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RELATION OF ENDOTHELIAL DYSFUNCTION AND ADIPOKINE LEVELS TO INSULIN RESISTANCE IN METABOLIC SYNDROME PATIENTS

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Obese metabolic syndrome (MS) patients were categorised into three groups: 44 with type 2 diabetes mellitus (T2DM)(D); 20 with T2DM and coronary artery disease (CAD) (DC), and 26 with MS alone (M). Eighteen healthy subjects were selected as controls (C). Insulin resistance (IR) was assessed by HOMA-IR. Adiponectin, tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and interleukin-8 (IL-8) concentrations were measured by xMAP technology. Endothelin-1 (ET-1) was determined by ELISA. We used laser Doppler imaging for evaluating cutaneous endothelium-dependent vasodilatation in the hand. D and DC groups had significantly elevated IR compared with M or C group (P < 0.01). TNF-α, IL-6, IL-8, MCP-1 and ET-1 levels in DC were significantly elevated compared with other groups (P < 0.001). IL-6, IL-8, MCP-1 and ET-1 in D group were higher than those in C group (P < 0.05). TNF-α, IL-6, IL-8, MCP-1 and ET-1 concentrations were correlated with HOMA-IR indexes and adiponectin levels. All patients had lower adiponectin concentrations than controls (P < 0.001), but there were no differences between the patient groups. Only D and DC groups demonstrated a significant and similar decrease in LDI-Ach marker compared to C group (P < 0.001). LDI-Ach values were significantly correlated with HOMA-IR indexes and adiponectin levels (P < 0.001). Our findings show that obese MS patients have significantly increased HOMA-IR, TNF-α, IL-6, MCP-1 and IL-8 levels, decreased adiponectin concentration, and endothelial dysfunction, but the presence of T2DM and CAD in these patients is associated with more pronounced endothelial dysfunction and increased production of inflammatory cytokines and chemokines.

Key words: *metabolic syndrome, insulin resistance, endothelial dysfunction, adiponectin, inflammatory cytokines.*

INTRODUCTION

The pathophysiological basis of the metabolic syndrome (MS) is multiple and complex. Obesity is characterised by microvascular alterations and can affect microvascular function. There is increasing evidence that microvascular function is a potential factor explaining the clustering of several components of MS (Serne *et al*., 2007). Microvascular dysfunction may affect both peripheral vascular resistance (Antonios *et al*., 1999) and insulin-mediated glucose disposal (Clark *et al*., 2003), thereby contributing to hypertension and insulin resistance (IR). Studies have shown that obesity-related insulin resistance is associated with increased production of proinflammatory cytokine, e.g., tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) and that the vasculature is an important target of TNF-α (Youd *et al*., 2000).

TNF-α elevation impairs insulin sensitivity and increases blood pressure through mechanisms that are not completely understood but do involve microvascular function (Youd *et al*., 2000). TNF-α down-regulates the expression of endothelial nitric oxide synthase (eNOS) (10- Rask-Madsen and King, 2007) and up-regulates endothelin-1 (ET-1) expression in human endothelial cells (Mohamed *et al*., 1995). More importantly, adipose tissue-derived TNF-α may suppress insulin-mediated hemodynamic and metabolic effects through inhibition of insulin receptor substrate (IRS)-1 phosphorylation (Williams *et al*., 2002).

The chemokines monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) appear to be involved in atherosclerosis and obesity (Charo *et al*., 2004), besides elevated concentrations of MCP-1 and IL-8 are associated with the incidence of T2DM (Herder *et al*., 2006). It has been postulated that MCP-1 and IL-8 might be an important step in recruitment and activation of peripheral blood monocytes and leucocytes in atherosclerotic lesions (Herder *et al*., 2006) and adipose tissue (Wellen and Hotamisligil, 2003). In particular elevated levels of MCP-1 and IL-8 are involved in the pathogenesis of diseases associated with excess amount of adipose tissue e.g. cardiovascular disease (Shin *et al*., 2002).

Adiponectin is a multifunctional protein produced by adipose tissue and is known to have protective role against the development of insulin resistance, dyslipidemia, and atherosclerosis (Schaffler *et al*., 2005). Obesity is associated with decreased insulin sensitivity (Bruun *et al*., 2003) and hypoadiponectinemia (Haluzik *et al*., 2005).

The aim of the study was to assess endothelial function (ET-1 level, endothelial-dependent vasodilatation) and adipokine levels, including adipocyte derived inflammatory cytokines (TNF-α, IL-6) and chemokines (MCP-1, IL-8), and their relation to insulin resistance in obese MS patients who were categorised as having type 2 diabetes mellitus (T2DM) or both T2DM and coronary artery disease(CAD), or neither.

MATERIAL AND METHODS

Subjects. Obese MS patients with dyslipidemia were divided into three groups (Table 1): 44 patients with T2DM (D); 20 patients with T2DM and CAD (DC), and 26 patients with neither T2DM nor CAD (M). Eighteen healthy subjects were selected as controls (C). The study groups were matched for age and sex. Metabolic syndrome was diagnosed according to the International Diabetes Foundation criteria with specific reference to a European population (Alberti *et al*., 2005). MS patients were not included if their systolic blood pressure was ≥ 160 mm Hg or diastolic ≥ 95 mm Hg and if they were treated with antihypertensive drugs other than angiotensin-converting enzyme inhibitors. Diabetes was defined as a reported history of diabetes and treated with antidiabetic drugs. Duration of T2DM was 8 ±5 yrs and glycated hemoglobin, HbA_{1c} in diabetics was less than 7.5%. Diabetics were without insulin therapy and pronounced diabetic complications. The diagnosis of CAD was substantiated by coronary angiography. Digital coronary angiography was performed by means of a GE Medical System X-ray digital angiography system. Results of coronary angiography were accepted as positive if stenosis ≥ 50% of at least one of the three main epicardial branches of coronary arteries was detected. Patients with acute coronary syndrome and also patients who had evidence of peripheral vascular disease or cerebral ischemia were excluded. Other exclusion factors were acute inflammatory condition or chronic inflammatory states such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis, inflammatory bowel disease, and other diseases which are known to be associated with significant changes of cytokines, including surgery or trauma within the preceding 30 days. Malignancy, alcoholism and smoking were also exclusion criteria. We did not include patients who were taking COX-2 inhibitors, nonsteroidal antiinflammatory agents or corticosteroids, or had been taking them within the preceding 30 days. All subjects gave their informed consent to the protocol, which was approved by the local Medical Ethics Committee of the University of Latvia for Biomedical Research.

Clinical and laboratory investigations. Blood samples for cytokines and other blood tests were taken after a 12-h fast. Samples for determination of cytokines and chemokines were collected without anticoagulant and were allowed to coagulate for 20 to 30 min at room temperature. Sera were separated by centrifugation at 1600 x g for 20 min. All specimens were immediately aliquoted, frozen, and stored

Table 1

ANTHROPOMETRIC AND LABORATORY PARAMETERS OF STUDY GROUPS

	Healthy controls	Metabolic syndrome patients			*P*
	(C)	– (M)	T2DM (D)	T2DM and CAD (DC)	
N (F/M)	18 (10/8)	26 (14/12)	44 (24/20)	20 (12/8)	NS
Age, years	54 ± 10	52 ± 9	55 ± 7	56 ± 8	NS
Body mass index, $kg \cdot m^{-2}$	25.3 ± 3.4	35.0 ± 5.2	35.3 ± 6.1	42.9 ± 3.6	< 0.001
Waist, cm	90 ± 10	112 ± 14	111 ± 16	123 ± 9	< 0.001
Hypertension, %	0 (n = 0)	77 (n = 20)	82 (n = 36)	90 (n = 18)	< 0.001
Systolic blood pressure, mm Hg	125 ± 12	134 ± 10	132 ± 8	136 ± 9	NS
Diastolic blood pressure, mm Hg	80 ± 5	83 ± 6	84 ± 5	85 ± 6	NS
Triglycerides, $mmol \cdot l^{-1}$	1.58 ± 0.91	2.76 ± 0.78	2.95 ± 0.89	3.14 ± 1.02	< 0.001
HDL-cholesterol, $mmol \cdot l^{-1}$	1.19 ± 0.16	0.77 ± 0.12	0.71 ± 0.20	0.62 ± 0.28	< 0.001
LDL-cholesterol, $mmol \cdot l^{-1}$	3.81 ± 0.88	4.90 ± 0.66	5.16 ± 0.95	5.72 ± 1.12	< 0.001

M, male; F, female; data are expressed as number (n), or means ±SD.

NS, not significant ($P > 0.05$ compared to all group).

Table 2

HOMA-IR AND ADIPONECTIN, INFLAMMATORY CYTOKINES, CHEMOKINES AND ENDOTHELIAL DYSFUNCTION MARKERS IN STUDY GROUPS

	Healthy controls (C)	Metabolic syndrome patients			*P*
		– (M)	T2DM (D)	T2DM and CAD (DC)	
HOMA-IR	1.30 ± 0.29	3.87 ± 1.86*	6.00 ± 3.07$^{*,+}$	7.24 ± 5.14$^{*,+}$	< 0.001
Adiponectin (μg/ml)	35.7 ± 15.3	22.7 ± 11.4*	17.4 ± 8.7*	16.1 ± 11.0*	< 0.001
TNF-α (pg/ml)	3.86 ± 0.82	5.62 ± 3.17	5.96 ± 2.75	13.04 ± 7.54$^{*,+,\&}$	< 0.001
IL-6 (pg/ml)	1.84 ± 1.17	3.05 ± 1.79	4.24 ± 3.26*	8.47 ± 6.92$^{*,+,\&}$	< 0.001
IL-8 (ng/ml)	2.84 ± 0.83	4.57 ± 2.25	5.52 ± 3.31*	10.43 ± 5.98$^{*,+,\&}$	< 0.001
MCP-1 (ng/ml)	238 ± 98	325 ± 124	373 ± 87*	534 ± 163$^{*,+,\&}$	< 0.001
ET-1 (fmol/ml)	0.14 ± 0.31	0.34 ± 0.46	0.65 ± 0.46$^{*,+}$	1.51 ± 0.71$^{*,+,\&}$	< 0.001
LDI-Ach (%)	347 ± 107	329 ± 73	144 ± 93$^{*,+}$	137 ± 72$^{*,+}$	< 0.001

Data are presented as mean ± SD.

$^{*}P < 0.01$ vs. controls; $^{+}P < 0.05$ vs. M; $^{\&}P < 0.05$ vs. D.

at –80 ^{o}C. xMAP multiplex immunobead assay technology was used to test TNF-α, IL-6, MCP-1, IL-8, and adiponectin by Luminex200 analyzer (Luminex Corp., Austin, TX). ET-1 was measured by ELISA (Biomedica Gesellschft, Vienna, Austria) (Williams *et al*., 2002). For quantification of insulin resistance, we used the homeostasis model assessment (HOMA-IR = fasting glucose × fasting insulin/22.5). The HOMA-IR values have been shown to correlate well with values obtained using the "gold standard" clamp technique (Bonora *et al*., 2000). Fasting concentrations of lipids, insulin, and glucose were analysed by standard methods.

Blood flow measurements. We used laser Doppler imaging (LDI; moorLDI2, Moor Instruments Ltd., UK) in conjunction with iontophoretic application of 1% acethylcholine (LDI-Ach) solution for evaluating cutaneous endothelium-dependent vasodilatation on the dorsum of the hand (Turner *et al*., 2008).

Statistical analysis. After testing the normality of data distribution, statistical differences between four groups were assessed by one-way ANOVA using Fisher's multiple comparison test. Data were recorded as the means ±SD and two-tailed values of $P < 0.05$ were considered to be significant. Correlation analyses were performed using one-factor linear regression analysis. All analyses were performed using STATISTICA 6.0 software (StatSoft Inc, USA).

RESULTS

All patient groups had significantly elevated HOMA-IR indexes compared with the group of controls, at the same time the value of HOMA-IR in the diabetic group was higher than that in the group of patients with MS alone (D 6.00 ± 3.07 vs M 3.87 ± 1.86, $P < 0.01$), but did not differ from the group with T2DM and CAD ($P > 0.05$) (Table 2).

The levels of TNF-α, IL-6, IL-8, MCP-1 and ET-1 in patients who had both T2DM and CAD were significantly higher compared with those in other groups ($P < 0.001$). These biomarkers did not differ between the patients with MS alone and control subjects, but in the group of diabetics, the levels of IL-6, IL-8, MCP-1, and ET-1 were significantly higher compared with the group of controls ($P < 0.05$) (Table 2). Besides, TNF-α, IL-6, IL-8, MCP-1 and ET-1 concentrations were significantly correlated with HOMA-IR indexes ($P < 0.01$) (Table 3). All patients had lower adiponectin concentrations than control subjects ($P < 0.001$), but there were no differences between the patient groups. A strong correlation between adiponectin concentrations and HOMA-IR indexes (r = –0.49, $P < 0.001$) (Figure 1) was established. The levels of TNF-α, IL-6, IL-8, MCP-1 and ET-1 were also significantly correlated with adiponectin concentrations ($P < 0.01$) (Table 3).

MS patients with T2DM and those who had both T2DM and CAD showed a significant and similar decrease in LDI-Ach marker compared with healthy controls ($P < 0.001$) (Table 2). LDI-Ach values were significantly correlated with HOMA-IR indexes and adiponectin levels r = –0.58 and r = 0.43, $P < 0.001$) (Table 3).

Table 3

CORRELATIONS OF INFLAMMATORY CYTOKINES, CHEMOKINES AND ENDOTHELIAL DYSFUNCTION MARKERS WITH HOMA-IR AND ADIPONECTIN IN TOTAL STUDY CLINICAL MATERIAL

	HOMA-IR		Adiponectin (μg/ml)	
	r	*P*	*r*	*P*
TNF-α (pg/ml)	0.31	< 0.01	-0.39	< 0.001
IL-6 (pg/ml)	0.30	< 0.01	-0.37	< 0.001
IL-8 (ng/ml)	0.34	< 0.001	-0.40	< 0.001
MCP-1 (ng/ml)	0.60	< 0.001	-0.47	< 0.001
ET-1 (fmol/ml)	0.53	< 0.001	-0.49	< 0.001
LDI-Ach (%)	-0.58	< 0.001	0.43	< 0.001

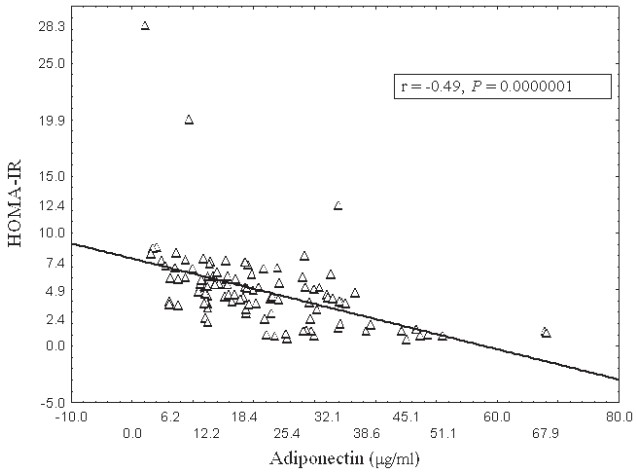

Fig. 1. Correlation of HOMA-IR and adiponectin in total study clinical material.

DISCUSSION

Despite the presence of pronounced insulin resistance (HOMA-IR) in all three groups of obese MS patients, only the group of patients with both T2DM and CAD demonstrated approximately a 3-fold increase in the circulating inflammatory cytokines TNF-α and IL-6 (adipokines) concentration. This group also showed more pronounced endothelial dysfunction (decreased ET-1 level and endothelium-dependent vasodilatation). Insulin resistance in the group of diabetics was similar to that in patients with T2DM and CAD, and the levels of adiponectin and endothelium-dependent vasodilatation did not differ between these two groups. Regarding adipokines, only inflammatory chemokines MCP-1 and IL-8 demonstrated significantly different levels when compared between the groups of obese MS patients with T2DM and those who had both T2DM and CAD and healthy controls. At the same time, in patients with MS alone, the only biomarker that differed from the group of controls was adiponectin.

Taken together, different "stages" of metabolic syndrome are associated with differently pronounced changes of aforementioned biomarkers, insulin resistance, and endothelial dysfunction. Moreover, our study revealed several associations between the variables (Table 3), including already confirmed correlations — between HOMA-IR values and MCP-1 and IL-8 levels (Bruun *et al*., 2003), and also an inverse correlation between adiponectin and HOMA-IR (Bilgili *et al*., 2008).

Many factors are able to cause insulin resistance and endothelial dysfunction. Among them, inflammatory cytokines are of particular importance as T2DM is associated with a low-grade inflammation with increased levels of inflammatory cytokines such as TNF-α, IL-6, MCP-1, and C-reactive protein (Wellen and Hotamisligil, 2005). Increased production of TNF-α is associated with obesity-related insulin resistance (Ijzerman *et al*., 2006) and circulating TNF-α levels are associated with reduced whole body glucose uptake and skin capillary recruitment (Ijzerman *et al*., 2006). TNF-α has been proposed to impair vasodilator effects of insulin in the skeletal muscle microcirculation. Furthermore, in endothelial cells TNF-α has been shown to impair insulin-mediated activation of eNOS (Kim *et al*., 2001). This is achieved through inhibition of insulin-mediated activation of phosphoinositide 3-kinase (PI3K), and protein kinase B, which are crucial steps in insulin-mediated eNOS activation (Zeng *et al*., 2000). With insulin resistance, insulin action through the PI3K/Akt pathway is blunted (Kim *et al*., 2006), leading to decreased NO production. insulin resistance may actually enhance insulin's mitogenic actions, leading to increased production of adhesion molecules and ET-1 and thereby predisposing insulin-resistant patients to hypertension and atherosclerosis.

Obesity significantly contributes to the development of the proinflammatory milieu, where adipose tissue functions as an endocrine organ secreting a variety of proinflammatory factors including chemokines IL-8 and MCP-1 (Sell *et al*., 2006). Elevated plasma concentrations of these adipokines in obese patients may contribute to the insulin-resistant state (Sell *et al*., 2006). Adiponectin is the only adipokine known to be down-regulated in obesity. It is known to prevent the impairment of insulin signaling and thus plays a crucial and causal role in obesity-linked insulin resistance and MS (Yamauchi and Kadowaki, 2008). Adiponectin-regulated cytokines are IL-8 and MCP-1. Also, adiponectin prevents the impairment of insulin signaling (Sell *et al*., 2006).

Adiponectin and MCP-1 are secreted by adipocytes and seem to be implicated in the pathogenesis of insulin resistance associated with obesity (Kasuga, 2006). MCP-1 influences the function of adipocytes (Weisberg *et al*., 2006), is a recruitment factor for macrophages, and may be a crucial link among chemokines between adipose tissue inflammation and insulin resistance (Dahlman *et al*., 2005). It has been found that MCP-1 inhibits insulin-stimulated glucose uptake as well as the adipocyte expression of metabolically important genes (Sartipy and Loskutoff, 2003). Studies have shown that adiponectin levels are negatively correlated with MCP-1 (Schinner *et al*., 2008), and this agrees with the results of our study. There is evidence that already moderate elevation of body mass index (BMI), which is potentially associated with a slight increase in MCP-1, may contribute to insulin resistance in skeletal muscle and possibly underlies early steps in the development of MS (Sell *et al*., 2006). MCP-1 is a candidate of special interest because it is highly effective in inducing insulin resistance in skeletal muscle cells. Therefore, it has been suggested that MCP-1, which is regulated by adiponectin and which is clearly associated with the obese state and diabetes, may represent a molecular link between obesity and skeletal muscle insulin resistance (Sell *et al*., 2006).

Circulating IL-8 is increased in obese compared with lean subjects and is associated with measures of insulin resistance, development of atherosclerosis, and cardiovascular disease. An increased release of IL-8 from visceral adipose tissue compared with subcutaneous adipose tissue has been found (Bruun *et al*., 2004). This observation reveals that the

elevation in circulating levels of IL-8 in obese subjects is primarily due to the release of IL-8 from non-fat cells from adipose tissue. The high levels of IL-8 released from human adipose tissue and accumulation of this tissue in obese subjects may account for some of the increase in circulating IL-8 observed in obesity (Bruun *et al*., 2004). Hence inhibition of IL-8 by adiponectin may have important antiinflammatory, antiatherogenic, and cardiovascular protective effects (Moreau *et al*., 1999). Studies have also shown that the concentration of IL-8 in the circulation correlates with measures of adiposity e.g., BMI and fat mass, indicating a possible relationship between this adipose tissue-derived chemokine and the obese state (Straczkowski *et al*., 2002), where inflammatory cells and/or endothelial cells within the adipose tissue matrix are responsible for the correlation between IL-8 and BMI (Bruun *et al*., 2004).

Increased fat accumulation, especially in the visceral depot, has been demonstrated to be highly associated with a decrement in insulin sensitivity (Kissebah and Krakower, 1994) as well as an increment in development of cardiovascular disease (Despres *et al*., 2001). Increasing evidence suggests that adipokines may participate in the pathogenesis of obesity-related health complications (Ravussin and Smith, 2002), e.g., IL-8 released from the adipose tissue may have endocrine effects on other tissues (Bruun *et al*., 2004). It has been found that adiponectin down-regulates IL-8 (Kobashi *et al*., 2005) and our findings demonstrate that adiponectin levels are negatively correlated with IL-8.

Our findings indicate that obese MS patients with T2DM and CAD have more pronounced endothelial dysfunction (increased ET-1 level and decreased endothelial-dependent vasodilatation) and increased concentrations of inflammatory cytokines TNF-α and IL-6 (adipokines), and chemokines MCP-1 and IL-8 (adipokines) simultaneously with both higher insulin resistance and lower adiponectin concentrations than patients with MS alone or healthy subjects, which suggests that obese state with MS *per se* contributes to a more vicious proinflammatory milieu for developing T2DM and coronary atherosclerosis.

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ENDOTELIĀLĀS DISFUNKCIJAS UN ADIPOKĪNU PĀRMAIŅU SAISTĪBA AR INSULĪNA REZISTENCI METABOLĀ SINDROMA PACIENTIEM

Pētījumā iesaistītie metabolā sindroma (MS) pacienti, tika iedalīti sekojošās grupās: 26 pacienti ar MS (M), 44 ar 2-tipa cukura diabētu (T2DM) (D) un 20 ar T2DM un koronāro sirds slimību (CAD). Astoņpadsmit veseli cilvēki izveidoja kontroles grupu (C). Insulīna rezistence (IR) tika novērtēta ar HOMA-IR. Adiponektīna, tumoru nekrozes faktora-alfa (TNF-α), interleikīna-6 (IL-6), monocītu hemoatraktantā proteīna-1 (MCP-1) un interleikīna-8 (IL-8) koncentrāciju noteikšanai izmantojām xMAP tehnoloģiju, bet endotelīna-1 (ET-1) koncentrāciju noteicām ar ELISA. Lai novērtētu endotēlija-atkarīgo vazodilatāciju plaukstas ādā, izmantojām lāzerdoplerogrāfijas attēldiagnostiku kopā ar 1% acetilholīna transdermālu jontoforēzi (LDI-Ach). D un DC grupā IR bija būtiski lielāka, salīdzinot ar M un C grupu ($P < 0.01$). TNF-α, IL-6, IL-8, MCP-1 un ET-1 koncentrācijas DC grupā bija būtiski lielākas, salīdzinot ar visām pārējām pētījuma grupām ($P < 0.001$), bet IL-6, IL-8, MCP-1 un ET-1 koncentrācija D grupā būtiski atšķīrās no koncentrācijas C grupā ($P < 0.05$). Turklāt TNF-α, IL-6, IL-8, MCP-1 un ET-1 koncentrācijas statistiski ticami korelēja ar HOMA-IR rādītāju un adiponektīna koncentrāciju. Adiponektīna koncentrācijas neatšķīrās starp pacientu grupām, bet bija būtiski zemākas, salīdzinot ar kontroles grupu ($P < 0.001$). Vienīgi D un DC grupā bija būtisks un līdzīgs LDI-Ach rādītāja samazinājums ($P < 0.001$). Rādītāju LDI-Ach un HOMA-IR vērtības savstarpēji būtiski korelēja ($P < 0.001$). Mūsu pētījuma rezultāti liecina, ka adipoziem MS pacientiem būtiski pieaug IR un TNF-α, IL-6, IL-8 un MCP-1 koncentrācijas, bet samazinās adiponektīna koncentrācijas, un ir endoteliālā disfunkcija (palielinātas ET-1 koncentrācijas un samazināta LDI-Ach), savukārt, T2DM un CAD klātbūtne šiem pacientiem saistīta ar vairāk izteiktu endoteliālo disfunkciju un iekaisuma citokīnu un hemokīnu pieaugumu.