Adipocytokines and their relationship with symptomatic atherosclerotic peripheral arterial disease

Adipocitokinele şi arteriopatia aterosclerotică periferică simptomatică

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Abstract

Atherosclerosis – a focal arterial process rather than a disease in itself, is the main factor causing ischemic cardiopathy, cerebrovascular accidents or peripheral arterial disease (PAD) and represents the major cause of morbidity and mortality in developed countries. The aim of this study was to determine clinical characteristics extended by possible determination of new atherogenesis markers in patients with the PAD due to atherosclerosis. We measured resistin and leptin serum levels (as proatherogenic markers), and adiponectin (as an antiatherogenic marker) in a patients with PAD. The study is an analytic observational case-control study, performed on a group of 70 patients with atherosclerotic PAD.

The mean value of resistin (14.9±9 pg/ml) and leptin in male: 1429.5 (189-9714) pg/ml and female: 3045 (217-9785) pg/ml patients included in the PAD group was higher, as compared to the control group. No statistically significant difference was found between the two groups for resistin (p=0.766) and for leptin in females (p=0.131). Difference was signifficant in male group for leptin (p=0.020). The results of our study confirm the fact that hypoadiponectinemia can be associated with atherosclerotic PAD (p=0.003). We also tried to establish correlations between these adipocytokines and the risk factors involved in PAD. A percentage of 76.4% of the patients had at least one risk factor. Serum levels of adiponectin and leptin were significantly changed in the arteriopathy patients with coronary artery disease, with no statistically significant difference in adipocytokines levels in the obese arteriopathy patients.

Keywords: *adipocytokines, atherosclerosis, peripheral arterial disease, risk factors.*

Rezumat

Ateroscleroza, un proces arterial focal mai degrabă decât o boală în sine, principalul factor care determină apariţia cardiopatiei ischemice, a accidentelor vasculare cerebrale sau a arteriopatiei cronice obliterante (ACO), reprezintă principala cauză de morbiditate şi mortalitate în ţările dezvoltate. Scopul studiului nostru a

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constat în extinderea caracteristicilor clinice la pacienții cu ACO de natură aterosclerotică prin posibila determinare de noi markeri ai aterogenezei. Am determinat nivelurile serice ale rezistinei şi leptinei (ca şi markeri proaterogenici) şi ale adiponectinei (ca şi markeri antiaterogenici) pe pacienți cu ACO. Studiul a fost analitic observațional, de tip caz-control şi s-a efectuat pe un lot de 70 de pacienți cu ACO aterosclerotică.

Valorile medii ale rezistinei (14.9 ± 9 pg/ml) şi ale leptinei la pacienții de sex masculin: 1429.5 (189-9714) pg/ml şi feminin: 3045 (217-9785) pg/ml incluşi în lotul cu ACO au fost mai ridicate, comparativ cu lotul martor, dar fără semnificație statistică (p=0.766) pentru rezistină şi pentru leptină la femei (p=0.131). Rezultatele studiului nostru au confirmat faptul că hipoadiponectinemia poate fi asociată cu PAD ateroclerotică (p=0.003). De asemenea, am încercat să stabilim corelații între aceste adipocitokine şi factorii de risc asociați ACO. Un procent de 76.4% a pacienților au prezentat cel putin un factor de risc. Nivelurile serice ale adiponectinei şi leptinei au fost semnificvativ modificate la pacienții coronarieni cu ACO, nivelurile acestor adipocitokine nefiind modificate semnificativ la pacienții obezi cu arteriopatie.

Cuvinte cheie: *adipocitokine, ateroscleroză, arteriopatie cronica obliterantă, factori de risc.*

Introduction

Adipocytokines are bioactive mediators released from the cells of the adipose tissue. Currently, it has been recognized that white adipose tissue also acts as an endocrine organ. This tissue secretes pro and anti-inflammatory protein factors, known as adipocytokines. These adipocytokines include hormones involved in energy balance (e.g., leptin, adiponectin), glucose tolerance and insulin sensitivity (adiponectin, resistin), classical cytokines (e.g., tumoral necrosis factor α (TNF-α), IL-6 interleukin-6), and proteins involved in lipid metabolism (e.g., lipoprotein lipase, retinol binding protein), vascular haemostasis (e.g., plasminogen activator inhibitor-1 (PAI-1) and angiotensinogen) and in inflammation and stress responses (such as haptoglobin and metallothionein) (1-3). Obesity is associated with accelerated atherosclerosis and increased rates of cardiovascular death (4). The most abundantly expressed in the adipose tissue are leptin and adiponectin. The physiological role of leptin is very complex, it could be an important mediator of the relationship among obesity, overweight and atherosclerosis. Leptin might operate through effects on blood pressure, platelet aggregation, lipid metabolism and vascular function. Adiponectin is another potential mediator of atherosclerosis, it modulates the inflammatopry response of endothelial cells and influences the proliferation of vascular smooth cells (5, 6).

According to data in recent literature, leptin, adiponectin, and resistin are all involved in endothelial dysfunction. Resistin is expressed primarily in inflammatory cells, monocytes, recombinant resistin upregulating cytokines and adhesion molecule expression on human endothelial cells, suggesting a potential role in atherosclerosis (7, 8). However, the relationship between resistin and inflammation, insulin resistance, and atherosclerosis in humans remains largely unexplored.

Atherosclerosis implies many intertwined pathologic processes, including lipid imbalances, platelet aggregation, thrombosis, endothelial dysfunction, inflammation, oxidative stress, vascular smooth cell activation, alteration of matrix metabolism, arterial wall remodeling and genetic factors (9). Inflammation appears to be involved at all stages of atherosclerosis. It is involved in the formation of early fatty streaks, when the endothelium is activated and expresses chemokines. Cells involved in the atherosclerotic process include vascular (endothelial and smooth muscle) cells, monocytes/macrophages, lymphocytes (T, B, NKT), dendritic cells, and mast cells. They secrete or are stimulated by soluble factors including peptides, glycoproteins, proteases, and a set of cytokines (10). Given accumulating evidence that atherosclerosis could be an inflammatory condition, the relationship between adipocytokines and atherosclerosis may be important to elucidate.

Table 1. Demographical and clinical characteristics of the patient

	Quantifier	PAD patients (n=70)	Controls (n=70)	p
Male / female	ratio	60 / 10	56 / 14	0.370
Age (years)	mean±std. dev	63.36 ± 10.94	62.30 ± 10.70	0.564
ABI (mmHg/mmHg)	median (range)	0.70 (0.30-0.90)	0.80(0.50-7.0)	**0.000**
Diabetes mellitus	number(%)	13 (18.6%)	22 (32.4%)	0.079
Arterial hypertension	number(%)	41 (58.6%)	46 (65.7%)	0.384
Coronary artery disease	number(%)	41 (58.6%)	34 (50.7%)	0.358
Current smoking	number(%)	50 (71.42%)	43 (61.4%)	0.210
BMI (kg/m^2)	mean±std. dev	25.6 ± 4.13	25.42 ± 4.5	0.76
Obesity (BMI>30)	number(%)	25 (35.7%)	16 (23.2%)	0.105

Atherosclerosis is a pathological condition that underlies several important adverse vascular events including coronary artery disease (CAD), stroke, and peripheral arterial disease, which are responsible for most of the cardiovascular morbidity and mortality. Peripheral arterial disease (PAD) can be considered as less studied manifestation of systemic atherosclerosis, with potentially fatal or mutilating consequences, such as major amputations (11, 12). The goal of our study was to determine the level of adipocytokines, interpreting their variations depending on the manifestations of atherosclerosis in patients with PAD.

Material and method

Subjects and design

The study is an analytic observational case-control study, performed on a group of 70 patients with atherosclerotic lower limb peripheral arterial disease hospitalized at the Surgical Clinic No. II of Cluj-Napoca and a control group including an equal number of healthy patients.

From a clinical perspective, the study was undertaken on a series of 70 consecutive patients with clinically evident lower limb atherosclerosis and PAD, and a control group of 70 subjects, without symptomatic PAD. The criteria for selection and inclusion in the study were in accordance with international consenses and were based on clinical and biochemical data, and imaging. The patients' characteristics are summarized in *Table I*.

Diagnosis of intermittent claudication was established in accordance with World Health Organization criteria/Rose Questionnaire (13, 14). Ankle-brachial arterial pressure ratio was calculated for each lower limb. The lowest ankle-brachial index (ABI) for each lower limb was used in the analysis. PAD was considered present where the ABI was < 0.9 for at least one lower limb (15, 16).

Patients were considered as hypertensive in accordance with Joint British Societies' guidelines (17). Diabetes mellitus (DM) was defined according to the American Diabetes Association (17, 18).

Subjects were divided into current or former smokers and non-smokers. A patient was considered to be a smoker if he or she had been smoking more than 10 cigarettes per day for at least 10 years. The body mass index (BMI) in kg/m^2 was also calculated.

A clinical trial protocol was developed, in accordance with the ethical principles regarding research involving humans and approved by the Ethics Board of the institution where the research was conducted. The clinical trial protocol was applied identically to all patients. Participation in the trial was voluntary and confidentiality was absolute.

Table 2. Biochemical data for peripheral arterial disease patients and control group

	Quantifier	PAD patients (n=70)	Controls (n=70)	p
Total cholesterol (mg/dL)	mean±std. dev	197.4 ± 51.8	186.8± 46.4	0.331
HDL-cholesterol (mg/dL)	mean±std. dev	46.2 ± 17.16	51.8 ± 18.2	0.063
Triglycerides (mg/dL)	mean±std. dev	129.9 ± 49.6	131.0 ± 91.8	0.953
Fasting glucose (mg/dL)	mean±std. dev	110. 3± 35.4	127.4 ± 60.5	**0.043**
Resistin (pg/ml)	mean±std. dev	14.9 ± 9.0	13.0 ± 6.0	0.766
Adiponectin (pg/ml)	median (range)	1011.0 (19-4046)	1376.0 (76-5999)	**0.003**
Leptin (pg/ml) - Female	median (range)	3045.0(217-9785)	389(213-8609)	0.131
Leptin (pg/ml) - Male	median (range)	1429.5(189-9714)	806(184-7794)	**0.020**

Laboratory methods

All patients had tests performed in order to evaluate disease severity and possible dysfunctions of other organs. The following were assessed: complete blood count, lipid profile, fasting blood glucose (and oral glucose tolerance test with blood glucose measurement after 2 hours in patients without previous carbohydrate metabolism disorders), urea, creatinine, hepatic transferases. The total serum level of cholesterol was determined by an automated enzymatic method from fasting blood samples. Total cholesterol > 200 mg/dL was used as a cutoff value for the lipid profile according to Adult Treatment Panel III guidelines (19).

Apart from standard biochemical parameters, we examined new markers: resistin, leptin and adiponectin. Blood was drawn after at least 14 h of fasting and the samples were transported in cooled containers to laboratory. Serum aliquots for measurement of resistin, leptin and adiponectin were frozen and stored at -800C. Serum levels of resistin, adiponectin and leptin were measured by using the ELISA - sandwich test, using Quantikine® reagents (R&D Systems, USA): Human Resistin, Human Leptin, and Human Adiponectin.

Statistical analysis

After testing all continuous variables for normality of data using Kolmogorov-Smirnov Test, data analyses were performed using Student T test or Mann-Whitney U Test, according to distribution. Normally distributed variables were expressed as mean± standard deviation, while non-normally distributed variables were expressed as median (minimum-maximum). Qualitative data was tested using Chi square/ Fisher Exact Test, based on standard application criteria. Results were considered significant for $p<0.05$. Statistical package SPSS 17.0 was used for all data analyses.

Results

Evaluation of risk factors: smoking, diabetes mellitus (DM), cardiovascular-associated diseases, coronary artery disease, arterial hypertension (AHT), hypercholesterolemia and obesity, by using anamnestic methods and paraclinical examinations which were then statistically processed, was the primary goal of our study. A percentage of 77.3% of the patients had at least one risk factor, and particularly: 32.85% DM; 58.57% AHT; 57.14% CAD, with a very high percentage (71.42%) of smokers (*Table 1*).

Biochemical characteristics, including variations of adipocytokines in the patients of the studied groups are presented in *Table 2*.

The mean plasma value of resistin in patients included in the PAD group was higher, as compared to the control group, but with no statistically significant difference between two groups

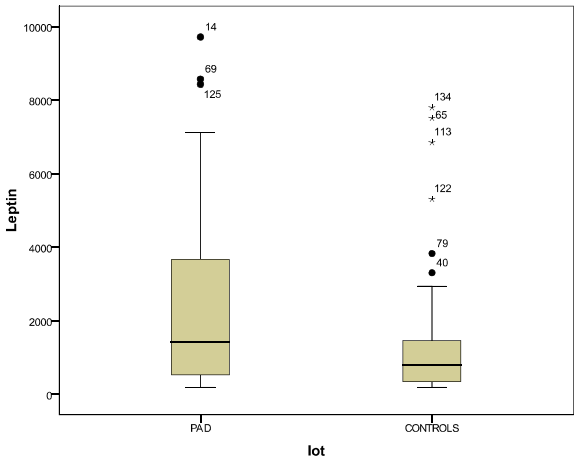

Figure1. Serum leptin concentration (pg/ml) in male patients with peripheral arterial disease comparatively with control group

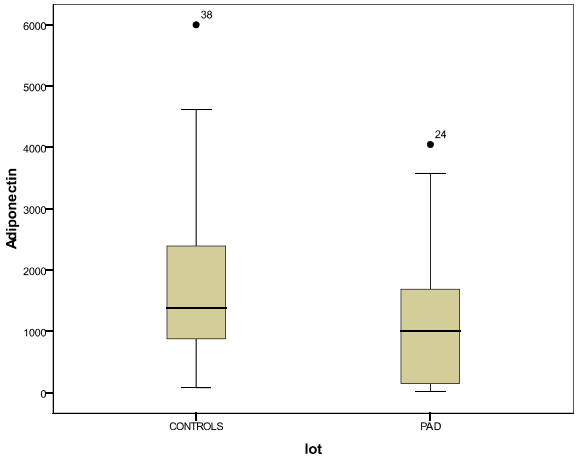

Figure 2. Serum adiponectin concentration (pg/ml) in patients with peripheral arterial disease comparatively with control group

(p=0.766). The mean concentration of resistin in patients with PAD and DM was 12.2±5.3 pg/ml, similarly with the value in control group with DM (12.3±5.4 pg/ml). Note that in the group of patients with PAD and DM, 7.96% of patients had higher resistin levels than 26.4 pg/ml (maximum limit), all of these patients being smokers, with associated cardiovascular diseases.

Serum values of leptin were assessed according to gender, as normal values are 2205-

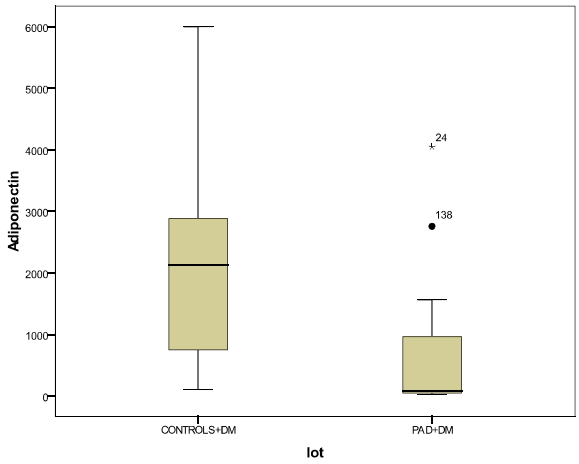

Figure 3. Serum adiponectin concentration (pg/ml) in patients with diabetes mellitus associated to peripheral arterial disease and control group

11149 pg/ml with an average of 4760 in men, and 3877 – 77273 pg/ml with an average of 20676 in women. In female patients included in the PAD group the median value of serum leptin was 3045 (217-9785) pg/ml, while in the control group the median value was 389 (213-8609) pg/ml (p=0.131). A maximum value of 24846 pg/ml was recorded in a smoker patient with high blood pressure and PAD stage IV, in critical ischemia, with coronary and cerebral atherosclerosis required major amputation. In male patients the median value of leptin was higher compared to the control group, with statistically significant difference (p=0.020) (*Figure 1*).

The median value of plasma adiponectin in patients included in the PAD group was of 1011 (19-4046) pg/ml, as compared to 1376 (76-5999) pg/ml in the control group (p=0.003) (*Figure 2*). The value of adiponectin in patients with PAD and associated DM was of 86 (24-4046) pg/ml (*Figure 3*). In obese patients of the PAD group, the median value of adiponectin was of 857 (19-4046) pg/ml, as compared to the median value of adiponectin in normal-weight patients of the same group – 1062 (24-2880) pg/ml (p=0.485).

Serum levels of each biomarker were analyzed in the subgroups of hypertensive, coronary artery disease or overweight.

There were statistically significant differences between serum levels of adiponectin in hypertensive PAD patients (p=0.005), the average being significantly lower when compared to the hypertensive controls. There was no difference between serum levels of resistin in PAD hypertensive and the hypertensive controls (p=0.619). Leptin plasma levels recorded significantly different values for the two groups (p=0.015). No statistically significant differences were observed in serum adiponectin, leptin and resistin levels between obese PAD patients and corresponding controls (p = 0.162, p=0.419 and 0.101, respectively).

Within patients with coronary artery disease, there were no statistically significant differences in serum levels of resistin (p=0.844), between PAD and controls. The adiponectin plasma levels were significantly lower (p=0.049) and leptin levels were significantly higher (p=0.047) in PAD patients with CAD, comparatively with controls with CAD.

Discussions

The adipocyte is an active endocrine secretory cell that releases free fatty acids and produces several cytokines including TNF-α, interleukins (IL), PAI-1, resistin, leptin, adiponectin, visfatin, omentin, chemerin (20). It has become increasingly evident that white adipose tissue–derived cytokines mediate between obesity-related exogenous factors and the molecular events that lead to metabolic syndrome, inflammation, and cardiovascular diseases (21, 22). Atherosclerosis, the main cause of death in industrialized countries, is currently at the origin of most cardiovascular diseases affecting the adult population (23). Starting from the assumption that among people aged over 55 atherosclerotic PAD is a known indicator of systemic atherosclerotic disease (24) and literature in this area is scarce (25 - 27), we have decided to undertake the present study.

Understanding the mechanisms underlying the atherosclerotic process, which is a form of inflammatory response to factors damaging the vessel wall from a pathologist's point of view, allowed the identification of many inflammatory response markers that are crucial in atherogenesis. A new, recently revealed inflammatory marker, closely connected with atherosclerosis, is resistin produced by adipocytes. This molecule takes part in atherogenesis through complex pathophysiological pathways of the inflammatory response which finally lead to activation of endothelial cells and smooth muscle proliferation (28, 29). Additionally, resistin mRNA was found to be important in the inflammatory response of cells constituting atherosclerotic plaque (30).

In our study, the mean value of plasma resistin in PAD patients, with or without associated DM, was higher than the normal mean value, a fact suggesting its possible participation in atherogenesis, along with its well-known effects of inducing insulin resistance. Moreover, we have found elevations of serum levels of resistin in patients with PAD and associated CAD or obesity, but statistically insignificant. There are no published studies that focus only on resistin level in PAD patients. Golledge et al. (31) have prospectively assessed serum levels for lipids, C-reactive protein, adiponectin, leptin, resistin and osteoprotegerin (OPG) in the patients presenting with life style-limiting intermittent claudication in order to establish the presence of cardiovascular risk factors, obesity and metabolic syndrome. Their findings suggest that metabolic syndrome is an important determinant of endothelial function in patients with PAD, and OPG may be a useful biomarker of this effect (31).

The reports published so far on the relationship between resistin levels and progression, severity and prognosis of patients with CAD in most cases confirmed, the usefulness of resistin in the routine diagnostic process, as its level indicates the severity of the inflammatory response connected with atherogenesis (32 - 34).

On the other hand, Pilz et al. (35) did not show any correlation between resistin level and severity of atherosclerosis in a group of

1100 patients, though high level of this adipocytokine was a strong and independent predictor of non-fatal cardiovascular events in this group (32, 35). Our results are similar. Serum resistin concentrations were not significantly higher in patients with PAD as compared to controls. The lack of relationship between our studied parameters and atherosclerosis severity in the arteries of the lower limbs can be explained by very advanced, quite homogeneous lesions hindering blood flow in these arteries.

Interest in the biology of white adipose tissue has increased dramatically since the discovery of leptin in 1994 (21). It has been proposed that leptin could play a role in the pathogenesis of atheromatous plaques acting synergistically with other inflammatory mediators (21, 36). The proposed proatherogenic actions of leptin are supported by the demonstration that, *in vitro*, leptin stimulates the proliferation and hypertrophy of vascular smooth muscle cells and the production of matrix metalloproteinase 2 by these cells, promotes vascular production of proliferative and profibrotic cytokines, increases the secretion of the proatherogenic lipoprotein lipase by cultured human and murine macrophages, and enhances platelet aggregation (21, 37). Moreover, leptin has been shown to induce mitochondrial superoxide production and monocyte chemoattactant protein-1 (MCP-1) expression in aortic endothelial cells, thus playing an important role in the early phase of atherosclerosis by initiating monocyte/ macrophage recruitment to the vessel wall (21, 38). Some *in vivo* animal studies also suggest an important role for leptin in the pathogenesis of atherosclerosis and it has been shown that leptin administration decreases the activity of the antiatherogenic enzyme paraoxonase in plasma, aorta, renal cortex, and medulla of rats (21, 37).

In our study serum values of leptin, are increased in PAD patients, especially in male patients. These data suggest the possible role of leptin in the developement of peripheral atherosclerosis in the light of the results yielded in patients of the PAD group, as the values were significantly higher than normal mean values. These results do not support the presence of a clinically relevant relationship between leptine and PAD in the general population, with the exception of male patients.

Leptin can influence the production of other adipokines involved in inflammation and metabolic regulation. Zamboni et al have recently suggested that leptin could be an independent predictor of adiponectin gene expression (21, 30).

Adiponectin has an antiatherogenic effect on endothelial cells, inhibits the proliferation of vascular smooth muscle cells, and suppresses the conversion of macrophages to foam cells. There is a general consensus about a putative protective role of adiponectin from an inflammatory state, at least at an endothelial-vascular level, although not uniquely (21, 40). Low levels of adiponectin have been linked to inflammatory atherosclerosis in humans, suggesting that normal adiponectin levels are required to maintain a noninflammatory phenotype on the vascular wall (40). In addition, many works with animal models and a great amount of *in vitro* laboratory data published in recent years have shown that adiponectin plays a pivotal role in atherosclerosis physiopathology (21, 41). Adiponectin knockout mice develop vascular disorders of inflammatory type in response to mechanical injury, with neointimal thickening and increased proliferation of vascular smooth muscle cells that can be attenuated by restoration of physiologic levels of adiponectin (41).

Serum levels of adiponectin in our PAD patients are lower than the levels in the control subjects, which suggests the role of hypoadiponectinemia in the development of PAD. Significant differences were etablished between PAD patients and DM, CAD or AHT associated, highlighting the previous statement. These findings are confirmed by the results of Dieplinger et al (42, 43). Low plasma adiponectin levels are significantly correlated with endothelial dysfunction (20, 44). These results

suggest that low adiponectin levels may be a useful marker for early-stage atherosclerosis. Hypoadiponectinemia correlates significantly and independently with CAD (20, 45). In addition, low adiponectin plasma levels are associated with progression of coronary artery calcification in type 1 diabetic and nondiabetic subjects independently of other cardiovascular risk factors (20, 46). Plasma adiponectin levels are an inverse predictor of cardiovascular outcome in patients with end-stage renal disease (20, 47).

Pathogenesis of atherosclerosis involves a complex series of events that result in final atherosclerotic plaque formation. Knowledge of cellular components of the arterial wall, the role of growth factors in the cell cycle under normal and pathological conditions, the role of risk factors in arterial wall lesions are of great importance in understanding atherosclerotic vascular disease pathogeny, and are subject to further research (48). The measurement of serum proatherogenic (resistin, leptin) and antiatherogenic (adiponectin) adipocytokines might be useful for the assesement of the endothelial dysfunction (9).

The results of our study have confirmed that hypoadiponectinemia can be associated with atherosclerotic PAD and increased levels of leptin and adiponectin in the CAD patients with PAD. A possible limitation of the results in this study could be the failure to establish the causal relationship that leads to these oscillations of adipocytokines in atherosclerotic PAD. Continuation of this study on larger population cohorts is our goal for the future, allowing resolution of the potential causal relationship between adipocytokinemia and atherosclerotic PAD.

Conclusions

At present, it is evident that adipokines play multiple relevant roles in the body, and the increasing research effort in this area reveals the complex adipokine-mediated interaction among white adipose tissue, metabolic disorders, and cardiovascular diseases. Proinflammatory cytokines produced by white adipose tissue: resistin and leptin are likely to be promising targets for controlling the reduction of inflammation in order to prevent the progression of early or already established atherosclerotic lesions. It is generally too early to suggest wellsupported therapeutic hypotheses. Our results suggest the role of hypoadiponectinemia in the development of PAD, confirming the antiatherosclerotic effect of adiponectin. Controlling the hypoadiponectinemia is an interesting target for future research in reducing the morbidity and mortality of atherosclerotic PAD. The necessity of a different approach to patients with PAD as well as the requirement for distinction between PAD variants made the previous diagnostic and therapeutic management insufficient in many cases. The key element of the management strategy seems to be medical history, interpretation of selected, routine imaging studies and novel biochemical markers such as adiponectin, resistin, leptin.

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Abbreviations

ABI – ankle-brachial index,
AHT – arterial hypertension,
BMI – body mass index,
CAD – coronary artery disease,
DM – diabetes mellitus,
ELISA - Enzyme-Linked Immunosorbent Assay,
HDL – high-density lipoprotein,
IL-6 - Interleukin-6,
MCP-1 - Monocyte Chemoattactant Protein-1,
mRNA - messenger ribonucleic acid,
OPG – osteoprotegerin,
PAD – peripheral arterial disease,
PAI-1 - Plasminogen Activator Inhibitor-1,
TNF-α - Tumor Necrosis Factor-A.

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