

Value of C-reactive protein and IL-6 measurements in type 1 diabetes mellitus

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Abstract

Introduction: The role of non-specific inflammation in β -cell loss in type 1 diabetes (T1DM) is unclear. In the present study, inflammatory markers were determined in patients with type 1 diabetes mellitus (T1DM) and related to conventional risk factors for cardiovascular disease. To determine the levels of high sensitive CRP (hs-CRP) and interleukin-6 (IL-6) as markers of inflammation in type 1 diabetes mellitus and to investigate their determinants in type 1 diabetes and their correlation with conventional risk factors for cardiovascular problems.

Material and methods: Plasma hs-CRP and IL-6 were measured in 70 young patients with type 1 diabetes mellitus. Forty patients were well controlled on intensive insulin therapy (group I), while 30 patients were not controlled on in spite of intensive insulin therapy (group II). These results were compared to those of 30 age- and sex-matched healthy children. All subjects were assessed for a family history of type 2 diabetes and history of premature cardiovascular illness, presence of hypertension, body mass index (BMI), HbA_{1c} and serum lipids, all of which are considered to be risk factors for cardiovascular problems.

Results: Diabetic patients had significantly higher levels of hs-CRP and IL-6 than the non diabetic control group (p -value < 0.001 , < 0.05 respectively). The uncontrolled diabetes group showed significantly higher levels of the two markers than the controlled diabetic group (p -value < 0.000). Serum levels of IL-6 correlated positively with hs-CRP ($r = 0.926$, $p < 0.0001$). Hs-CRP and IL-6 correlated positively with HbA_{1c}, BMI, total cholesterol ($p < 0.000$), LDL ($p < 0.0001$) and triglycerides ($p < 0.000$), whereas HDL showed a negative correlation to both hs-CRP and IL-6 ($p < 0.000$). There was a significantly higher incidence of hypertension, a family history of cardiovascular disease and a family history of type 2 diabetes in the uncontrolled diabetes group ($p < 0.000$). Hs-CRP and IL-6 were significantly higher in patients with a family history of type 2 diabetes mellitus ($p < 0.005$).

Conclusions: In type I diabetes there is an increase in the inflammatory markers, hs-CRP and IL-6 denoting subclinical chronic inflammation. These markers correlate significantly with conventional risk factors for vascular disease, namely hypertension, dyslipidemia, high body mass index and glycemic control. Type 1 diabetes patients with a positive family history of type 2 diabetes mellitus have higher hs-CRP and IL-6 levels indicating that they are more prone to cardiovascular complications.

Key words: inflammatory markers, type 1 diabetes mellitus, C-reactive protein (hsCRP), interleukin-6, hypertension.

Introduction

The autoimmune response leading to type 1 diabetes (T1DM) is closely associated with overproduction of T helper-1 (Th1) cytokines which activate macrophage production of proinflammatory mediators interleukin-6 (IL-6) and TNF- α [1]. IL-6 is produced also by a variety of cells such as adipocytes, which produce 30% of the circulating IL-6, fibroblasts and endothelial cells [2]. It mediates damage to micro- and macro-vascular tissues, altered insulin secretion either directly or through stimulation of free fatty acid production and altered glucose homeostasis [3]. High sensitive C-reactive protein (hs-CRP) is an acute-phase protein and a marker of non-specific inflammation synthesized in the liver. The biosynthesis of CRP is largely regulated by IL-6 [4]. Plasma markers of inflammation, such as CRP and IL-6 are positively associated with risk of vascular disease in non diabetic individuals [5]. Recently, inflammation has been considered, at least in part, to lead to the development and progression of atherosclerosis [6]. Diabetes is an important risk factor for atherothrombosis, an association which is not explained by conventional risk factors. Atherosclerosis is the leading cause of morbidity and mortality in T1DM [7]. Increased concentrations of the circulating inflammatory markers IL-6 and hs-CRP have been reported in patients with type 2 diabetes [8] and in patients with type 1 diabetes aged > 30 years [9].

There have been few studies to determine the level of hs-CRP and IL-6 in young patients with T1DM. In the present study, we investigated the level of hs-CRP and IL-6, the relation to their determinants and to conventional risk factors for cardiovascular problems in young patients with type 1 diabetes.

Material and methods

Patients

Seventy patients with T1DM who were attending the Diabetes Clinic of the Children's Hospital, Cairo University were included in the study. T1DM was diagnosed according to the World Health Organization criteria [10]. All patients had been treated with daily intensive insulin therapy since diagnosis. Regular doses of insulin with a mean dose of (1 IU/kg/day ranging from 0.5-1.5 IU/kg/day) in the form of intermediate and short acting insulin.

The patients were classified into two groups: group I included forty patients who had controlled diabetes on insulin therapy. Controlled diabetes was defined as a fasting glucose \leq 80 mg/dl and a mean glycated hemoglobin < 8% during the last 6 months. Group II included 30 patients with type I diabetes on insulin therapy, but whose diabetes was not well controlled. Uncontrolled diabetes was defined as a fasting glucose over \geq 120 mg/dl and a mean

glycated hemoglobin \geq 8% over the past 6 months. Group III were 30 age-matched healthy, non diabetic, normotensive and non-obese children, confirmed to have no known disease as CVD or any other condition, acute or chronic, who were presenting to the general outpatient clinic for a routine check up. These made up the control group.

Exclusion criteria were as follows: a duration of diabetes less than 2 years, patients participating in any intervention studies at the time of this study, patients with symptoms of infection, malignancy, systemic somatic illness other than DM and patients having been subjected to surgery or suffering trauma in the preceding four weeks or those taking drugs that might interfere with the results. Since these children were going to be venepunctured for various other tests (controls were going to have a CBC performed), and many parents could not read or write an informed verbal consent was deemed sufficient. This was obtained from the parents of all subjects included in the study.

Clinical work up

All patients underwent a complete clinical assessment, thorough medical history with emphasis on family history of cardiovascular illness or problem, history of type 2 diabetes within the family and examination including anthropometric measurements. Height and weight were measured according to standard procedure [11]. Height was measured using a Harpenden stadiometer and the mean of 3 consecutive readings was taken. Body mass index was defined as weight in kilogram/(height in meter)² [kg/m²]. Corresponding normative data for Egyptian children and adolescents were used. Overweight was defined as between the 90th and 95th BMI percentiles and obesity as > 95th percentile.

Systolic and diastolic blood pressures, calculated from at least three measurements, was measured with the Riva-Rocci method (which is based on Korotkoff sounds) using a standard mercury sphygmomanometer and an appropriately sized cuff. Normative BP levels for children and adolescents published by the National High Blood Pressure Education Program (NHBPEP) Working Group on High Blood Pressure in Children and Adolescents served as reference values [12].

According to the NHBPEP, hypertension is defined as systolic BP and/or diastolic BP \geq 95th percentile. Blood pressure values between the 90th and 95th percentiles are designated as prehypertensive.

Assays

At the time of assessment blood samples were collected from 8 to 9 A.M. after an overnight fast. The sera were separated from the venous blood

within 30 min and kept frozen at -80°C up to 3 months prior to analysis. $\text{HbA}_{1\text{c}}$ was measured with a reference range from 4 to 6%. Lipid assays in sera were determined by a colorimetric enzymatic method.

The serum hs-CRP assay was based on the fast agglutination procedure, allowing a direct detection on kit slide (Biosystems S.A., R&I, Barcelona Spain). The reagent is a latex particle suspension coated with specific anti-human CRP antibodies that agglutinate in the presence of serum CRP. The CRP-latex preparation is adjusted to detect a sensitive amount between (6-250 mg/l). The positive sera were titred by serial two of -two dilutions in 9 mg/l saline solution. The serum titer was defined as the highest dilution showing a positive result. The serum concentration of hs-CRP [mg/l] was calculated by multiplying the detected titer by the limit of sensitivity, which is 6 mg/l. Elevated serum CRP was defined as more than 3.0 mg/l.

Serum levels of IL-6 were determined using the ELISA technique, with the R&D Systems (Minneapolis, MN, USA) commercial kits, according to the manufacturer's instructions. In this technique, a specific monoclonal antibody is adsorbed onto a plate. After addition of the serum sample where the mediator to be determined is placed, the material is incubated, and this is the moment when the antigen molecules will bind to the antibodies adsorbed onto the plate. All unbound material is washed away. Next, a new antibody specific for an antigenic determinant linked to the plate is added, and an Ab-Ag-Ab-enzyme complex is obtained (sandwich technique). The material is washed again to remove the unbound antibodies. After this, a substrate with the property of turning into a different color when in contact with the enzyme is added in proportion to the amount of the mediator present in the sample (antigen). The reading is performed in a plate reader (BioRad,

Tokyo, Japan) at 450 nm and compared to a standard curve obtained with known concentrations of the recombinant mediators. The detection limits of the essays were 0.09 pg/ml.

Statistical analysis

Statistical analysis was performed using software (SPSS 10.0; SPSS: Chicago, IL). Variables were expressed as mean \pm standard deviation (SD). Mean values were compared among patients using Student's *t* test and ANOVA was used to compare variables between all groups. The Pearson's test was used to test correlations between variables. The correlation was considered significant if the corresponding *p* value was < 0.05 .

Results

Patients with uncontrolled DM tended to have higher systolic and diastolic blood pressures (Tables I) as well as higher incidences of hypertension and positive family histories for type 2 DM or premature CVD (Table II).

Levels of total cholesterol, LDL cholesterol and triglycerides were higher in diabetics than in controls, whereas levels of HDL cholesterol were significantly lower in diabetics than in controls. These differences were more pronounced in uncontrolled diabetics (Table III). Levels of hs-CRP and IL-6 correlated positively with levels of total cholesterol, LDL cholesterol and triglycerides and negatively with levels of HDL cholesterol (Tables IV, V).

Levels of hs-CRP and IL-6 were significantly higher in patients with T1DM (groups I and II) when compared with controls (Figure 1).

Levels of hs-CRP and IL-6 were significantly higher in patients with T1DM who had a positive family for type 2 DM, no. = 42 patients than in those without a positive family history no. = 28 patients (Figure 2). Hs-CRP levels correlated positively with those of IL-6 (Figure 3). Hs-CRP and

Table I. Descriptive data and comparison of the mean of the study groups

Variables	Group I controlled DM (n = 40)	Group II uncontrolled DM (n = 30)	Group III control (n = 30)	P-value
Age [years]	8.4 \pm 3.84	10.17 \pm 2.73	9.78 \pm 3.58	0.08
Sex (F/M)	15/25	11/19	14/16	0.3
DM duration [years]	4.8 \pm 1.95	5.8 \pm 2.7	–	0.073
BMI [kg/m ²]	21.065 \pm 0.25 ^a	26.92 \pm 1.1984 ^b	21.6 \pm 0.498 ^c	< 0.01 (a,c) 0.000 (b,c)
Mean systolic BP [mm Hg]	110 \pm 6.7 ^a (100-140)	128 \pm 7.6 ^b (105-135)	107 \pm 5.1 ^c (100-110)	0.001 (b,c) 0.142 (a,c)
Mean diastolic BP [mm Hg]	75 \pm 6.5 ^a	80 \pm 5.6 ^b	73 \pm 5.9 ^c	0.01 (a,b) 0.001 (b,c)
BP amplitude [mm Hg]	45 \pm 5.4 ^a	55 \pm 7.9 ^b	40 \pm 6.2 ^c	0.001(a,b) (b,c) 0.01 (a,c)

Table II. Descriptive data of the study groups

Chi-Square	Group I controlled DM (n = 40) (%)	Group II uncontrolled DM (n = 30) (%)	P-value
Presence of hypertension	4/40	14/30	0.000
Positive family history of type 2 DM [%]	18/40 (30%)	24/30 (63.3%)	0.000
Family history of premature CVD [%]	2/40 (0.05%)	14/30 (46.6%)	0.000

Table III. Laboratory data and comparison of the mean of the study groups

Variables	Group I controlled DM (n = 40)	Group II uncontrolled DM (n = 30)	Group III control (n = 30)	P-value
HbA _{1c} [%]	6.6 ±0.21	10.3 ±0.96	3.7 ±0.41	< 0.001
Total cholesterol [mg/dl]	191.1 ±15.3	332.1 ±15.825	163.6 ±8.2	< 0.000
HDL cholesterol [mg/dl]	67.7 ±3.16	62.62 ±3.1	71.57 ±1.11	< 0.000
LDL cholesterol [mg/dl]	70.4289 ±3.1	137.11 ±5.25	74.48 ±6.8	< 0.001
Triglycerides [mg/dl]	71.43 ±0.65	110.81 ±3.4	52.3 ±3.6	< 0.000
Hs-CRP [mg/l]	3.0 ±0.78	7.9 ±1.1	0.7 ±0.14	< 0.000
IL-6 [pg/ml]	1.7 ±0.3	3.5 ±0.5	0.5 ±0.1	0.05

Table IV. Correlation of hs-CRP and lipid profile:
no. = 100

	Pearson correlation (r)	P-value
CRP and total cholesterol	0.935	< 0.0001
CRP and triglycerides	0.960	< 0.0001
CRP and HDL	-0.804	< 0.0001
CRP and LDL	0.881	< 0.001

Table V. Correlation of IL-6 and lipid profile: no. = 100

	Pearson correlation (r)	P-value
IL-6 & total cholesterol	0.9	< 0.0001
IL-6 & triglycerides	0.940	< 0.0001
IL-6 & HDL	-0.817	< 0.0001
IL-6 & LDL	0.824	< 0.0001

Mean systolic BP of girls and boys with type 1DM was prehypertensive ($\geq 90^{\text{th}}$ percentile), while diastolic BP of diabetic subjects were normotensive ($< 90^{\text{th}}$ percentile) as well as systolic BP and diastolic BP of non-diabetic controls. In group I, 4/40 were hypertensive. In group II, 14/30 were hypertensive, while in group III none were hypertensive. Subjects with uncontrolled diabetes had a significantly higher incidence of type II diabetes mellitus and premature cardiovascular diseases in their families with χ^2 p-value < 0.05 . The levels of inflammatory markers are significantly higher in cases with positive family history of T2DM, but are not significantly different for family history of premature CVS or presence of hypertension

IL-6 levels correlated positively with levels of HbA_{1c} (Figure 4). Body mass index was significantly higher in T1DM patients when compared with both control group and group of well controlled diabetics. Hs-CRP and IL-6 levels correlated positively with BMI (Figure 5).

Discussion

In this study, we measured two inflammatory markers, namely hs-CRP and IL-6 in T1DM (two years after the onset of their diabetes) and compared them to levels in normal subjects. The two year cut off point was chosen to avoid the autoimmune inflammatory process present in T1DM patients at the onset of their disease. T1DM patients showed significantly higher level of hs-CRP and IL-6 than did the controls. Higher levels of CRP [13] and of IL-6 [2] have been described in type 1 diabetes. This elevation might be related to activation of macrophages, increased oxidative stress, or induction of cytokines. Although the mechanism of T1DM is still unknown it is now accepted to be a chronic immunoinflammatory disorder [14].

Elevated hs-CRP concentrations have also been described in children who later developed T1DM and were found to be elevated in children with positive islet antibodies even before development of hyperglycemia [14].

Higher levels of IL-6 have been described in type 1 diabetes, however, some studies reported normal [15] and even decreased levels of IL-6 [16]. IL-6 elevation was thought to be due to hyperglycemia and the formation of advanced glycation end products and endothelial dysfunction [17]. There is evidence that all three of these may be factors in the etiology of type 1 diabetes [14].

Our study showed a positive correlation between elevated plasma level of IL-6 and elevated plasma level of hs-CRP which confirms a previous study [18].

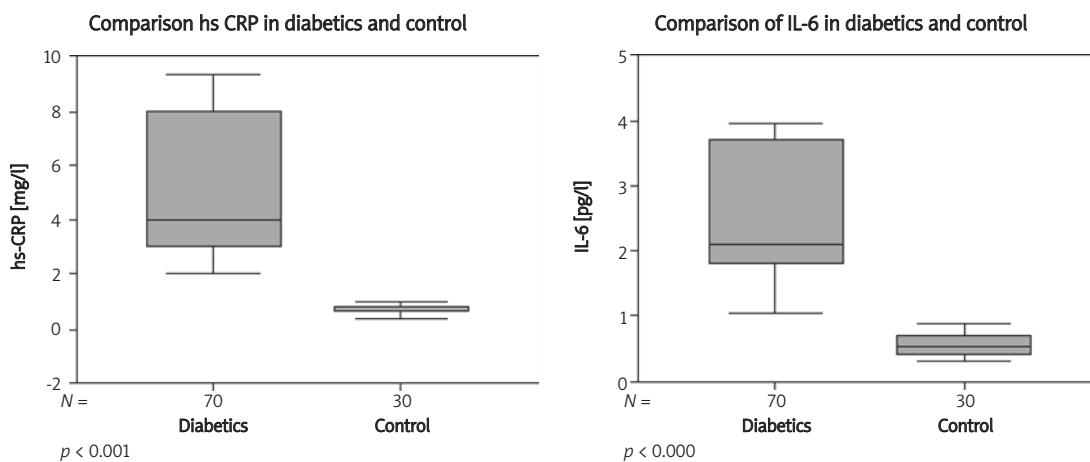


Figure 1. Inflammatory markers in diabetics and controls

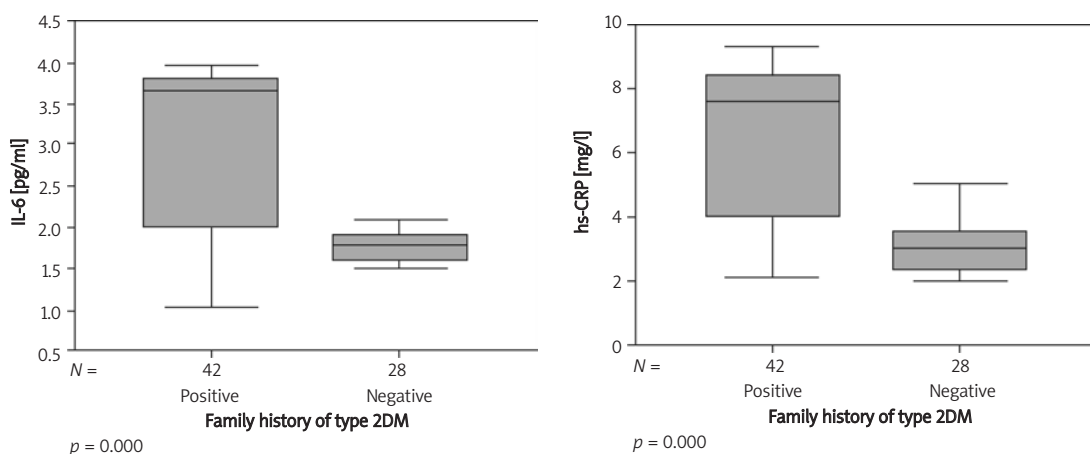


Figure 2. Inflammatory markers in cases with and without a family history of type 2 diabetes

This could be explained by the production of CRP in the liver being stimulated by inflammatory cytokines such as IL-6 [6].

Recently, inflammation has been implicated in the development and progression of atherosclerosis. From the pathological viewpoint, all stages i.e. initiation, growth and complications of the atherosclerotic plaque, may be considered as inflammatory responses to vascular endothelial injury. Being the major cause of mortality and morbidity in patients with T1DM [19] it is very important to study and monitor markers of inflammation to define patients at higher risk of vascular complications. There is a correlation between elevated concentrations of CRP and carotid intimal media thickness (i.e. early stage atherosclerosis) in these patients [20].

IL-6, which is secreted by macrophages and lymphocytes, is an important cytokine that can initiate events leading to atherogenesis by induction

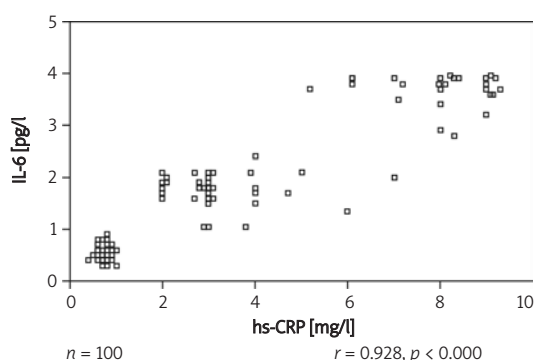


Figure 3. Correlation of hs-CRP with IL-6

of adhesion molecules, monocyte-endothelial interactions, and inflammation injury to the blood vessels [5, 21].

Glycemic control, BMI, LDL cholesterol, HDL cholesterol, triglycerides, and systolic blood pressure

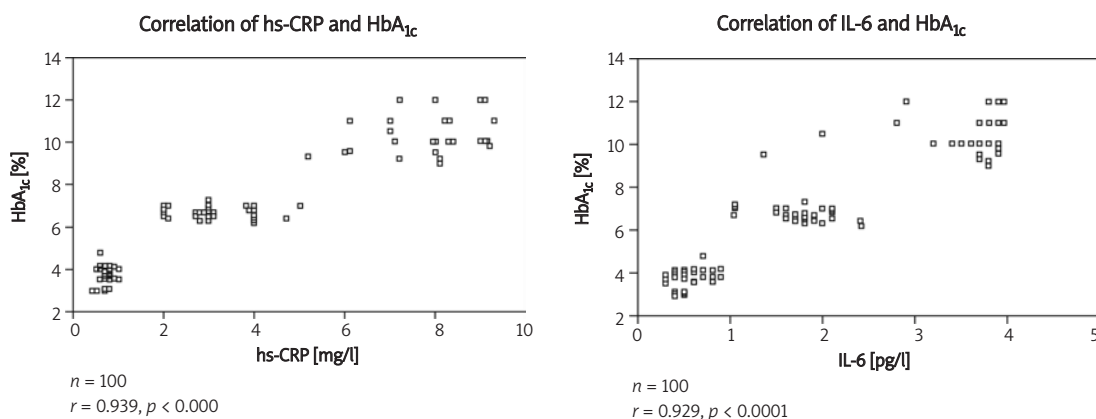


Figure 4. Correlation of inflammatory markers with HbA_{1c}

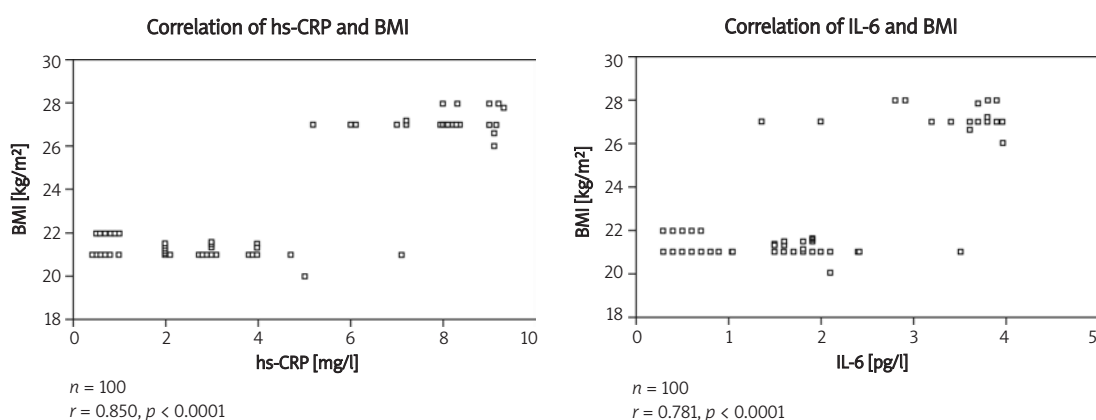


Figure 5. Correlation of inflammatory markers with body mass index

were defined as the determinants of inflammatory activity in type 1 diabetes [17, 22].

We found higher serum levels of hsCRP and IL-6 in patients with uncontrolled diabetes compared to the controlled diabetic group as well as a positive correlation of both studied inflammatory markers and HbA_{1c} which supports other studies [2, 23]. This can be explained by the fact that HbA_{1c} reflects the biological activities of hyperglycemia and advanced glycation end products, all of which can induce inflammation [24]. Hyperglycaemia has an indirect influence on atherosclerosis through lipid changes. It increases potentially atherogenic forms of small VLDL and small dense LDL which are susceptible to glycation and oxidation. However, chronic hyperglycaemia may be a separate risk factor for accelerated macroangiopathy [25].

Body mass index is commonly accepted as an indicator of overweight and obesity which are frequently associated with elevated BP, increased prevalence of type 2 diabetes, metabolic syndrome or increased carotid intimal thickness [26].

The mean BMI adjusted for age and gender in both the controlled diabetic group and the non

diabetic group was below the 90th percentile, while in the uncontrolled diabetic group it was above the 95th percentile. The BMI was significantly higher in uncontrolled diabetics, who in turn had significantly higher CRP and IL-6. It is possible that increased local adipose tissue inflammation associated with obesity and the secretion by the adipocytes of a number of bioactive proteins collectively termed adipocytokines, one of which is IL-6, are causative factors. Adipocytokines may have an important role in insulin resistance [27].

The uncontrolled diabetes group had a significantly higher incidence of a positive family history of type 2 DM. This may indicate that patients of type 1 DM with a positive family history of T2DM are more difficult to control than patients with a negative family history of T2DM.

A strong family history of type 2 DM affects bioavailability of nitric oxide and glycemic burden, even in the nondiabetic range which can contribute to endothelial dysfunction. It has been postulated that abnormalities of endothelial function may contribute to atherosclerosis before development of overt diabetes [28]. Serum levels of hs-CRP and

IL-6 are significantly higher in patients with a positive family history of type 2 DM, which is supported by other studies [29, 30]. Therefore, patients of T1DM with a positive family history for T2DM must be closely observed for inflammatory markers and for complications.

Our study showed high serum levels of TG, total cholesterol and LDL and low levels of HDL in T1DM which was more pronounced in the uncontrolled than in the controlled diabetics. This is supported by other studies that showed that optimal diabetes control may lead to average means of TC, LDL-C, HDL-C and TG on a level with the non-diabetic population or even better [31]. All showed a significant correlation with the inflammatory markers (hs-CRP, IL-6) which was positive for all serum lipids except for HDL which showed a negative relation. Diabetes induced abnormalities in fatty acid metabolism have the potential to influence macrophage cytokine release inducing up-regulation of proinflammatory cytokines [3]. Such unfavorable changes in lipid profile are thought to facilitate the formation of foam cells in the arterial wall, and may thereby increase the inflammatory state in type 1 diabetic individuals [17]. The association of CRP with insulin resistance, adipocytokines, and resistin reveals close links between inflammation, CVD, and adipose tissue. These findings provide an exciting therapeutic opportunity in cardiovascular disease by targeting various proinflammatory cascades in adipocytes [27].

High systolic pressure in the vascular tree may damage the endothelial cells and vascular tissue, thereby inducing an inflammatory response [32]. Our study showed a significantly higher prevalence of hypertension and family history of premature cardiovascular disease in the uncontrolled diabetic group. However, levels of inflammatory markers didn't differ between hyper- and normotensive patients. Associations between inflammation and systolic blood pressure were found in some population studies [17, 30].

Furthermore, patients with a family history of premature cardiovascular disease were not found to have significantly higher levels of hs-CRP and IL-6. The control of factors that determine inflammation namely optimization of glycemic control, BMI, control of dyslipidemia, and systolic blood pressure are well established therapeutic targets in diabetes and they may be beneficial in forestalling diabetic vasculopathies and lowering premature mortality in young adults with diabetes.

Further studies are needed to investigate the role of inflammatory markers in diabetic nephropathy, to correlate it to microalbuminuria and Doppler index for vascular endothelial dysfunction. In order to allow comparison with non-diabetic individuals, it is important to have excellent reference values

specified for sex, age and pubertal development. Due to difficulties in blood and tissue sampling in large healthy control populations, well defined reference values are often lacking [33].

Elevated levels of inflammatory cytokines, inflammatory prostaglandins and prostaglandin synthase 2 (COX2) have been described in children before or after the onset of T1DM. A study in adults with type 2 diabetes demonstrated that treatment with aspirin, a COX2 inhibitor, resulted in reduced CRP, insulin resistance, and serum triglycerides despite a lack of change in body weight [34]. If inhibitors of the COX2 enzyme are used to try to prevent T1DM in humans, monitoring levels of CRP may be helpful [14].

Although abdominal obesity has been linked with markers of inflammation [35], we did not measure this in our patients.

In conclusion, these results indicate that in T1DM there is an increase in the inflammatory markers hs-CRP and IL-6, denoting subclinical chronic inflammation. These markers are strongly correlated with conventional risk factors for vascular disease such as hypertension, dyslipidemia, high body mass index, glycemic control and a positive family history of 2 diabetes suggesting strategies for the treatment of T1DM and prevention of complications.

In patients with childhood-onset type 1 diabetic, the most important factors for CVD are a family history of type 2 diabetes mellitus (T2DM) and hypertension [36]. Therefore, detection of inflammatory markers in families with a strong family history of diabetes mellitus can help in the early diagnosis of cardiovascular risk in young diabetics. Patients with a positive family history for type 2 DM need close observation of their glycemic control and for the development of complications as they are at increased risk for both bad control and cardiovascular complications. Early and effective prevention of cardiovascular disease will improve lifestyle with the emphasis on disease prevention.

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