

CORRELATIONS BETWEEN OBESITY ANTROPOMETRIC MARKERS, ADIPOCYTOKINES AND MONOCYTES OXIDATIVE STRESS STATUS IN TYPE 2 DIABETIC PATIENTS

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Abstract

Adipose tissue is linked to cardiovascular and metabolic complications of obesity by increased local production of adipocytokines that may lead to increased oxidative stress and endothelial dysfunction.

The aim of this study was to evaluate the pro-oxidant and antioxidant balance on peripheral mononuclear cells in overweight/obese diabetic patients.

Two groups of subjects were selected: one with type 2 diabetes mellitus (group DM, n=60) and a control group (group M, n=30). The first group was divided in two subgroups, according to their body mass index (overweight group, with BMI 25-29.9 kg/m² and obese group, with BMI ≥ 30 kg/m²).

The monocyte capacity to release oxidative radicals and to neutralize them was determined by measuring the NADPH oxidase activity and, respectively, the activity of the intracellular antioxidant enzyme paraoxonase2 (PON2). Microalbuminuria was determined by measuring the urinary albumin excretion rate, while serum leptin and adiponectin levels were determined by ELISA methods.

All the determined parameters were modified significantly in the diabetic patients *versus* control. Comparing the obese diabetic patients with the overweight diabetics, increased NADPH oxidase activity ($p=0.005$) was measured. PON2 activity was similar in subgroups, but much lower than in control ($p<0.001$). Serum leptin (for $p<0.05$) was correlated with the visceral fat ($r=0.55$), with the monocyte NADPH oxidase activity ($r=0.39$) and with microalbuminuria ($r=0.31$), while adiponectin was negatively correlated with the level of visceral fat ($r=-0.27$).

This study emphasizes the monocyte's pro-oxidant/antioxidant imbalance in obese diabetic patients. Low monocyte PON2 activity and high NADPH oxidase activity are influenced by the obesity degree and by serum adipocytokines levels.

Rezumat

Țesutul adipos, prin producerea de adipocitokine determină un proces oxidativ crescut care stă la baza complicațiilor cronice vasculare din diabet.

Scopul prezentului studiu a fost evaluarea statusului pro-oxidativ/antioxidativ în celulele mononucleare periferice și corelarea acestuia cu markeri ai obezității.

Două grupuri au fost selectate: un grup de pacienți cu diabet de tip 2 (grupul DM; n=60) și un grup martor (grup M; n=30). Primul grup a fost împărțit în funcție de valoarea indicelui de masă corporală (subgrupul de supraponderali, cu IMC 25-29,9 kg/m² și subgrupul de obezi cu IMC ≥30 kg/m²).

În monocitele circulante, capacitatea de producere a radicalilor liberi versus capacitatea de neutralizare a lor a fost determinată prin măsurarea activității NADPH oxidazei și respectiv prin activitatea intracelulară antioxidantă a enzimei paraoxonaza2 (PON2). Microalbuminuria a fost determinată prin măsurarea ratei de excreție urinară, iar concentrațiile serice de leptină și adiponectină au fost determinate prin metoda ELISA.

Toți parametrii determinați au fost modificați semnificativ la pacienții diabetici față de control. Comparând pacienții obezi diabetici cu diabeticii supraponderali, a fost determinată o activitate crescută a NADPH oxidazei (p=0.005). Activitatea PON2 a fost similară pe subgrupuri, dar mult mai mică față de control (p<0.001). Leptina serică (p<0.05) s-a corelat cu grăsimea viscerală (r=0.55), cu activitatea NADPH oxidazei (r=0.39) și cu microalbuminuria (r=0.31), în timp ce, adiponectina s-a corelat negativ cu grăsimea viscerală (r=-0.27).

Acest studiu subliniază dezechilibrul pro-oxidant/antioxidant din monocitele circulante ale pacienților diabetici obezi. Activitatea scăzută a PON2 și activitatea crescută NADPH oxidazei monocitare sunt influențate de gradul obezității și de concentrația serică a adipocitokinelor.

Keywords: Monocyte; NADPH oxidase; Paraonase2; Adipocytokines

Introduction

In association with obesity and the metabolic syndrome, diabetes is considered a typical vascular disease, being an independent risk factor for atherosclerosis [1, 2]. Patients with diabetes mellitus have a 2-3 times higher risk for cardiovascular morbidity and mortality [3]. Among the underlying mechanisms that can account for this acceleration of atherosclerosis in diabetes, abnormalities in monocyte/macrophage (mo/ma) function contribute significantly to the development of the atheroma plaque, its progression and even rupture [4]. Monocytes by means of their respiratory burst (NADPH oxidase activity), are an important source of free radicals that promote multiple atherogenic pathways in the vascular wall [5]. In diabetes mo/ma are more numerous in the atheroma plaque [6] and display an enhanced respiratory burst [7, 8]. This abnormality is related to the higher blood concentration of both triglycerides [9], fatty acids [10] and glucose [11] found in these patients.

Recently, high density lipoprotein (HDL) has also been shown to both protect low density lipoprotein (LDL) from oxidation and to attenuate the biological effects of oxidized LDL *in vitro* [12]. This antioxidant activity is largely due to the presence of the enzyme paraoxonase1 (PON1) within the HDL particle. By preventing the oxidation of HDL and LDL, PON1 could slow down

the initiation and progression of atherosclerotic lesions and, in this way protect against cardio-vascular diseases (CVD) [13]. The recent identification of another member of paraoxonase (PON) gene family, PON2, present in macrophages, opens up new perspectives on the interactions involving the various paraoxonases [14].

Oxidative stress leading to macrophage foam cell formation is the hallmark of the early atherosclerotic lesion [15, 16]. Lipid peroxidation occurs not only in the lipoproteins but also in the arterial cells, including macrophages [17]. These "oxidized macrophages" contain more peroxides and less glutathione and have an increased capacity to oxidize LDL [18]. Serum PON1 was found to decrease macrophage oxidative stress [19,20] but is itself decreased under oxidative stress conditions [21].

Unlike PON1, PON2 mRNA activity appears to be up-regulated in response to oxidative stress. Treatment of mouse peritoneal macrophages with various oxidative stress-inducing agents resulted in an increase in PON2 mRNA and lactonase activity, probably as a compensatory mechanism against oxidative stress [22]. PON2 is also able to lower the intracellular oxidative stress of a cell and to prevent the cell-mediated oxidation of LDL. Cells over-expressing PON2 are less able to oxidize LDL and show considerably less intracellular oxidative stress when exposed to either H₂O₂ or oxidized phospholipids. Since PON2 is ubiquitously expressed not just in cells of the artery environment but in tissues throughout the body, it is likely that PON2 plays a role in reducing intracellular or local oxidative stress [23]. In humans, lower levels of PON2 mRNA are seen in monocyte-derived macrophages of hypercholesterolemic individuals when compared to individuals with normal cholesterol levels. Administration of atorvastatin was able to reverse this effect [24].

The adipose tissue secretes several biologically active substances called adipocytokines including leptin and adiponectin [25, 26].

Leptin has a variety of important central and peripheral actions to regulate energy balance and metabolism, fertility and bone metabolism that are mediated by specific cell surface leptin receptors [27, 28]. Adiponectin is produced exclusively by white adipose tissue and in contrast to the other adipokines, its expression is reduced in obese subjects [29]. Adiponectin has anti-atherogenic action by down regulating the expression of endothelial adhesion molecules that participate in the recruitment of macrophages to inflammatory lesions, which is crucial for the development of atherosclerosis [30]. The net effects of these adipocytokines released by adipose tissue are complex and not yet completely understood.

The aim of this study was to evaluate the pro-oxidant and antioxidant status on peripheral mononuclear cells in obese diabetic patients and to correlate the values

with markers of cardiovascular risk (microalbuminuria, low adiponectin) and with the body mass index (BMI). The monocyte capacity to release oxidative radicals and to neutralize them was determined by measuring the NADPH oxidase activity and, respectively, the activity of the intracellular antioxidant enzyme paraoxonase2 (PON2).

Materials and Methods

Study Subjects

The study was approved by the ethical commission of the „N. Paulescu” National Institute of Diabetes, Nutrition and Metabolic Diseases, Bucharest, Romania, and the informed consent was obtained from all participants.

Blood was collected from 60 (29 male/31female) diabetic patients attending of the “N. Paulescu” National Institute of Diabetes, Nutrition and Metabolic Disease after 12 hours of overnight fasting. Morning urine samples were collected in a container for analysis of creatinine and albumin excretion rate. Blood samples were also taken from a group of 30 sex-matched nondiabetic healthy volunteers recruited from the university and medical hospital staff (control group, lot M).

Type 2 diabetic patients were divided into two subgroups according to their body mass index (BMI) as follows: group 1 = overweight patients with a BMI between 28.4- 29.9 kg/m² and group 2 = obese patients with a BMI \geq 30 kg/m² (30-40).

The *inclusion criteria* in the group with type 2 diabetes were: positive diagnosis of type 2 diabetes, age 40–70 years, fasting blood glucose level >7 mmol/L (>126 mg/dL), HbA_{1c} $\geq 6.5\%$, duration of diabetes > 3 years, previous oral anti diabetic therapy or appropriate diet therapy. Type 2 diabetes was defined according to the criteria of American Diabetes Association [31].

The *exclusion criteria* included the following: urinary infection, congestive heart failure, myocardial infarction, or stroke in the past 6 months; epilepsy or other severe diseases; liver disease, serum creatinine concentration $>120\mu\text{mol/L}$ (1.36mg/dL), or macroalbuminuria, proliferative retinopathy or severe maculopathy; excessive alcohol consumption (>20 g/day) and night work as previously described.

We performed anthropometric measurements: weight, height, visceral fat using a resistometric assessment (Body composition analyzer-MC-980 from Tanita), waist and hip circumferences. The BMI was calculated as weight in kilograms divided by height in square meters. Albumin excretion rate was determined by measuring the albumin excretion rate from three non consecutive first morning urine samples. Normal albumin excretion rate was $<30\text{mg/g}$ creatinine and microalbuminuria was defined as albumin excretion rate (AER) in

the range of 30 and 299 mg/day [31]. The ACR (albumin to creatinine ratio) was calculated as follows: urinary albumin concentration (mg/L)/urinary creatinine concentration (mg/dL). The mean value of each patient's three ACRs was used to indicate the level of albumin excretion [31].

Reagents

PBS (Dulbecco's Phosphate Buffered Saline), Ficoll-Paque™ Plus, D-glucose, Trypan Blue, lucigenin (N,N'-dimethyl-9,9'-biacridinium dinitrate, LG), PMA (phorbol 12-Myristate 13-Acetate), dihydrocoumarin (DHC) were purchased from Sigma Chemical Co., St.Louis, MO, USA.

Analytical methods

Routine blood tests including total cholesterol, glucose, HDL-cholesterol (high density lipoprotein; HDLc), triglycerides (TG), serum creatinine, urea, uric acid and glycosylated hemoglobin (reference range 4.0-6.0%) were measured using standard HPLC techniques on the day of sampling from fasting venous blood samples. LDL-cholesterol (low density lipoprotein; LDLc) was calculated according to the Friedewald equation [32]. Leptin and adiponectin levels were respectively determined using the EIA-2395 and EIA-4177 kits, ELISA methods (DRG Instruments GmbH, Germany). The coefficient of variation (CV) was respectively 5.95% and 7.4%.

The oxidative stress status was evaluated by measuring the respiratory burst on peripheral blood mononuclear cells and the antioxidant enzyme PON2 activity.

Isolation of peripheral blood mononuclear cells (PBMC) and Respiratory Burst: PBMC were isolated by density centrifugation on Ficoll-Paque™ Plus (1.0077g/mL). After centrifugation at 630g for 30 min the mononuclear cells (PBMC) were collected, washed twice and resuspended in 1mL PBS. Cell viability by Trypan Blue exclusion was $\geq 90\%$.

The ability to produce a respiratory burst was monitored by lucigenin-enhanced chemiluminescence [33]. In short, to PBMC (0.3×10^6 cells) resuspended in phosphate-buffered saline, dark-adapted lucigenin (final concentration $0.143 \mu\text{mol/L}$) was added. After monitoring spontaneous chemiluminescence for 15 min, the respiratory burst was initiated by adding of $100 \mu\text{L}$ PMA (final concentration $5.4 \mu\text{mol/L}$) and the maximum chemiluminescence peak was recorded (Luminometer TD 20/20, Turner Designs). Chemiluminescence production was expressed as Relative Chemiluminescence Units (RLU).

Paraoxonase 2 lactonase activity towards dihydrocoumarin (DHC) was determined at 270nm using a continuously recording UV/VIS spectrophotometer (CECIL,-CE 1010). Protein concentration was determined using the method of Bradford [34]. $100 \mu\text{L}$ cells suspension were added to $1880 \mu\text{L}$ Tris buffer

(25mmol/L Tris/HCl, 1 mmol/L CaCl₂, pH=7.6) containing 1mmol/L DHC. One unit of lactonase activity is equal to 1 μ mol of DHC hydrolyzed/mL/min using the extinction coefficient of 1295M⁻¹cm⁻¹ [35].

Statistical Analysis

Results were expressed as mean \pm SD (standard deviation)/SEM (standard error of the mean). Data were analysed using the statistical package SPSS version 16.0. Differences between groups were analysed using Student's t-test and two-tailed p-values <0.05 were considered as statistically significant. Differences between groups were calculated using two-way ANOVA. Multiple linear regression and Pearson correlations were applied to identify the relationship between the various parameters.

Results and Discussion

Subjects' clinical and biochemical characteristics

The clinical characteristics and routine biochemistry tests of the healthy subjects and diabetic patients are shown in Table I. Notably, diabetic patients compared to controls had higher BMI, atherogenic index, homeostasis model assessment of insuline resistance (HOMA-IR), ACR, urea and uric acid levels. Serum leptin levels were found to be higher, while serum adiponectin levels were lower in diabetic patients when compared with controls (Table II).

Table I
Clinical and biochemical characteristics of the healthy subjects and diabetic patients

Parameters	Healthy subjects (lot M) (n=30)	Diabetic patients (n=60)	Diabetic patients		p-value group 2 vs group 1	p-value diabetic patients vs mator
			Group 1 (n=20)	Group 2 (n=40)		
Age (years)	52.28 \pm 7.16	55.63 \pm 8.13	56.75 \pm 7.53	55.08 \pm 8.45	NS	NS
Body mass index (kg/m ²)	23.1 \pm 2.24	33.29 \pm 3.78	29.10 \pm 0.52	35.39 \pm 2.81	<0.001	<0.001
Duration of diabetes (years)	-	5.88 \pm 3.08	5.2 \pm 2.5	6.2 \pm 3.31	NS	-
Sex (female/male)	14/16	31/29	10/10	19/21	NS	NS
Smokers/Nonsmokers	12/18	17/43	7/14	10/30	NS	NS
Systolic blood pressure (mmHg)	118 \pm 5.21	135 \pm 7.74	142 \pm 4.01	132 \pm 6.78	NS	<0.001
Diastolic blood pressure (mmHg)	70 \pm 4.7	82 \pm 5.45	85 \pm 3.66	80 \pm 5.6	NS	<0.001
Serum total cholesterol (mg/dL)	179 \pm 20	201.95 \pm 34.07	202.55 \pm 31	201.65 \pm 36	NS	<0.001
Serum HDL cholesterol (mg/dL)	54 \pm 4	45.25 \pm 9.01	47.36 \pm 8	44.19 \pm 10	NS	<0.001
Serum LDL cholesterol (mg/dL)	99 \pm 19	122.12 \pm 32.96	122.28 \pm 32	122.04 \pm 34	NS	<0.001
Serum triacylglycerol (mg/dL)	129 \pm 14	172.86 \pm 72.49	164.5 \pm 102	177 \pm 53	NS	<0.001
Atherogenic Index	3.3 \pm 0.33	4.6 \pm 1.07	4.39 \pm 1.11	4.7 \pm 1.04	NS	<0.05
FPG (mg/dL)	84 \pm 9	155 \pm 23	145 \pm 22	161 \pm 27	<0.05	<0.001
HbA _{1c} (%)	4.94 \pm 1.4	7.86 \pm 0.84	7.75 \pm 0.97	7.87 \pm 0.78	NS	<0.001
HOMA-IR (arbitrary units)	1.26 \pm 0.33	5.39 \pm 3.4	3.87 \pm 1.8	6.15 \pm 3.76	<0.001	<0.05
ACR (mg/g)	5.36 \pm 1.75	22.77 \pm 14	21.56 \pm 10	23.37 \pm 15.45	NS	<0.001
Urea (mg/dL)	30.88 \pm 5.36	34.29 \pm 8.36	36 \pm 7	33 \pm 9	NS	<0.05
Uric Acid (mg/dL)	4.18 \pm 0.62	5.96 \pm 1.43	5.53 \pm 1.35	6.17 \pm 1.44	NS	<0.05

Data are expressed as mean \pm SD. NS = not significant; FPG = fasting plasma glucose; ACR = albumin to creatinine ratio; HOMA-IR = homeostasis model assessment of insuline resistance; HbA_{1c} = glycosylated hemoglobin

Table II
The serum adipocytokines levels

Parameters	Healthy subjects (lot M) (n=30)	Diabetic patients (n=60)	Diabetic patients Group 1 (n=20)	Diabetic patients Group 2 (n=40)	p-value group 2 vs group 1	p-value diabetic patients vs martor
Serum Leptin (ng/mL)	5.23±1.68	11.77±3.2	8.91±2.86	13.2±2.47	<0.001	<0.001
Serum Adiponectin (ng/mL)	14.16±1.48	9.08±7.78	10.44±2.96	8.39±2.44	<0.05	<0.001

Oxidative stress status

As shown in Figure 1, the reactive oxygen species (ROS) production and the concentration of antioxidant enzymes PON2 was different in the two diabetic groups. Whereas, the respiratory burst (RB) increased in the two groups when compared to the control group (0.654 ± 0.059 ; 1.023 ± 0.053 vs 0.452 ± 0.032 RLU lot M, $p < 0.05$), antioxidant enzymes PON2 decreased (0.047 ± 0.008 ; 0.066 ± 0.006 and respectively 0.237 ± 0.035 U/mg proteins; $p < 0.001$).

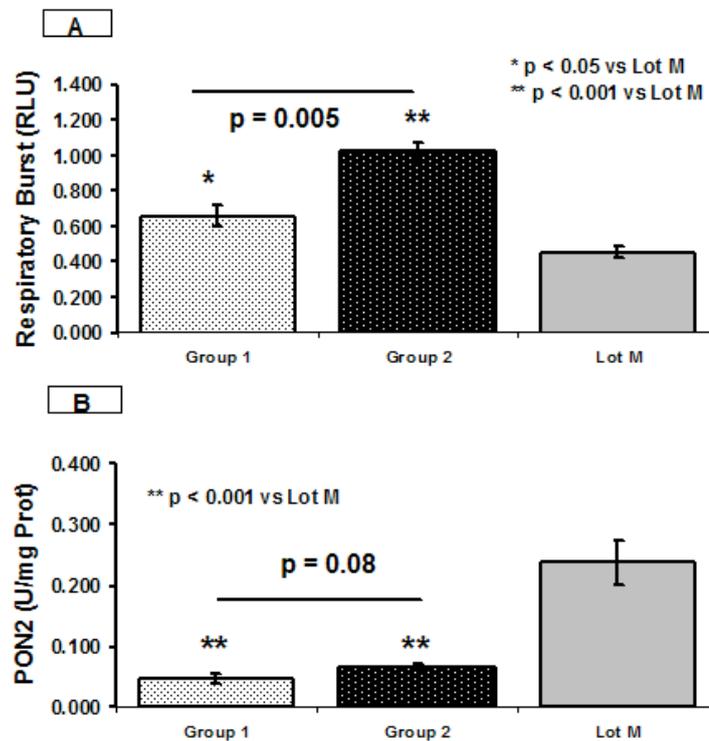


Figure 1

The measured markers of oxidative stress. Respiratory burst (A) and lactonase PON2 enzymatic activity (B) in the healthy subjects (lot M) and diabetic patients (groups 1 and 2).

The correlations lower than -0.26 and higher than 0.26 are considered significant and are presented in Table III.

Table III
The correlations between the studied parameters (r values)

Antropometric Factors	Leptin	Adiponectin	Respiratory Burst	PON2
BMI (kg/square meter)	0.66	-0.33	0.49	NS
Visceral Fat (%)	0.55	-0.26	0.31	0.26
Waist Circumference (cm)	0.28	NS	NS	NS
Hip Circumference (cm)	0.45	-0.34	0.41	NS

NS = not significant

The association between obesity and diabetes has been well interpreted by Paulescu in 1920 [36] in a short and conclusive paragraph: „Often, the obese people became glycosuric, as the two disturbances (obesity and diabetes) are two successive phases of the same pathogenic process“. This association has been found by us in a large cohort of newly discovered diabetics in a proportion of almost 90% [37]. In addition, diabetes mellitus was well documented to be a true vascular disease.

Our study aimed to investigate if obesity, in particular the visceral fat accumulation, can affect monocyte/macrophage functions and their antioxidant enzymes capacity thus affecting their proatherogenic potential.

In obesity, enlargement of adipocyte size can lead to activation of adipocytes that begin to secrete cytokines which attract macrophages from the adipose tissue [38]. This is a stimulus for preadipocytes and resident endothelial cells to produce MCP-1/CCr2 (Monocyte Chemoattractant Protein-1/CC motif chemokine receptor-2) that stimulate macrophage recruitment and infiltration into the adipose tissue [39]. So, in obese, prediabetic/diabetic subjects, the macrophage infiltrated white adipose tissue becomes an important source of increased reactive oxygen species (ROS) production. Elevated ROS appear to upregulate mRNA expression of NADPH oxidase, establishing a vicious cycle that augments oxidative stress in white adipose tissue and blood [40]. Visceral fat, compared with white adipose tissue, registers a higher degree of macrophages infiltration and its secretion of proinflammatory cytokines is greater (eg. IL-6) [41], augmenting the systemic oxidative stress.

Vascular ROS are linked to impaired endothelial function and progression of atherosclerosis. Also, under diabetic conditions, ROS levels are increased in many tissues and organs, and especially, NADPH-derived ROS could play a critical role in leading to the progression of β -cell dysfunction in type 2 diabetes [42].

Oxidative stress could induce inflammation during development of insulin resistance. The molecular pathways that link inflammation and insulin resistance include a variety of cytokines and adipocytokines such as tumor necrosis factor - α (TNF- α), interleukin-6 (IL-6), MCP-1, leptin, and adiponectin [43].

In this study, the BMI was positively correlated with the values for leptin and negatively with the values for adiponectin. Moreover, respiratory burst (NADPH oxidase activity) and adiponectin were correlated with the visceral fat level and with waist circumference (positive for respiratory burst and respectively negative for adiponectin (Table II and III).

Leptin has certain structural similarities to classical cytokines. Acting on monocytes, leptin induces the release of other cytokines (like TNF- α , IL-6). Moreover it leads to increased proliferation and differentiation of monocytes. Acting on neutrophils, leptin leads to an increase of CD11b expression as well increased neutrophil chemotaxis and oxidative burst [44]. In our study, we found a positive correlation between serum leptin levels and RB ($r=0.39$, $p<0.05$) in diabetic patients. And more, our data underline that the RB of activated mo/ma is increased in diabetes and is associated with an abnormal antioxidant activity of PON2. We measured a significantly decreased activity for the antioxidant enzyme PON2 in diabetic patients (group 1-overweight and group 2-obese) *versus* the control group (Figure 1B). On contrary, the NADPH oxidase activity is increased in both groups 1 and 2 *versus* normal subjects (Figure 1A). From the above mentioned data (Table 1) we could conclude that a decrease in the intracellular mo/ma antioxidant capacity could be an important player in inducing chronic vascular complications of diabetes. A pro-oxidant status created by these double-pathogenic mechanisms can contribute to the endothelial dysfunction.

Endothelial cells serve as a metabolically active barrier between the lumen and the vessel wall and play a pivotal role in vascular homeostasis. Loss of normal endothelial cells function is thought to be an early marker of development of chronic micro and macro vascular diseases.

The main link between obesity-diabetes-vascular dysfunction could be the oxidative stress [45-50]. Increasing evidence show that local production of reactive oxygen species (ROS) has a pivotal role, in both beta

cells and endothelial dysfunction [51]. Such changes could be mediated by increased NADPH oxidase activity in vascular endothelium. Evidence of increased local vascular generation of ROS in diabetes is also supported by the nitro-tyrosine staining present in vascular structures [52].

Endothelial dysfunction can be manifested by an increase in the urinary excretion rate. Endothelial dysfunction is the central event in initiating the process of vascular diseases including diabetic renal disease. We found positive correlation between ACR and HbA_{1c} and glycemia ($r=0.57$ and 0.50 respectively), with HOMA-IR ($r=0.30$) and with leptin ($r=0.31$).

Conclusions

The contribution of the oxidative stress mechanisms induced by both, increased body fat and hyperglycaemia, could be one of the vascular risk factors operating early in the natural history of diabetes. The appropriate treatment of the oxidative stress might represent a mean by which we can prevent the progression of normoalbuminuric patients into a microalbuminuric stage or in the regression of the albumin elimination rate from microalbuminuric stage into the normal status.

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