Expression of adipocytokine genes and their content in various adipose tissue sites in patients with heart diseases

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Abstract

Background Adipose tissue (AT) is an endocrine and paracrine organ that synthesizes biologically active adipocytokines, which affect inflammation, fibrosis, and atherogenesis. Epicardial and perivascular fat depots are of great interest owing to potential effects on the myocardium and blood vessels.

Objective To assess expression and secretion of adipocytokine genes in adipose tissue in patients coronary artery disease (CAD) and patients with aortic or mitral valve replacement.

Methods The study included 84 patients with CAD and 50 patients with aortic or mitral valve replacement. Adipocytes were isolated from subcutaneous (SAT), epicardial (EAT), and perivascular AT (PVAT) samples. Isolated adipocytes were cultured for 24 h after which, gene expression and secretion levels of selected adipokines and cytokines in the culture medium were determined.

Results The study parameters differed depending on the adipose tissue location. EAT adipocytes in CAD patients were characterized by a pronounced imbalance in the adipokine system. EAT had the lowest adiponectin gene expression and secretion, regardless of nosology and high expression levels of the leptin gene, its receptor, and interleukin-6 (IL-6) were detected. High leptin and IL-6 levels resulted in increased pro-inflammatory activity, as observed in both EAT and PVAT adipocytes, especially in individuals with coronary artery disease.

Conclusion The "protective" potential of adipose tissue depends on its location.

Background

Atherosclerosis and cardiovascular disease (CVD) remain one of the main causes of death in industrialized countries, despite significant achievements in modern medicine [1]. Moreover, the increasing incidence of obesity is likely to aggravate this problem. Adipose tissue (AT) is universally regarded as an endocrine and paracrine organ that synthesizes biologically active molecules. Adipocytokines are involved in various processes, including inflammation, fibrosis, and atherogenesis. It has been shown that local fat depots, such as epicardial (EAT) and perivascular AT (PVAT), are associated with increases in cardiovascular events, independent of traditional risk factors [2]. In addition, reports have shown differences in the matrix RNA (mRNA) expression of adipokines in epicardial and subcutaneous AT (SAT) [3]. Since different fat depots are characterized by their unique adipocytokine and secretome, studies on the role of AT adipocytokines at different locations and CVD are relevant.

EAT is of particular interest owing to its close location to the coronary arteries and its possible paracrine action on them [4]. When inflammatory processes are activated, the epicardium becomes a site of impaired adipogenesis, which leads to secretion of pro-inflammatory adipokines and fibrosis of the atrial and ventricular myocardium. PVAT is also of interest, since its heterogeneity determines the functions of ATs – protective or atherogenic. EAT and PVAT have been shown to be associated with coronary artery disease (CAD) [5]. However, the mechanisms by which adipocytokines affect the pathogenesis of CVD are

still not fully understood. Thus, it is necessary to further study the expression of adipokine genes in AT at various locations to develop potential therapeutic interventions against pathological activation of AT in CVD.

Materials And Methods

The study was performed at the Federal State Budgetary Institution's Research Institute for Complex Issues of Cardiovascular Diseases.

Study participants

Inclusion criteria consisted of the following: CAD presence of indications for coronary artery bypass grafting (CABG) (based on coronarography data); patient age to 75 years; and patient consent for the research. Exclusion criteria were as follows: patient age greater than 75 years; presence of type 1 and type 2 diabetes mellitus (DM) in the history and/or revealed during the hospitalization examination; presence of MI; presence of clinically significant comorbidities (anemia, kidney and liver failure, oncological and infectious-inflammatory diseases in the period of exacerbation, autoimmune diseases; and refusal of the patient to conduct the study.

Study population

The study included 84 patients with CAD and indications for direct myocardial revascularization by CABG (Group 1), including 21 women (25%) and 63 men (75%), with an average age of 64.0 (57.0; 68.0) years (Table 1).

Men predominated among the surveyed individuals. In the patient history, hypertension, smoking, angina pectoris, and burdened heredity for cardiovascular pathology were more often recorded. In total, 57 patients had a previous history of myocardial infarction (MI), and 6 people had a history of stroke.

The comparison group included 50 patients with aortic or mitral valve replacement (Group 2), with an average age of 60.4 (56.45; 65.35) years (Table 3) without coronary atherosclerosis (according to coronary angiography). Individuals in Group 2 were characterized by a low percentage of patients with cardiovascular risk factors.

Cells collection

Biopsies of 3–5 g of SAT, EAT, and PVAT were obtained during aortocoronary bypass surgery and aortic or mitral valve replacement. SAT samples were obtained from the subcutaneous tissue of the lower angle of the mediastinal wound. EAT samples were obtained from the right heart (right atrium and right ventricle), and PVAT were obtained from the mammary-coronary vascular sinus, located in the anterior mediastinum (ascending part of the aorta).

Adipocytes were isolated from ATs under sterile conditions in a laminar flow hood (BOV-001-AMS MZMO, Millerovo, Russia) using the method described earlier [6]. Adipocytes were counted in a Goryaev's chamber. Cell viability was evaluated according to the method described by Suga et al [7]. Adipocytes (20×10^5) were seeded into a 24-well plate (Greiner Bio One International GmbH, Kremsmünster, Austria), and the volume in each well was adjusted to 1 mL with culture medium. Incubated for 24 hours at a temperature of $(37 \pm 1)^\circ\text{C}$ in an atmosphere of 5% CO₂ and 10% oxygen. Following, the culture medium was carefully taken from the bottom of the wells for subsequent determination of adipokines and cytokines by enzyme immunoassay.

Laboratory assays

RNA extraction

Total RNA purification from isolated adipocytes was performed using the commercial RNeasy® Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with some modifications described previously [8]. Extracted RNA was stored at -70°C .

The quantity and quality of purified RNA were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) by measuring the light absorbance at 280 nm, 260 nm, and 230 nm and calculating the 260/280 (A₂₆₀/280) and 260/230 (A₂₆₀/230) ratios. The integrity of the RNA was determined by electrophoresis in agarose gel, followed by visualisation using the Gel Doc™ XR+ System (Bio-Rad, Hercules, CA, USA).

cDNA synthesis

Single-stranded cDNA was synthesised using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems). Reverse transcription was performed using the program suggested by the kit's manufacturer. The quantity and quality of synthesised cDNA were assessed using a NanoDrop 2000 Spectrophotometer. Samples were stored at -20°C .

Real time quantitative polymerase chain reaction (qRT-PCR)

Expression of the adiponectin, leptin, leptin receptor (leptin R) and interleukin-6 (IL-6) genes was evaluated by quantitative real-time polymerase chain reaction (qPCR) using TaqMan™ Gene Expression Assays (ADIPOQ Hs00605917_m1, LEP Hs00174877_m1, LEPR Hs00174497_m1, IL6 Hs00174131_m1, Applied Biosystems, USA) on a ViiA 7 Real-Time PCR System (Applied Biosystems, USA). As a negative control, we used 20 µL of reaction mix with no cDNA template. For each sample and negative control, three technical replicates were prepared. Results were normalized for three reference genes, HPRT1 (hypoxanthine phosphoribosyltransferase-1), GAPDH (glyceraldehyde-3-phosphatedehydrogenase), and B2M (beta-2-microglobulin). To assess the effectiveness of PCR, amplification graphs and standard curves were analyzed using QuantStudio™ Real-Time PCR Software v.1.3 (Applied Biosystems). The

expression of the studied genes (normalised quantification ratio, NRQ) was calculated by the Pfaffl method and is represented on a logarithmic (log10) scale as the fold change compared to that in control samples.⁹

ELISA

The concentration of adiponectin, leptin and leptin R (Human Total Adiponectin/Acro30, DRP300; Human Leptin, DLP00; Human Leptin R, D0BR00) in the culture medium of adipocytes was determined by the enzyme immunoassay using test systems from R&D Systems, Inc. (Minneapolis, MN, USA) and IL-6 using the test system Interleukin-6 BMS213-2 from eBioscience (Vienna, Austria). The free leptin index (FLI) was defined as the ratio of the level leptin to leptin R.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and Statistica software, 6.1 (Dell Software, Inc., Round Rock, TX, USA). The Kolmogorov–Smirnov test was used to verify the normal distribution of data, for non-normally distributed variables, data were presented as median and 25th and 75th quartiles (Me, Q1; Q3). Differences between the three groups were compared using one-way analysis of variance (ANOVA) for continuous variables. Categorical variables are expressed as percentages and compared using chi-squared test or Fisher's exact test. P value of less than 0.05 was considered statistically significant.

Results

Adiponectin gene expression and concentration of adiponectin in the daily adipocyte culture of various fat depots

Adipocytes in the EAT expressed the lowest amount of the adiponectin gene relative to adipocytes in different locations in both groups (Fig. 1).

In Group 1 patients, adiponectin gene expression in the EAT was significantly lower than in the culture of SAT and PVAT (1.2 times ($p=0.038$) and 1.5 times ($p=0.027$), respectively). In Group 2, adiponectin gene expression in EAT adipocytes was also lower compared to adipocytes in the SAT and PVAT (in 1.4 ($p=0.001$) and 1.5 times ($p=0.002$), respectively). Moreover, in Group 2, the mRNA level of adiponectin in EAT adipocytes was higher than in Group 1 by 1.2 times ($p=0.031$). High levels of adiponectin mRNA were observed in the culture of PVAT. Further, adiponectin gene expression in cultures of SAT and PVAT adipocyte was not statistically different between Group 1 and Group 2 patients (Fig. 1).

The concentration of adiponectin in daily culture of EAT adipocytes was lower than in SAT in both groups (1.3 times ($p=0.004$) and 1.13 times ($p=0.012$), respectively) (Table 2).

Moreover, adiponectin levels in Group 2 were 1.4 times higher than in Group 1 individuals ($p=0.011$). In PVAT adipocytes of Group 1 patients revealed the lowest level of adiponectin secretion compared to

adipocyte cultures at different locations. By contrast, in Group 2, adiponectin levels in the PVAT exceeded that of fat depots at a different location and Group 1 patients 1.8 times ($p=0.001$). Adiponectin concentrations in cultures of SAT adipocyte was not statistically different between Group 1 and Group 2 patients (Table 2).

Leptin gene expression and concentration of leptin in the daily adipocyte culture of various fat depots

High levels of leptin gene expression in patients of both groups were observed in a culture of EAT adipocytes compared with adipocytes at a different location (Fig. 2). In Group 1, leptin gene expression was 1.9 times higher than in Group 2 ($p=0.004$). Thus, in Group 1, leptin mRNA levels in the EAT exceeded that of the SAT and PVAT by 2.1 times ($p=0.003$) and 1.5 times ($p=0.002$), respectively, and by 1.4 ($p=0.019$) and 1.6 times ($p=0.012$), respectively, in Group 2.

Moreover, in Group 2, the lowest level of leptin mRNA was detected in the PVAT culture, which was 2 times ($p=0.008$) lower than in Group 1. However, leptin gene expression in SAT adipocyte cultures did not show statistically significant difference, independent of nosology (Fig. 2).

Leptin concentration was also the highest in the culture of EAT adipocytes in both groups, while in Group 1, it exceeded that of Group 2 by 1.2 times ($p=0.038$) (Table 2). However, statistically significant differences compared with SAT were only found in individuals in Group 2 (1.2 times, $p=0.041$). The lowest leptin concentration was observed in PVAT adipocytes compared to adipocyte cultures of other locations, and statistically significant differences were found relative to the EAT. Leptin levels in the PVAT were 1.1 times ($p=0.044$) lower than that of the EAT in Group 1 and 1.5 times ($p=0.003$) lower in Group 2. Further, leptin concentration in the PVAT adipocyte supernatant of Group 1 was 1.5 times higher than Group 2 ($p=0.002$). There were no statistically significant differences in leptin concentration in the culture of SAT adipocytes between groups (Table 2).

Leptin receptor gene expression and concentration of in the daily adipocyte culture of various fat depots

The expression of the Leptin R gene in a culture of EAT adipocytes in patients of both groups exceeded that of the SAT by 2 times ($p=0.001$ in Group 1 and $p=0.003$ in Group 2), but was lower than in the PVAT by 1.6 in Group 1 ($p=0.028$) and 1.7 times in Group 2 ($p=0.034$) (Fig. 3). The highest mRNA levels of the Leptin R were found in the PVAT, regardless of nosology. In Group 1, Leptin R gene expression was 1.6 times ($p=0.022$) lower than that of Group 2. In the presence of CAD, this parameter was higher in both SAT by 3.2 ($p=0.0003$) and in Group 2 by 3.3 ($p=0.024$). Thus, SAT adipocytes were characterized by the lowest level of the Leptin R gene expression when compared to other fat depots in all examined patients.

The concentration of Leptin R in the culture of EAT adipocytes in Group 1 exceeded SAT by 1.3 times ($p=0.017$), but was lower than the PVAT by 1.2 times ($p=0.025$) (Table 2). In Group 2, the highest concentration of Leptin R was observed in the EAT and PVAT as compared to the SAT (1.2 times, $p=0.013$, $p=0.024$ respectively). PVAT adipocytes of Group 1 were characterized by the highest secretion level of

the Leptin R compared with SAT and EAT (1.5, $p=0.011$ and 1.2, $p=0.025$ times, respectively), while there were no statistically significant differences between groups.

Moreover, regardless of nosology, the culture of PVAT adipocytes had the lowest index of free leptin, reflecting peripheral leptin resistance. In the culture of SAT adipocytes, the free leptin index was maximal in comparison to EAT and PVAT adipocytes (Table 2).

IL-6 gene expression and concentration of IL-6 in the daily adipocyte culture of various fat depots

It is known that adipocytes produce up to 30% of circulating IL-6 and are the second most important source of this cytokine [10]. It has recently been demonstrated that plasma levels of IL-6 correlate with the area of epicardial and abdominal visceral AT in patients with coronary artery atherosclerosis, suggesting a potential effect of these fat depots on the development of atherosclerosis through paracrine rather than systemic effects [11]. Thus, both volume and localization of fat depots may affect IL-6 production by adipocytes [12].

In measuring pro-inflammatory IL-6 gene expression in a 24-h culture of adipocytes at various locations, maximum levels were observed in the EAT relative to the SAT and PVAT in Group 1 (2.1 times, $p=0.0012$ and 1.4 times, $p=0.0024$ respectively) and Group 2 relative to the PVAT (1.9 times, $p=0.0002$). Moreover, IL-6 mRNA levels in the EAT of Group 1 exceeded that of Group 2 by 1.4 times ($p=0.0013$) (Table 3). Further, expression of the IL-6 gene in PVAT adipocytes in Group 1 was 1.9 times ($p=0.0001$) higher than Group 2. Subcutaneous adipocytes in both groups did not show differences in IL-6 gene expression.

The concentration of IL-6 in the EAT adipocyte culture also exceeded the parameters of other fat depots, regardless of nosology (Table 3). Thus, in patients with CAD, the concentration of IL-6 in the EAT was 2.5 times and 1.8 times higher than in the SAT and PVAT, respectively. In Group 2, it was 1.5 times and 1.8 times higher, respectively. Moreover, the concentration of IL-6 in the EAT and PVAT in Group 1 exceeded the level of Group 2 by 1.4 times ($p=0.022$ and $p=0.018$, respectively). IL-6 secretion in SAT adipocytes did not differ between groups.

Thus, EAT adipocytes in the examined patients showed the highest level of IL-6, while patients with CAD exceeded that of patients with aortic or mitral valve replacement. The IL-6 gene expression in PVAT adipocytes in Group 1 was higher than Group 2, while there was no difference in the subcutaneous adipocytes of patients.

Discussion

Among reasons underlying the development and progression of CVD, increasing attention has been paid to the endocrine and paracrine functions of local fat depots. It is believed that the metabolic and hormonal activity of visceral fat is significantly higher than that of subcutaneous fat. Moreover, adipocytes secrete adipokines and proinflammatory cytokines, the level of which correlates with obesity [13]. Further, fat deposits around the heart and blood vessels can play an important role in the

pathogenesis of CVD owing to their anatomical proximity relative to vascular structures and the myocardium.

The results of our study indicate that mRNA levels and the content of adipocytokines showed differences, depending on the location of the fat depots. Adiponectin gene expression levels in the culture of EAT adipocytes was the lowest as compared to adipocytes in other location in patients of both groups. In Group 2, the level of adiponectin mRNA in EAT adipocytes was higher than in CAD patients. According to the literature, there is no general consensus regarding the differences in this indicator in CAD and with aortic or mitral valve replacement. Bambacea et al studied adiponectin gene expression in AT in patients with CABG and patients with valve replacement and found significantly lower levels in the EAT compared to the SAT, similar to our results. In addition, higher expression of the adiponectin gene was observed in patients without CAD [3]. Similar data were demonstrated by Iacobellis et al [14] In the Eiras et al study, a reduced level of adiponectin mRNA was shown in the EAT relative to the SAT and in patients without CAD [15]. However, other authors have shown no differences between patients with CAD and with aortic or mitral valve replacement. For example, Iglesias et al. showed that adiponectin gene expression in the EAT was lower than in the SAT, but there were no statistically significant differences between patients after CABG and with valve replacement [16]. The authors explain this by gender characteristics, since adiponectin mRNA levels in the EAT were higher in women.

PVAT is also actively involved in atherogenesis, and it synthesizes proinflammatory molecules that are involved in the formation of unstable plaques that are prone to rupture and atherothrombosis and the progression of CAD [5, 17]. Due to this close interaction, the PVAT is the first fat depot that recognizes and reacts to emerging changes in homeostasis through the synthesis and production of adipocytokines [18, 19]. Adiponectin plays a crucial role, since its protective and anti-inflammatory properties are known. Our results demonstrate that PVAT had the highest level of adiponectin gene expression. Moreover, adiponectin was higher in Group 2 patients. Similar data were demonstrated by Cybularz In this study, the mRNA level also depended on body mass index (BMI). Specifically, in patients with a BMI above 30 kg/m², it was higher than those with a BMI between 25 and 30 kg/m² [20]. Reduced adiponectin gene expression in combination with high expression of the leptin gene may be associated with activation of key atherogenic pathways, contributing to the progression of CAD [21].

Based on the literature, it can be assumed that the levels of adipocytokine mRNA in the AT are not always a complete reflection of their concentration [22]. In this study, PVAT adipocytes of patients with CAD were characterized by the highest level of adiponectin gene expression, yet the lowest concentration in the supernatant compared to adipocyte cultures of other locations. One of these reasons for this difference may be the low expression of adiponectin receptors in these fat depots. In a study by Guo et al assessing the role of adiponectin and its receptors *in vivo*, they found that angiotensin II (Ang II)-induced hypertension led to a significant decrease in the expression of both adiponectin and its AdipoR1 and AdipoR2 receptors in perivascular adipocytes and vascular cells [23]. Nasci et al investigated the effect of infliximab on adiponectin gene expression in PVAT and showed a decrease in the level of adiponectin mRNA, AdipoR1, and AdipoR2 in mice with type 1 DM. Taken together, the findings suggest that the PVAT

is a site of adiponectin/AdipoR dysregulation, and secondly, it is most susceptible to proinflammatory signals [24].

Another reason may be long-term processes of posttranslational modification and oligomerization of adiponectin. The observed high level of adiponectin in PVAT is due to the longer processes of "maturation" of adiponectin in CAD, which occur in the endoplasmic reticulum (ER) of adipocytes and are controlled by special proteins-chaperones ER and Ero1-L α . It is assumed that part of the synthesized adiponectin undergoes decay or proteolysis, and the delayed release of adiponectin is due to disruption of proteins-chaperones that regulate adiponectin secretion from the cell [25]. In addition, Bauchea et al in transgenic mice showed that adiponectin was to be able to suppress its own production and expression of its AdipoR2 receptor [26]. Further, in Kadowaki et al, we demonstrated a decrease in the number of adiponectin receptors in metabolic syndrome and obesity [27].

Leptin, the most studied adipokine marker of obesity, participates in the regulation of atherogenesis, thrombogenesis, and vascular revascularization. Further, it stimulates inflammatory processes, oxidative stress, and vascular smooth muscle cell hypertrophy, playing a role in the pathogenesis of hypertension, CAD, and type 2 DM and the development of their complications [28]. More recently, the ability of cardiomyocytes to express leptin and its receptors has been demonstrated, which is of clinical interest. Further, the high gene expression of leptin in the adipocyte culture of both groups is consistent with the data of other groups [29]. Polyakova et al showed that in men, regardless of the presence of coronary heart disease, the leptin gene expression in the EAT was significantly higher than the SAT [30]. Further, the authors explain the observed differences by the predominance of men among patients with CAD, and more severe atherosclerotic lesions of the coronary arteries in male patients [31]. However, Iglesias et al in their study showed that leptin gene expression in the EAT was lower than the SAT [16]. Increased leptin gene expression in the EAT culture of patients can have a negative effect on both adipocytes and cardiomyocytes by activating inflammatory and atherosclerotic processes [32].

An increase in the risk of cardiovascular events is associated with both an increase in leptin levels and with resistance of leptin receptors [33]. Few studies have indicated a decrease in the gene expression of the Leptin R in the hypothalamus, hepatocytes, muscle, and AT in obesity [34, 35]. Leptin receptors gene expression in cell cultures may depend on leptin concentration. It was shown that the addition of exogenous leptin to the cell culture reduced the expression of its receptors, while the decrease in the concentration of circulating leptin during prolonged fasting was accompanied by an increase in receptor mRNA levels [36, 37]. In addition, it is currently hypothesized that increased concentrations of the leptin R inhibit leptin effects, while reduced concentrations of the leptin receptor reflect low membrane expression of the receptor. These data partially explain changes in leptin sensitivity that are associated with changes in leptin R levels in metabolic disorders [38].

According to our data, the higher expression of the leptin R gene in the culture of EAT adipocytes in patients was observed compared to the SAT. Further, in patients with CAD, it was highest in PVAT

adipocytes. Data on the expression of the leptin R in the human AT are scarce. Studies have shown that the content of the leptin R depends on the type of AT and differs in obese and non-obese individuals [39].

In addition, peripheral leptin resistance was evaluated in this study, characterized by changes in the activity of leptin genes and its receptor using the FLI as a reliable marker of leptin resistance. It was demonstrated that the expression of genes for short and long leptin receptor isoforms (OB-Ra and OB-Rb, respectively) in AT was reduced in obesity [40]. In studies on cell cultures, it has been shown that the gene expression of leptin receptors decreases when exogenous leptin is added, but when the concentration of circulating leptin decreases during prolonged fasting, the leptin R mRNA level increases [41]. Therefore, a decrease in the level of the leptin R can serve as both a reflection and cause of disruption of leptin transport to the hypothalamus and changes in its biological influence. An increase in the FLI indicates an imbalance in the leptin-receptor system and is a reflection of compensatory mechanisms in peripheral leptin resistance in adipocytes of the SAT, in contrast to adipocytes of the PVAT characterized by a minimum FLI, regardless of nosology.

Thus, these results indicate that the "protective" potential of AT depends on its localization. Thus, EAT adipocytes of CAD patients are characterized by a pronounced imbalance in the adipokine system: a low level of adiponectin gene expression against a high level of leptin gene expression and secretion compared to adipocytes of other localization.

IL-6 has been shown to produce various types of cells, including fibroblasts, monocytes, smooth muscle cells of the coronary arteries, endothelial cells, and cardiomyocytes. However, adipocytes are also able to secrete proinflammatory IL-6 [42]. About 30% of IL-6 in the body is synthesized by AT, and its production is increased in overweight individuals [43]. IL-6 is involved in the regulation of energy balance of fat and muscle tissues, triggering regenerative mechanisms of immune protection (activation and differentiation of T-cells, maturation of B-cells, synthesis of C-reactive protein in the liver, increased hematopoiesis), and regulation of fat intake into AT. In addition, IL-6 is able to limit the inflammatory response by inhibiting the synthesis of a number of proinflammatory cytokines, including tumor necrosis factor- α (TNF- α) [44]. Its effect on cardiomyocytes is ambiguous, since IL-6 has both protective (antiapoptotic) and negative (induction of hypertrophy) effects [42]. In vitro studies have shown that isolated visceral fat synthesizes more IL-6 than subcutaneous fat, and increased IL-6 production has been observed in adipocyte hypertrophy.

We found that IL-6 gene expression in the daily culture of EAT adipocytes was the highest in comparison to the SAT and PVAT in both CAD and with aortic or mitral valve replacement. Moreover, the level of IL-6 mRNA in the EAT and PVAT in individuals with CAD was higher than in patients with malformations. These identified features are consistent other studies. For example, Shibasaki et al studied SAT and EAT samples obtained during open-heart surgery and found that the level of IL-6 mRNA in the EAT was significantly higher than in the SAT. Moreover, the gene expression of IL-6 and leptin in the EAT was higher in patients with CAD, and the levels of adiponectin mRNA were comparable in both groups. In the SAT, IL-6 mRNA and leptin gene expression levels were moderately higher in patients with CAD compared

to those without CAD. However, there were no differences in plasma cytokine levels between the two groups. Based on this, the authors concluded that the mRNA levels of inflammatory cytokines, adipokines, neurohumoral factors, and their receptors increased in the EAT, independent of their plasma levels [45].

In this study, it was found that IL-6 content in the culture of EAT adipocytes was higher than in other fat depots, regardless of nosology. However, IL-6 levels in the PVAT adipocyte culture in Group 1 exceeded that of Group 2, while SAT adipocytes did not show differences in IL-6 secretion between the groups.

Increased IL-6 gene expression and secretion in EAT and PVAT adipocytes can contribute to the high level of leptin gene expression and content observed in these types of AT. It is known that leptin has a stimulating effect on immune cells and is able to regulate the production of pro- and anti-inflammatory cytokines. Thus, *in vivo* and *in vitro* studies have shown that elevated leptin levels activate neutrophils and chemotaxis in polymorphonuclear leukocytes, increase monocyte/macrophage activity and phagocytosis, increase lymphocyte proliferation, and produce IL-6 and TNF- α monocytes, which are independent predictors of MI development and adverse prognosis in patients with CAD [46]. In addition, some studies have noted homology in the structure of leptin and IL-6 receptors, also suggesting an interaction between leptin and circulating factors, through which cytokines inhibit binding of the leptin OB-Rs receptor to block its signaling activity in cell culture [47].

Experimental *in vitro* findings demonstrated that secretory products of adipocytes increase the secretion of inflammatory cytokines by macrophages and other immune cells, and pro-inflammatory cytokines, in particular IL-6, increase the transcription of leptin, which was confirmed by *in vivo* studies [48]. In addition, the release of IL-6 from human adipocyte culture is inhibited by glucocorticoids and stimulated by insulin and catecholamines. Pro-inflammatory stimuli such as TNF- α and IL-6 itself can enhance IL-6 production *in vitro*. The content of IL-6 in AT is hundreds of times higher than in the plasma, suggesting important auto and paracrine regulatory functions in this tissue [49].

Conclusion

Thus, increased production of leptin and IL-6 and decreased production of cardioprotective adiponectin leading to activation immune cell and inflammatory, manifested primarily in EAT and PVAT adipocytes, especially in individuals with CAD, which creates favorable conditions for the development and progression of atherosclerosis. Moreover, leptin is able to affect the vascular wall and activate platelet aggregation, increasing the risk of thrombosis. Adipocytokine imbalance in epicardial adipocytes is a prognostically unfavorable sign in this category of patients. Therefore, the study of EAT revealed the pathogenetic significance of changes in the adipokine and cytokine status of adipocytes in patients with CAD.

Abbreviations

CVD, cardiovascular disease; AT, adipose tissue; EAT, epicardial adipose tissue; PVAT, perivascular adipose tissue; mRNA, matrix RNA; SAT, subcutaneous adipose tissue; CAD, coronary artery disease; CABG, coronary artery bypass grafting; DM, diabetes mellitus; MI, myocardial infarction; cDNA, coding DNA; qPCR, quantitative real-time polymerase chain reaction; leptin R, leptin receptor HPRT1, hypoxanthine phosphoribosyltransferase-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; B2M, beta-2-microglobulin; IL-6, interleukin-6; FLI, free leptin index; ANOVA; Me, median; Q1, 25th quartiles; Q3, 75th quartiles; BMI, body mass index; ER, endoplasmic reticulum; TNF- α , tumor necrosis factor- α .

Declarations

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Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Availability of data and materials

The data that support the findings of this study are not available due to containing information that could compromise research participant privacy/consent.

Ethics approval and consent to participate

The study protocol was approved by the Local Ethics Committee of the Federal State Budgetary Institution Research Institute for Complex Issues of Cardiovascular Diseases and was developed according to the World Medical Association's Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, 2000 edition, and the "GCP Principles in the Russian Federation", approved by the Russian Ministry of Health. Patients were included in the study after they provided written informed consent.

Consent for publication

No individual participant data were reported that would require consent from the participant to publish.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Clinical and anamnestic characteristics of patients with coronary artery disease and heart defects

Parameter	Group 1 (n=84)	Group 2 (n=50)	P
Men, n (%)	63 (75)	24 (48)	0.012
Body mass index, kg/m ²	29.41 (26.30;31.6)	26.59 (23.44;28.31)	0.078
Arterial hypertension, n (%)	81 (96.4)	12 (24)	0.003
Hypercholesterolemia, n (%)	20 (23.8)	5 (10)	0.024
Smoking, n (%)	58 (69.0)	3 (6)	0.0001
Anamnesis			
Family history of CAD, n (%)	50 (59.5)	16 (32)	0.018
History of myocardial infarction, n (%)	57 (67.9)	0	-
History of stroke, n (%)	6 (7.1)	0	-
Atherosclerosis of other pools, n (%)	13 (15.5)	0	-
No angina, n (%)	2 (2.4)	50 (100)	0.0001
Functional class I angina, n (%)	0	0	-
Functional class II angina, n (%)	39 (46.4)	0	-
Functional class III angina, n (%)	43 (51.2)	0	-
Chronic heart failure I functional class, n (%)	0	41 (82)	-
Chronic heart failure II functional class, n (%)	59 (70.2)	6 (12)	0.002
Chronic heart failure III functional class, n (%)	25 (29.7)	3 (6)	0.077
Chronic heart failure IV functional class, n (%)	0	0	-
Atherosclerosis of the 1st coronary artery, n (%)	6 (7.1)	0	-
Atherosclerosis of the 2nd coronary artery, n (%)	4 (4.8)	0	-
Atherosclerosis of three or more coronary artery, n (%)	74 (88.1)	0	-
Ejection fraction, %	50.0 (43.0;56.0)	52.5 (43.0;57.5)	0.125

Notes: Group 1 - patients with CAD and indications for direct myocardial revascularization by coronary artery bypass grafting, Group 2 - patients with aortic or mitral valve replacement.

Table 2 Concentration of adipokines and the free leptin index in the daily adipocyte culture of various fat depots in patients with coronary artery disease and with aortic or mitral valve replacement

Parameter	Subcutaneous adipocytes		Epicardial adipocytes		Perivascular adipocytes		P
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	
	1	2	3	4	5	6	
Adiponectin, ng/mL	16.44	19.11	12.36	16.95	11.25	20.41	$P_{1-3}=0.004$
	(14.1; 22.04)	(16.33; 21.73)	(11.65; 14.47)	(14.34; 18.56)	(10.72; 13.96)	(13.54; 23.04)	$P_{1-5}=0.003$
							$P_{2-4}=0.012$
							$P_{4-6}=0.025$
							$P_{3-4}=0.011$
Leptin, ng/mL				6.21		4.50	$P_{3-5}=0.044$
	7.57	5.30	7.67	(4.33; 7.34)	6.96	(2.81; 5.78)	$P_{2-4}=0.041$
	(6.94; 8.67)	(4.43; 6.62)	(5.95; 7.99)		(6.54; 7.12)		$P_{4-6}=0.003$
							$P_{3-4}=0.038$
							$P_{5-6}=0.002$
Leptin R, ng/mL		46.21		55.30		54.50	$P_{1-3}=0.017$
	37.67	(24.33; 67.34)	47.57	(14.43; 76.62)	56.96	(22.81; 75.78)	$P_{1-5}=0.011$
	(15.95; 57.99)		(26.94; 68.67)		(26.54; 77.12)		$P_{3-5}=0.025$

							$P_{2-4}=0.013$
							$P_{2-6}=0.024$
FLI	0.20 (0.13; 0.35)	0.11 (0.08; 0.18)	0.16 (0.14; 0.26)	0.11 (0.07; 0.31)	0.12 (0.09; 0.27)	0.08 (0.02; 0.18)	$P_{1-3}=0.012$ $P_{1-5}=0.003$ $P_{3-5}=0.001$ $P_{2-4}=0.002$ $P_{2-6}=0.002$ $P_{4-6}=0.034$

Notes: The data presented are the medians (25th quartiles, 75th quartiles), Group 1 - patients with CAD and indications for direct myocardial revascularization by coronary artery bypass grafting, Group 2 - patients with aortic or mitral valve replacement; P - significant differences as compared to subcutaneous adipocytes, ($p \leq 0.05$), FLI was defined as the ratio of the level leptin to leptin R.

Abbreviation: Leptin R, leptin receptor; FLI, free leptin index.

Table 3 Gene expression and IL-6 concentration in adipocyte culture of various fat depots in patients with coronary artery disease and with aortic or mitral valve replacement

Parameter	Subcutaneous adipocytes		Epicardial adipocytes		Perivascular adipocytes		P
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	
	1	2	3	4	5	6	
IL-6 gene expression, Delta Ct							P ₁₋₃ =0.001
	0.039	0.054	0.080	0.058	0.058	0.030	P ₃₋₅ =0.002
	(0.027;	(0.036;	(0.062;	(0.037;	(0.038;	(0.021;	P ₄₋
	0.058)	0.060)	0.084)	0.073)	0.066)	0.032)	P ₆ =0.0002
							P ₃₋
							P ₄ =0.0013
							P ₅₋
							P ₆ =0.0001
IL-6, pg/mL	12.42						P ₁₋
	(8.33;	14.81	30.77	22.26	16.92	12.49	P ₃ =0.0012
	15.69)	(10.24;	(20.11;	(17.45;	(13.38;	(9.71;	P ₃₋₅ =0.003
		18.56)	37.22)	25.12)	20.16)	17.17)	P ₂₋₄ =0.017
							P ₄₋₆ =0.002
							P ₃₋₄ =0.022
							P ₅₋₆ =0.018

Notes: The data presented are the medians (25th quartiles, 75th quartiles), Group 1 - patients with coronary artery disease and indications for direct myocardial revascularization by coronary artery bypass grafting, Group 2 - patients with aortic or mitral valve replacement; P - significant differences compared with subcutaneous adipocytes, ($p \leq 0.05$).

Abbreviation: IL-6, interleukin-6.

Figures

Figure 1

Adiponectin gene expression in various fat depots in the daily adipocyte culture of patients with CAD and with aortic or mitral valve replacement Notes: Group 1 - patients with coronary artery disease and indications for direct myocardial revascularization by coronary artery bypass grafting, Group 2 - patients with aortic or mitral valve replacement. Abbreviation: SAT, subcutaneous adipose tissue; EAT, epicardial adipose tissue; PVAT, perivascular adipose tissue.

Figure 2

Leptin gene expression in various fat depots in the daily adipocyte culture of patients with CAD and with aortic or mitral valve replacement Notes: Group 1 - patients with coronary artery disease and indications for direct myocardial revascularization by coronary artery bypass grafting, Group 2 - patients with aortic or mitral valve replacement. Abbreviation: SAT, subcutaneous adipose tissue; EAT, epicardial adipose tissue; PVAT, perivascular adipose tissue.

Figure 3

Leptin R gene expression in various fat depots in the daily adipocyte culture of patients with CAD and with aortic or mitral valve replacement Notes: Group 1 - patients with coronary artery disease and indications for direct myocardial revascularization by coronary artery bypass grafting, Group 2 - patients with aortic or mitral valve replacement. Abbreviation: SAT, subcutaneous adipose tissue; EAT, epicardial adipose tissue; PVAT, perivascular adipose tissue.