ORIGINAL ARTICLE

CHANGES IN THE SERUM PROINFLAMMATORY CYTOKINES IN PATIENTS WITH ELEVATED HOMA-IR AND TYPE 2 DIABETES MELLITUS

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Abstract

The first aim of this study was to find associations of two inflammatory markers (TNF-α and IL-6) with different metabolic variables in type 2 diabetic patients. The second aim in studying the proinflammatory cytokines was to perform a comparative study in diabetic patients divided according to the severity of insulin resistance. In this study, 138 type 2 diabetic patients, aged between 40 and 80, were enrolled. According to the HOMA-IR (the homeostatic model assessment estimated insulin resistance) values, the diabetic patients were divided in three groups: group 1 with HOMA-IR ≤ 3 (arbitrary units), group 2 with HOMA-IR between 3 and 5 (arbitrary units) and group 3 with HOMA-IR ≥ 5 (arbitrary units). The plasma values for proinflammatory markers (TNF-α, IL-6 and leptin), for surrogate markers of insulin resistance (insulin, C peptide and proinsulin) were determined by ELISA methods. Respiratory burst (RB) of isolated peripheral blood mononuclear cells (PBMC) was measured by chemiluminescence. In group 2 versus 1, RB ($p<0.05$), TNF-α ($p<0.03$) and uric acid ($p<0.02$) were higher and in group 3 versus 1, RB ($p<0.05$), TNF-α ($p<0.05$), IL-6 ($p<0.02$), leptin ($p<0.05$), proinsulin ($p<0.01$), C peptide ($p<0.001$) were higher. In diabetic patients, TNF-α was correlated with proinsulin ($r=0.65$, $p<0.05$), RB ($r=0.73$, $p<0.05$), IL-6 ($r=0.30$, $p<0.05$) and with the duration of diabetes ($r=0.77$, $p<0.05$), while IL-6 was correlated with RB ($r=0.28$, $p<0.05$) and with the duration of diabetes ($r=0.26$, $p<0.05$). In conclusion, in type 2 diabetic patients, serum TNF-α was strongly correlated with proinsulin and with monocyte respiratory burst. Patients with HOMA-IR higher than 3 (arbitrary units) had increased levels of proinflammatory cytokines, severe dyslipidemia and raised monocyte respiratory burst.

Rezumat

Primul obiectiv al acestui studiu a fost de a găsi relaţia dintre doi markeri inflamatori (TNF-α şi IL-6) cu diferite variabile metabolice la pacienţii cu diabet zaharat de tip 2. Al doilea obiectiv a fost desfăşurarea unui studiu comparativ privind nivelul seric al citokinelor proinflamatorii la pacienţii cu diabet zaharat de tip 2 (DZ tip 2) împărţiţi în funcţie de severitatea rezistenţei la insulină. Au fost incluşi 138 de pacienţi cu DZ tip 2, cu vârste cuprinse între 40 şi 80 ani. În funcţie de valorile HOMA-IR (marker al rezistenţei la insulină), pacienţii cu DZ tip 2 au fost împărţiţi în trei grupuri: grupul 1 cu HOMA-IR ≤ 3 (unităţi arbitrare), grupul 2 cu HOMA-IR 3-5 (unităţi arbitrare) şi grupul 3 cu HOMA-IR ≥ 5(unităţi arbitrare). Valorile plasmatice pentru markerii proinflamatori (TNF-α, IL-6 şi leptina) şi pentru markerii surogat ai rezistenţei la insulină (insulina, peptidul C şi proinsulina) au fost determinate prin metoda ELISA. Puseul respirator (RB-*respiratory burst*) a fost măsurat prin chemiluminescenţă. La grupul 2 *versus* grupul 1, RB ($p<0,05$), TNF-α ($p<0,03$) şi acidul uric ($p<0,02$) au fost mai mari, iar în grupul 3 faţă de 1, RB ($p<0,05$), TNF-α ($p<0,05$), IL-6 ($p<0,02$), leptina ($p<0,05$), proinsulina ($p<0,01$), peptidul C ($p<0,001$) au fost semnificativ crescute. La pacienţii diabetici, TNF-α a fost corelat cu proinsulina ($r = 0,65$, $p<0,05$), RB ($r = 0,73$, $p<0,05$), IL-6 ($r = 0,30$, $p<0,05$) şi cu durata diabetului ($r = 0,77$, $p<0,05$), în timp ce IL-6 a fost corelat cu RB ($r = 0,28$, $p<0,05$) şi cu durata diabetului ($r = 0,26$, $p<0,05$). În concluzie, la pacienţii cu DZ tip 2, concentraţia serică a TNF-α s-a corelat cu proinsulina şi cu valoarea puseului respirator monocitar. Pacienţii cu valori crescute ale rezistenţei la insulină au avut o concentraţie serică mai ridicată a citokinelor proinflamatorii, dislipidemie severă şi un puseu respirator monocitar mai mare.

Keywords: TNF-α, IL-6, HOMA-IR, respiratory burst, leptin

Introduction

Growing evidence supports the role of chronic low grade inflammatory syndrome as the main link between chronic disease (obesity, diabetes mellitus) and increased morbidity and mortality cardiovascular risk.

TNF-α is considered the link element between obesity, inflammation and insulin resistance, features which are present in type 2 diabetes mellitus (T2DM) [15, 43, 11]. TNF-α is a proinflammatory cytokine which is cleaved from the cell surface of monocytes, macrophages and T cells and is involved in lipid metabolism and adipocytes function [10]. It plays a causal role in obesity mediated insulin resistance. Experimental studies demonstrated that TNF-α inhibits insulin receptor signalling [12], glucose transport and has an important contribution to dyslipidaemia [1].

IL-6 is a polypeptide produced by T cells, monocytes and different tissues. It is a multifunctional cytokine which plays a key role in numerous physiological processes: haematopoiesis, B-cell plasmocytes proliferation, stimulating secretion of immunoglobulins and in hepatocytes differentiation [52]. IL-6 is also a major component of the inflammatory pathway [20] by regulating the synthesis of acute-phase proteins [2]. Increased levels of IL-6 were positively correlated with the onset and evolution of insulin resistance [7], as well as with the evolution of osteoporosis (46) and cardiovascular diseases [57].

It is known that pathogenic effects of high glucose, in concert with fatty acids are mediated by reactive oxygen species which elicit systemic inflammation, accelerating the progression of type 2 diabetes [34]. On the one hand it was demonstrated that markers of inflammation predict or/and are associated with T2DM [44] and on the other hand, lowering of blood glucose levels in type 2 diabetic patients is accompanied by reduced levels of inflammatory markers [50]. The crosstalk between inflammatory and metabolic signalling include c-Jun N-terminal kinase (JNK) and IkB kinase (IKKβ) as critical regulators of insulin action, kinases which are activated by TNF-α and other inflammatory and stress signals [25].

In patients with type 1 diabetes, hyperketonaemia was associated with increased levels of plasma lipid peroxides [30] and IL-6 [53], while TNF-α was involved in beta cell damage [8, 21] and in diabetic microvascular complications as: neuropathy [60], retinopathy [14] and nephropathy [38]. Also, in T2DM, the TNF-α level is used to monitor the response to thiazolidine drugs administration [37].

A recent study shows that TNF-α and IL-6 have strong predictive values in diabetic macrovascular complication development and the authors underline that hyperglycemia can trigger inflammation [58].

In patients with T2DM the gold standard to evaluate insulin resistance is the euglycaemic clamp, but the most frequently used indicator in clinical practice is HOMA-IR ("*The homeostatic model assessment estimated insulin resistance*") [35, 56].

The first aim of this study was to find associations of two inflammatory markers (IL-6 and TNF-α) with different metabolic variables in T2DM patients. The second aim was to perform a comparative study in diabetic patients divided according to the HOMA-IR values. These data will help physicians for a better management of diabetes mellitus with lower costs.

Materials and Methods

A total of 138 patients with T2DM according to the World Health Organisation (WHO) recommendations [3] were selected to participate in this study. HOMA-IR was calculated as the product of fasting plasma insulin (mmol/L) and fasting plasma glucose (mmol/L) divided by the constant 22.5 on two different days [35].

The patients aged between 40 and 80, were classified as follow: group 1 with HOMA-IR ≤ 3 arbitrary units (n=25), group 2 with HOMA-IR between 3 and 5 arbitrary units (n=28) and group 3 with HOMA-IR ≥ 5 arbitrary units (n=85). We included in the study T2DM patients with a duration of diabetes no longer than 6 years. Current smokers, alcohol drinkers (ethanol>20g/day) and pregnant women were not enrolled. Exclusion criteria were: haemodialysis treatment, acute ischemic cardiovascular disease, haemorrhagic strokes, urinary infections, epilepsy or other severe disease (e.g. hepatic failure, cancer or gangrene) and also, use of vitamins, minerals, or other supplements in the previous month. The study was approved by the Ethics Committee of the "N Paulescu" National Institute of Diabetes, Nutrition and Metabolic Disease and written informed consent was obtained from all participants before inclusion in the study.

For all subjects we performed anthropometric, clinic and biochemical measurements. After an overnight fast, blood samples (2x10mL) were collected into vacuum tubes. A volume of 10mL from each sample containing EDTA was immediately used for isolation of peripheral blood mononuclear cells (PBMC) for Respiratory Burst (RB) and the remained sample was centrifuged and the plasma was stored at -80°C until required.

Anthropometric measurements included: weight, height and waist circumference.

Routine blood tests including the concentration of fasting glucose, glycosylated haemoglobin HbA_{1c}, total cholesterol, HDL-cholesterol ("high density lipoprotein"; HDLc), total triglycerides, urea, creatinine, uric acid were measured using current biochemical methods on a Hospitex Diagnostics Eos Bravo Forte Analyser. LDL-cholesterol ("low

density lipoprotein"; LDLc) was calculated according to the Friedewald equation [23]. Serum concentrations of insulin, proinsulin, C peptide, leptin and adiponectin were determined by ELISA methods on Multiskan Ex-Thermo Electro Corporation using commercially available kits (EIA-2935, EIA-1560, EIA-1293, EIA-2395 and EIA-4177 respectively) from DRG Instruments GmbH, Germany. Cayman ELISA kits, no. 589201 and no. 583361 were used for TNF-α and IL-6 respectively, following the manufacturer's guidelines.

Isolation of peripheral blood mononuclear cells (PBMC) and Respiratory Burst: PBMC were isolated by density centrifugation on Ficoll-Paque™ Plus (1.0077g/mL) at 630g for 30 minutes. The mononuclear cells (PBMC) were collected, washed twice and resuspended in 1mL phosphate-buffered saline (PBS). Cell viability by Trypan Blue exclusion was ≥ 90%. The ability to produce a respiratory burst was monitored by a luminol-enhanced chemiluminescence method [4]. In short, to PBMC (0.3 x 10^6 cells) resuspended in phosphate-buffered saline, dark-adapted luminol was added. After monitoring spontaneous chemiluminescence for 15 min, the respiratory burst was initiated by adding of 100μL phorbol 12-Myristate 13-Acetate (PMA; final concentration 5.4 μmol/L) and the maximum chemiluminescence peak was recorded (Luminoskan Ascent® 392, LabsystemsEx-Thermo Electro Corporation). Chemiluminescence production was expressed as the Relative Chemiluminescence Units over time (RLUX60min). All reagents, PBS (Dulbecco's Phosphate Buffered Saline), Ficoll-Paque™ Plus, D-glucose, Trypan Blue, PMA, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, LM) were purchased from Sigma Chemical Co., St. Louis, USA.

Results were expressed as mean ± standard deviation (SD)/standard error of the mean (SEM). Differences between groups were analysed using Student t-test on independent samples and correlations between parameters were evaluated by the Pearson test. A value for $p<0.05$ was considered statistically significant.

Results and Discussion

Table I shows the clinical and biochemical characteristics of the patients included in the study. There were no significant differences between the studied groups regarding age, gender, blood pressure values and duration of T2DM.

Table I

The clinical characteristics and usual biological variables of T2DM subjects

Parameter	Diabetic patients (group T2DM; n=138)			
	Group 1 (n=25)	Group 2 (n=28)	Group 3 (n=85)	p-value
Age (Years)	58±1.43	54±1.51	57±1.07	NS
Sex (Male/Female)	14/11	15/13	46/39	NS
Duration of Diabetes (years)	2±0.5	1.57±0.43	1.81±0.18	NS
Weight (Kg)	86±2.80	88±2.77	94±2.11	$<0.05^{b}$
Waist Circumferance (cm)	104±1.55	106±1.64	110±1.25	$<0.05^{b, c}$
BMI (Kg/m^2)	30.50±0.61	32±0.84	33.50±0.53	$<0.05^{b}$
SBP (mmHg)	136±2.27	133±2.90	132±1.23	NS
DBP (mmHg)	80±2.10	79±2.11	77±1.02	NS
Fasting plasma glucose (mg/dL)	140±5.97	149±8.26	196±7.4	$<0.001^{b, c}$
HbA_{1c} (%)	7.20±0.25	7.48±0.26	8.30±0.20	$<0.05^{b, c}$
Serum total cholesterol (mg/dL)	199±6.91	204±6.38	212±5.93	NS
Serum HDL-cholesterol (mg/dL)	48±1.73	48±2.43	41±1.07	$<0.05^{b, c}$
Serum LDL-cholesterol (mg/dL)	122±6.36	128±0.47	131±5.45	NS
Serum triacylglycerol (mg/dL)	142±26	154±0.29	204±11	$<0.05^{b, c}$
Urea (mg/dL)	33±2.16	35±1.36	33±1.10	NS
Creatinine (mg/dL)	0.82±0.03	0.77±0.03	0.80±0.01	NS
Uric Acid (mg/dL)	5.59±0.31	6.58±0.27	6.35±0.20	$<0.05^{a,b}$
AST (U/L)	24±2.22	25±4.74	27±2.88	NS
ALT (U/L)	32±5.47	31±8.23	39±5.60	NS
GGT (U/L)	43±7.48	41±6.60	55±6.07	NS
Albumin (g/dL)	4.37±0.08	4.29±0.05	4.30±0.03	NS
HOMA-IR (arbitrary units)	2.22±0.11	3.90±0.12	9.70±0.55	$<0.001^{a, b, c}$

BMI-body mass index, WC-waist circumference, SBP-systolic blood pressure, DBP-diastolic blood pressure, AST-aspartate amino transferase, ALT-alanine aminotransferase, GGT-gamma glutamyl transferase. Data are expressed as mean ± SEM (standard error of the mean).[a, b] and [c] mean $p < 0.05$ or $p < 0.001$ when comparing group 1 to group 2, group 1 to group 3 and group 2 to group 3, respectively. NS = not significant

In Table II, results for variables measured by ELISA methods inT2DM patients are shown.

Comparing the groups, the values of the inflammatory markers (IL-6 and TNF-α) (Figure 1), of C peptide and uric acid were significantly

increased in the groups with more severe insulin resistance (groups 2 and 3) *versus* those with HOMA-IR < 3. No significant differences were recorded between groups 2 and group 3, regarding these four parameters. Leptin ($p<0.05$), proinsulin ($p<0.01$) and C peptide ($p<0.001$) were significantly increased in group 3 *versus* group 1 and dyslipidaemia (high triglyceridaemia and low HDL) was more severe.

In diabetic patients, TNF-α was correlated with proinsulin ($r=0.65$, $p<0.05$), RB ($r=0.73$, $p<0.05$), IL-6 ($r=0.30$, $p<0.05$) and with the duration of diabetes ($r=0.77$, $p<0.05$), while IL-6 was correlated with RB ($r=0.28$, $p<0.05$) and with the duration of diabetes ($r=0.26$, $p<0.05$).

Table II

The results for variables measured by ELISA methods inT2DM patients

Parameters	Diabetic patients (lot T2DM; n=138)			p-value
	Group 1 (n=25)	Group 2 (n=28)	Group 3 (n=85)	
Insulin (μU/mL)	6.53±0.35	11±0.47	22±1.68	<0.001[a, b, c]
Proinsulin (μU/mL)	9±1.62	14±3.10	18±1.77	<0.001[b]
C peptide (ng/mL)	2.70±0.33	4.73±0.50	5.60±0.36	<0.05[a, b]
Proins/Ins ratio	1.66±0.35	1.24±0.24	0.96±0.10	NS
Leptin(ng/mL)	12.62±2.47	15±3.03	18±1.59	<0.05[b]
Proins/Adiponectin ratio	1.28±0.28	2.27±0.56	3.96±0.57	<0.05[b, c]
IL-6 (pg/mL)	16±1.68	23±3.24	23±1.77	<0.05[a, b]
TNF-α (pg/mL)	7.54±1.60	12±1.94	12±1.67	<0.05[a, b]

Data are expressed as mean ± SEM (standard error of the mean).[a, b]and [c] mean $p< 0.05$ or $p<0.001$ when comparing group 1 to group 2, group 1 to group 3 and group 2 to group 3, respectively
NS = not significant

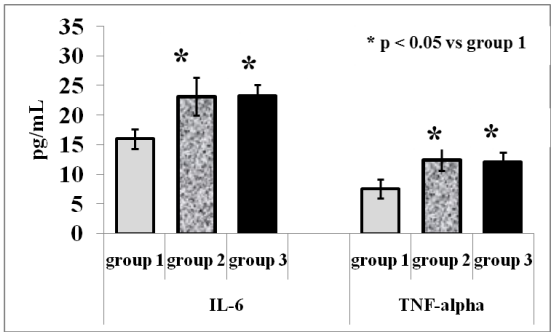

Figure 1.
IL-6 and TNF-α values in the studied groups

TNF-α is a proinflammatory cytokine which plays a causal role in obesity mediated insulin resistance, being extremely important in T2DM pathogenesis. It seems that adipocyte cell volume of both the subcutaneous and visceral fat depots may be determinants of TNF-α, sTNF-α R-2 (soluble serum TNF-α receptor 2) production and obesity-linked insulin resistance [55]. It is well known that waist circumference is a surrogate marker for insulin resistance [39], but in this study, the proinflammatory cytokines were correlated with BMI but not with the waist circumference and our result is in accordance with other studies [41, 33]. Most researchers demonstrated that higher levels of IL-6 and TNF-α are attributed to visceral fat [27, 28, 22]. But a very recent study, performed on subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) obtained from bariatric surgery, demonstrated that there is no association between gene expression in visceral fat and circulating level for proinflammatory cytokines and that abdominal SAT contributes more than VAT to the pro-inflammatory milieu associated with severe obesity [48].

TNF-α contributes to dyslipidaemia by negative effects on lipoprotein lipase activity, by inhibiting free fatty acid uptake and enhancing lipolysis [32, 36].

In the Insulin Resistance Atherosclerosis Study, the TNF-α was negatively correlated with HDL and positively with triglycerides and PAI-1(plasminogen activator inhibitor-1), and the correlation varied by ethnicity [40]. In diabetic patients with end stage renal disease, triglycerides were significantly higher in patients with higher HOMA-IR, whereas HDL cholesterol was significantly lower in those patients [9]. In our study the HOMA-IR values and not also the proinflammatory cytokines were correlated with dyslipidaemia. Groups 2 and 3, with high values for HOMA-IR had severe dyslipidaemia characterized by increased levels of triglycerides and decreased values for HDLc.

Both TNF-α and IL-6 can alter insulin sensitivity by triggering different key steps in the insulin signalling pathway. Phosphorylation of IRS-1 at Ser or The residue may explain insulin resistance under the action of TNF-α on insulinodependent tissue [19, 31]. The inflammatory markers are strong predictors of the development of diabetes mellitus and they have higher levels in subjects before diabetes mellitus onset [43, 15, 47, 45, 49]. Hyperglycaemic crises are associated with an increase in proinflammatory cytokines, markers of lipid peroxidation as TBARS (thiobarbituric acid reactive species), glutathione, cortisol and insulin possesses antiinflammatory actions [50].

Some authors demonstrated that an increase in glycaemic levels associated or not with an increase in proinflammatory cytokines such as IL-6 and TNF-α stimulates the transcription of several nuclear factor kappa light enhances of activated B cells (NF-kB) dependent proteins, which have proven their involvement in coagulation and inflammation. This effect is partly mediated by an increase in reactive oxygen species levels [29].

It was also reported that normoglycaemic obese patients, just as patients with T2DM present increased levels of C reactive protein (CRP), plasminogen activator inhibitor type 1 (PAI-1), free fatty acids (FFA), IL-6 and TNF-α [54, 21].

A recent study emphasizes that the state of subclinical inflammation defined by inflammatory scores including IL-6, TNF-α underlies whole-body insulin resistance and hyperglycaemia in T2DM [16]. Moreover, several potential factors present in diabetic patients as obesity, increased inflammatory cytokines and oxidative stress markers may influence the association between T2DM and cancer [17].

The Insulin Resistance Atherosclerosis Study, performed on a large multiethnic population (more than 1550 subjects) demonstrated increased levels of TNF-α in individuals with impaired glucose tolerance and type 2 diabetic patients. TNF-α was more closely associated with increased insulin resistance than with defects in beta cell function [40]. In a study on diabetic patients divided according to the diabetes duration a strong correlation between TNF-α and HOMA-IR was registered for those who had the illness for 6-10 years [51].

In our study the inflammatory cytokines, IL-6 and TNF-α were associated with HOMA-IR and they were correlated with the fasting proinsulin level and with the ratio proinsulin/insulin, known also as specific markers of insulin resistance. These values indicate a compensatory over functioning of beta cells to nullify the insulin resistance produced by TNF-α. Our previous studies have also shown that acute reactive proteins (haptoglobin and ceruloplasmin) are correlated with LDL cholesterol and atherogenic index and also with the level of leukocytes [55].

T2DM is characterized by an increased systemic oxidative stress and also, by an increased oxidative stress in leukocytes and monocytes [5] and is also correlated with the obesity degree and with serum adipocytokines levels [6]. Hyperglycaemia is the main trigger of NADPH oxidase activation, which contributes to superoxide production and this activation of monocytes is a major factor in the earliest events of atherosclerosis [26]. An i*n vitro* study demonstrated that the generation of superoxide (O_2^-) by monocytes from diabetic subjects versus control was higher and it was correlated with glycated haemoglobin of patients [18]. Almeida et al. [5] demonstrated that under basal culture condition, NADPH oxidase from different cells, including peripheral blood monocytes, is activated under stimulation with TNF-α and interferon-gamma. The researchers showed also that phorbol 12-myristate 13-acetate (PMA) stimulated superoxide release by the studied cells.

Glucose homeostasis and the activity of the TNF-α system may influence leptin secretion and production among overweight men [13]. Also, leptin can modulate TNF-α production and macrophage activation. TNF-α is overproduced in the adipose tissue of several rodent models of obesity and has an important role in the pathogenesis of insulin resistance in these species. However, its actual involvement in glucose metabolism disorders in humans remains controversial. In this study, the highest values for leptin were observed in group 3 formed by subjects with severe insulin resistance and increased BMI. Neither leptin nor uric acid were correlated with TNF-α or IL-6. In groups 2 and 3, uric acid had high significant values and it was negatively correlated with the ratio proinsulin/C peptide ($r= 0.21$, $p<0.05$). In the literature we found one study, performed on women with preeclampsia, which demonstrated that elevated serum uric acid levels were correlated with TNF-α and superoxide production by monocytes. This may contribute to an enhanced oxidative and inflammatory state [24, 42].

In this study, even the relations were weak (but with statistical significance), both cytokines TNF-α and IL-6 were correlated with the respiratory burst and with the duration of diabetes. One of the strongest correlations was between TNF-α and proinsulin. Also, we emphasized that diabetic patients with HOMA-IR higher than 3 (arbitrary units) have an increased inflammatory status, uricaemia, dyslipidaemia and high respiratory burst.

Conclusions

We found that the severity of HOMA-IR is associated with a proinflammatory status and an increased oxidative stress in patients with T2DM emphasizing a higher cardiovascular risk in these patients. This study can't explain the causal mechanisms among variables, but helps the physician to achieve a better management, targeting the cluster of pathogenic modifications in T2DM.

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