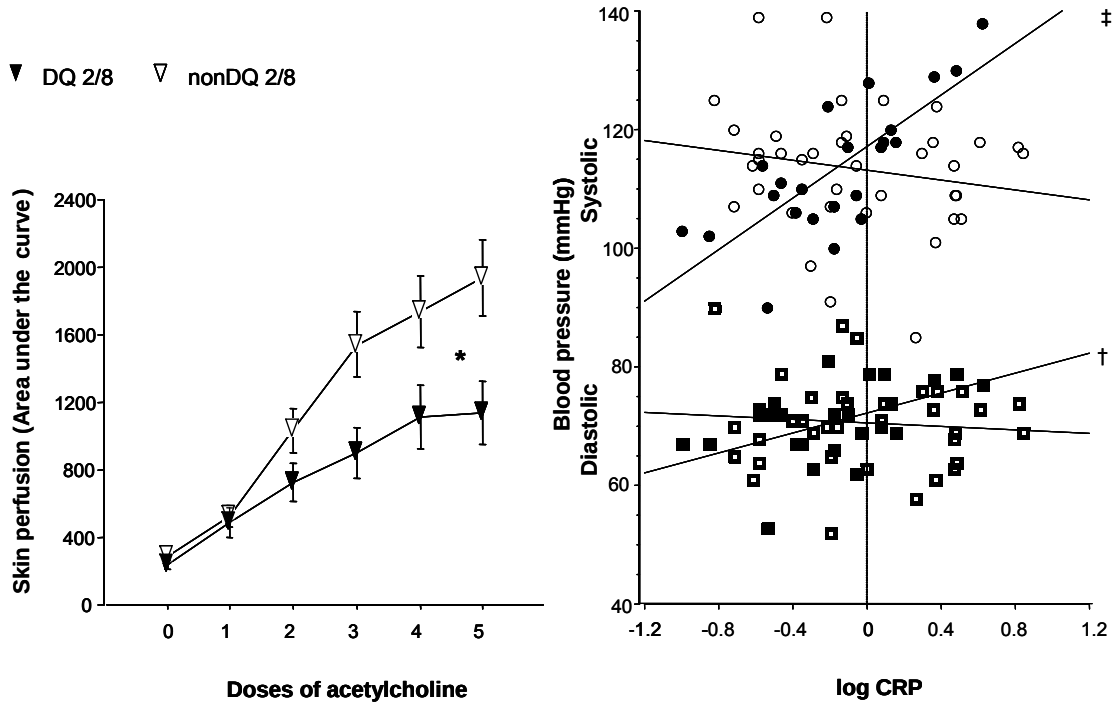

Figure 9: Skin perfusion (expressed as area under the curve) before and after cumulative doses of acetylcholine (left panel), and the relationship between blood pressure (systolic: circles; diastolic: squares) and log CRP (right panel) in diabetic patients with (*filled symbols*) and without (*empty symbols*) HLA-DQ 2/8. In left panel, data are expressed as mean±SE; * denotes p=0.01, † denotes p<0.01, r=0.50; ‡ denotes p<0.001, r=0.76, for systolic and diastolic blood pressure, respectively, in DQ 2/8 patients.

ETS and heart rate variability (Study IV)

Frequent exposure to ETS was reported by 16 patients, while occasional or no exposure was reported by 35 and 23 patients, respectively. The DQ 2/8 genotype was present in 25/74 (34 %) patients. Based on the presence of ETS exposure and the DQ 2/8 genotype, patients were divided into following groups: ETS- non-DQ 2/8: no or occasional exposure to ETS and negative for DQ 2/8; ETS+ or DQ 2/8: frequent exposure to ETS or positive for DQ 2/8; ETS+ DQ 2/8: frequent exposure to ETS and positive for DQ 2/8 genotype.

HFn was decreased in patients frequently exposed to ETS compared to patients with occasional or no exposure (Figure 10, left panel). LFn was increased in patients with frequent exposure to ETS compared to patients with occasional and no exposure (p=0.04 and p=0.03, respectively). LF/HF ratio was increased in patients with frequent exposure to ETS compared to patients with occasional and no exposure to ETS (Figure 10, right panel). There were no differences in SDNN, HF and LF between the groups.

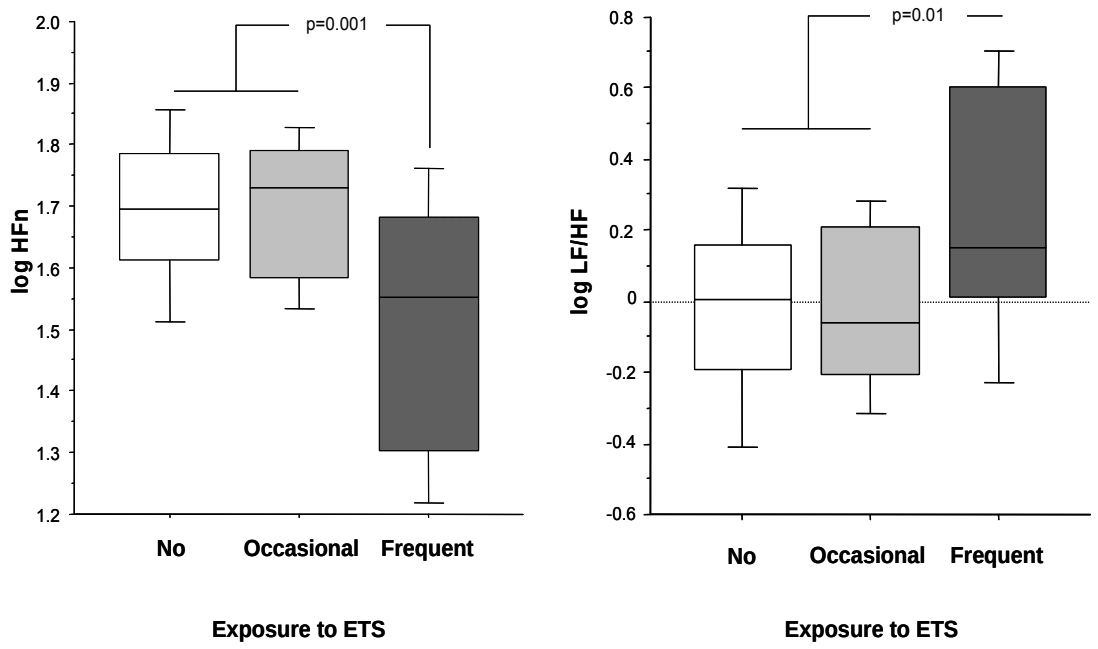

Figure 10: Box plot distribution of HFn (left panel) and LF/HF (right panel) in patients with frequent (n=14), occasional (n=23), and no (n=23) exposure to environmental tobacco smoke (ETS). The box plot displays the 25th percentile, median, and 75th percentile, as well as the 10th and 90th percentiles as horizontal lines outside the box.

When patients were further categorized in relation to DQ 2/8 and ETS, HFn was significantly decreased in patients with both compared with those with either one or none of these factors (p=0.01 and p=0.0003, respectively). Also, both LFn and LF/HF ratio were increased in patients with both factors compared to the other 2 groups.

Infection and microvascular dysfunction in animal model (Study V)

All single and repeatedly infected animals were positive for C. pneumoniae antibodies. There were no such antibodies in control animals. By PCR, C. pneumoniae DNA in the aorta and lung tissues showed a relatively similar distribution in the single infected and repeated infected animals. In contrast, all samples from control animals were free of C. pneumoniae DNA. The data are presented in Table 2.

Table 2: Presence of Chlamydia pneumoniae antibodies and DNA in the ascending aorta and lung samples.

	Single infected	Repeatedly infected	Controls
Serology	7/7	7/7	0/7
DNA in aorta	3/7	3/6	0/7
DNA in lung	2/6	1/6	0/7

One week after the last inoculation, baseline skin perfusion was increased in single-infected animals (13.7±2.8 PU) compared to non-infected (8.4±1.4 PU, p=0.05) and repeatedly infected (4.9±1.3 PU, p=0.007) animals. No significant difference in this regard was noted between non-infected and repeatedly infected animals (p=0.18). Maximal response to Ach was decreased in repeatedly infected animals (-6.4±5.5 PU) compared to single-infected (19.5±9.8 PU; p=0.04) and non-infected (28.6±8.9 PU; p=0.01) animals (Figure 11). There was no difference in response to Ach between non-infected and single-infected animals (p=0.46). In contrast, the maximal responses to SNP did not significantly differ between the groups (p>0.30).

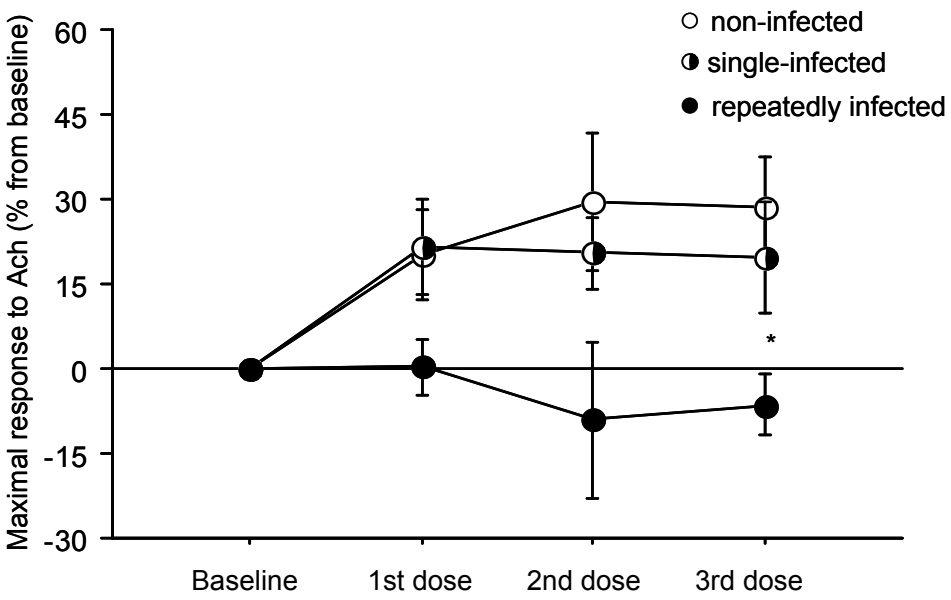

Figure 11: Microvascular response to acetylcholine one week after 3rd inoculation was decreased in repeatedly infected animals (*black circles*) compared to non-infected (*empty circles*) and single-infected (*half black circles*) animals. * denotes p<0.05. Data are expressed as mean±SE.

DISCUSSION

The development of cardiovascular disease in T1D, like in other disorders with cardiovascular risk, has a multifactorial etiology (75-77). Equal importance is nowadays attributed to both intrinsic and extrinsic factors (78-80).

In studies II, III, and IV, diabetic children and adolescents with HLA-DQ 2/8 appeared to have greater risk of functional cardiovascular disturbances (decrease in arterial and microvascular endothelium-dependent vasomotor function, and in HRV) than those without this genotype, independent of age, diabetes duration, BMI, and HbA_{1c}. These findings might be important since T1D remains an important source of cardiovascular morbidity at the adult age despite significant advancements in diabetes monitoring and therapy (81). Indeed, in almost 50 % of diabetics, their cardiovascular complications can not be explained by glycemic levels (82).

The genetic HLA-related susceptibility to T1D is recognized in up to 80 % of diabetic patients (46,83). Two HLA haplotypes, DQB1*0302-A1*0301 (DQ 8) and DQB1*0201-A1*0501 (DQ 2), seem to confer the highest risk for developing T1D (84), especially when both are present in the genotype (i.e. HLA-DQ 2/8) (85). This genotype may be detected in nearly 30 % of patients with T1D compared to 1 % in non-diabetic population (86).

Post-mortem studies near the onset of T1D have shown that HLA class II molecules may be abundantly expressed on vascular endothelial cells lining the capillaries and capillary sinusoids in the islets (87). The upregulation of HLA is paralleled by strong expression of adhesion molecules (i.e. ICAM-1) in the same endothelial areas (88). These events, seemingly induced by circulating proinflammatory mediators, facilitate homing and migration of inflammatory cells, such as T cells, across the vascular endothelium. Recent evidence suggests that similar changes may be found on the surface of endothelial cells of other vascular beds (52,89). In children with diabetes-risk HLA, signs of systemic endothelial cell activation were documented already before the clinical onset of T1D (90). There is some epidemiological evidence suggesting that diabetic microvascular retinopathy is more frequently encountered in patients with HLA-DQ (45,91). In atherosclerotic plaque, HLA class II molecules on surface of endothelial and smooth muscle cells colocalize with activated inflammatory cells (92,93).

Theoretically, given the upregulatory effects exerted by systemic inflammation on both vascular adhesion molecules and HLA molecules, their putative detrimental influences on the vascular wall would gain further consistency in an inflammatory environment. In patients with rheumatoid

arthritis (RA), another cardiovascular risk factor with important genetic susceptibility, the mortality from cardiovascular disease was found to be most increased in patients with RA-risk HLA and inflammatory activity (94,95). In study II, we observed further decrease in brachial artery's endothelium-dependent reactivity in HLA-DQ 2/8 patients with low-grade inflammatory activity (i.e. CRP > 1 mg/l). However, no such association could be demonstrated in microcirculation (study III). The meaning and causes of this discrepancy remain to be determined in future studies. There was however a moderate correlation between blood pressure and CRP only in HLA-DQ 2/8 patients. Microvascular endothelium plays an important role in the regulation of arterial blood pressure (96).

Infection remains the most common cause of inflammation. One intriguing epidemiological observation is that risk of developing T1D seems to increase with the number of infections experienced by an individual during the year preceding the onset of T1D (97,98). Although we currently lack knowledge about the precise underlying mechanism, there are other reports on similar association between infection recurrence and chronic disease such as multiple sclerosis and rheumatoid arthritis (99,100). In some animal studies, the development of atherosclerotic plaque was accelerated by repeated infection (30,101). In humans, the number of infections to which an individual had been exposed correlated with the extent of atherosclerosis and also with cardiovascular mortality (102,103). Vascular endothelial injury could be a possible mechanistic link between infection and various chronic inflammatory diseases including atherosclerosis. Infections cause vascular endothelial dysfunction, which may persist up to 1 year after infectious illness (26). Mild RTI seem to aggravate arterial endothelial dysfunction in young patients with T1D (104). In study I, increased frequency of RTI during the year preceding the investigation was associated with decrease in CAC. Decreased carotid artery elasticity was previously reported in young subjects with cardiovascular risk factors, and was suggested to predict cardiovascular events in high-risk patients (15,16). The elastic properties of the arterial wall are in part dependent on the functional integrity of endothelial cells. Using a mouse model susceptible to vascular disease, we demonstrated in study V increased propensity to in vivo-assessed microvascular endothelial dysfunction with increasing number of infectious episodes. The precise mechanisms need to be investigated in further study.

Although we did not observe any direct influence of ETS, the decrease in CAC in patients with frequent RTI was greatest in those with frequent exposure to ETS, suggesting a possible synergy of these two in the early development of arterial disease (study I). Given the important role of lipid peroxidation in atherosclerosis (105,106), the significant correlation between RTI frequency and oxLDL antibodies (study I) in patients with often

exposure to ETS is another finding that supports a synergy of RTI and ETS in arterial disease.

Studies in adults have shown that ETS may increase sympathetic activity (107,108). For instance, nicotine can activate sympathetic nervous system by increasing catecholamine release leading to increased heart rate and vasomotor tone (109,110). In animals, even short-term exposure to ETS decreases HRV and increases susceptibility to tachyarrhythmia, the latter by prolongation of sinus node recovery and by shortening the ventricular refractory period (111). It is possible, that the inflammatory response to ETS may be exacerbated by HLA, giving rise to increased circulatory levels of inflammatory mediators with subsequent influence of HRV. Other possible mechanisms by which ETS influences HRV may include alteration of cardio-respiratory reflexes (e.g. baroreflexes) and increased production of pro-inflammatory cytokines (109,112). In study IV, HRV was most impaired in ETS-exposed patients with HLA-DQ 2/8. Interestingly, macrophages from patients heterozygous for HLA-DQ alleles 0201/0302 appear to be hypersensitive to lipopolysaccharide, which is present in increased amount in cigarette smoke (113,114). Similarly, the higher levels of ICAM-1 in DQ 2/8 patients frequently exposed to ETS (study IV) might suggest increased propensity for ETS-mediated vasculopathy in the presence of this genotype.

Although appealing, there is no evidence to date of a direct interplay between infections and HLA in cardiovascular disease. Since infection promotes the inflammatory milieu needed for endothelial cell activation and upregulation of HLA, and since certain HLA seem to exacerbate endothelial dysfunction of both large and small vessels (studies II and III), these changes might in individuals with disease-susceptible genotypes contribute to more specific steps in which homing, transmigration and accumulation of inflammatory cells to certain tissues occur. In arteries, this process could provide an important ground for further development of changes typical to atherosclerosis, such as lipid oxidation, smooth muscle cells proliferation and formation of fatty streaks. Diabetes might in this context not be an exclusive prerequisite of accelerated atherosclerosis. At microcirculatory level, transendothelial migration of autoreactive T cells would create the premises for tissue inflammation and destruction. In pancreas, for instance, these microcirculatory changes could perhaps contribute to diabetes immunity by facilitating local presentation to the immune system of antigenic peptides of processed islet proteins.

Further studies are needed to provide additional mechanistic insights into the putative gene-environment interactions on the cardiovascular system in young patients with T1D, and to investigate eventual relationship to

cardiovascular complications later in life. Also, large-scale prospective studies on young populations presenting diabetes-risk (as well as other autoimmunity-risk) HLA are needed to elucidate whether these putative gene-environment influences on the cardiovascular system may be present already before the onset of the disease predisposed by these HLA. Such studies are presently underway at our center.

CONCLUSIONS

In young patients with T1D:

- Diabetes high-risk genotype HLA DQ 2/8 is associated with functional disturbances in both large arteries and microcirculation, and with atherogenic lipid phenotype (studies II and III).

- Frequent exposure to ETS is associated with disturbances in HRV, especially in those with diabetes-risk genotype HLA-DQ 2/8 (study IV).

- High frequency of RTI is associated with early signs of accelerated atherosclerosis in carotid artery, particularly in those regularly exposed to ETS (study I).

At least in a hypercholesterolemic millieu, the risk of developing microvascular dysfunction seems to rise with increasing number of infectious episodes (study V).

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PAPERS I - V

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