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Epicardial and subcutenious adipose tissue adiponectin gene expression in coronary artery disease patients

Aim	To determine the expression of adiponectin gene (*ADIPOQ*) and the content of high-molecular-weight adiponectin (HMWA) in epicardial (EAT) and subcutaneous adipose tissue (SCAT) in patients with ischemic heart disease (IHD).
Material and methods	Paired samples of EAT and SCAT and blood serum were withdrawn from patients with IHD after bypass surgery and 16 subjects without IHD (comparison group). Matrix RNA (mRNA) level was measured using real-time polymerase chain reaction. HMWA levels in EAT and SCAT were evaluated by Western blotting. Serum adiponectin concentration was measured immunoenzymatically. For all patients, echocardiography was performed to measure the EAT thickness; coronarography was performed to determine severity of coronary atherosclerosis.
Results	Serum adiponectin concentration was lower in IHD patients than in the comparison group ($p<0.001$). Levels of ADIPOO gene mRNA and HMWA in SCAT were lower in IHD patients than in the comparison group ($p=0.020$ and $p=0.003$, respectively). The HMWA level in EAT was lower with the EAT thickness of 8 mm compared to the HMWA level in IHD patients with EAT ≤8 mm ($p=0.034$).
Conclusion	The decreased serum concentration of antiatherogenic adiponectin and the reduced expression of *ADIPOQ* gene in SCAT (mRNA, HMWA) are associated with IHD.

Keywords	Epicardial adipose tissue; subcutaneous adipose tissue; adiponectin gene expression; adiponectin; atherosclerosis; ischemic heart disease
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Coronary artery disease (CAD) is a pathological condition characterized by the disturbance of myocardial blood flow, which is associated with hemodynamically significant atherosclerotic lesions of the coronary arteries.

White and brown fat are the predominant types of adipose tissue in mammals. White adipose tissue, which is the most common form of fat in the human body, is classified as subcutaneous and visceral fat. The regional distribution of white adipose tissue has been shown to be a strong predictor of metabolic health risks: the accumulation of fat in visceral adipose tissue increases the risk of type 2 diabetes and cardiovascular diseases [1]. Recent studies have shown that epicardial adipose tissue (EAT) is a type of visceral fat that may have a significant role in the development of coronary artery atherosclerosis and CAD [1–5].

Adipose tissue, EAT in particular, is now considered as an endocrine organ that secretes hormone-like agents – adipokines. Adipokines regulate carbohydrate and lipid metabolism, tissue sensitivity to insulin, blood clotting ability, and inflammatory responses. Their activity can be both systemic and local. It is particularly interesting to study EAT due to its anatomical proximity to the myocardium and coronary arteries and its potential local effect on the coronary arteries through paracrine mechanisms [5–8].

Adiponectin is one of the most important adipokines secreted by adipose tissue. It regulates energy homeostasis, being a part of the metabolism of fatty acids and glucose, and has anti-inflammatory and anti-atherogenic effects [9, 10]. Blood adiponectin reduces vascular inflammation and inhibits proatherosclerotic processes (monocyte adhesion, transformation of macrophages

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into foam cells, migration, and proliferation of smooth muscle cells) [11]. Low levels of plasma adiponectin are observed in obesity, type 2 diabetes mellitus, and cardiovascular diseases [12, 13].

Adiponectin circulates in the blood in trimer, hexamer and multimer forms. Multimer – i.e., high-molecular-weight – adiponectin (H-adn) has the most significant biological activity [14]. H-adn stimulates the production of nitrogen oxide II in the endothelial cells, participates in the suppression of oxidative stress and inhibits the development of inflammation. Low levels of plasma H-adn are associated with insulin resistance and high risk of cardiovascular diseases [15]. It has been established that high levels of H-adn and the H-adn/total adiponectin ratio are associated with a lower risk of the development of CAD [16].

It should be noted that it is still unclear what type of adipose tissue is responsible for regulating levels of blood-borne adiponectin in the human body, highlighting the relevance of studies designed to evaluate the expression of the *ADIPOQ* gene in different types of adipose tissue.

Studies concerning EAT *ADIPOQ* gene expression in obesity and related cardiovascular pathology are sparse due to difficulties in obtaining epicardial tissues. In two studies, a decrease in the messenger ribonucleic acid (mRNA) of the *ADIPOQ* gene in EAT was found in patients with metabolic syndrome and CAD [17, 18]. Iacobellis et al. established that levels of total adiponectin in EAT was lower in patients with CAD versus patients without CAD [19]. We found no studies investigating H-adn levels in both EAT and subcutaneous adipose tissue (SAT) in patients with CAD.

In this study, levels of mRNA of the *ADIPOQ* gene and H-adn in the paired EAT/SAT samples were evaluated in patients with CAD and patients without atherosclerotic coronary artery disease (reference group). The relationships of measured parameters with the level of total serum adiponectin, clinical and echocardiographic characteristics, and the severity of atherosclerotic coronary artery disease were also studied in patients with CAD.

Materials and Methods

Samples of EAT, SAT, and peripheral blood serum were collected from 74 patients with CAD during coronary bypass surgery (35% female and 65% male patients, mean age 61.8±8.5 years old). The reference group included 16 patients who underwent surgeries for valvular heart defects without clinical signs of CAD and atherosclerotic coronary artery disease (50% female and 50% male patients, mean age 67.6±15.3 years old). Coronary angiography was performed for all patients examined to confirm and evaluate the severity of coronary artery atherosclerosis or to show that there were no atherosclerotic lesions. The thickness of EAT was measured by echocardiography. The echocardiographic examination was performed on an expert ultrasound scanner (GE VIVID 7 Dimension). The main echocardiographic parameters were evaluated using a phased array messenger sector probe (M4S). Cardiac computed tomography and magnetic resonance imaging are the gold standards for estimating the thickness of EAT [20. 21]. At the same time, these examinations are time-consuming and expensive, require specially trained personnel, and expose patients to a high radiation load (computed tomography), which significantly limits the possibility of their extensive use in clinical practice to assess the severity of epicardial obesity [21]. The EAT measurements were made at three points and expressed in millimeters: above the apex of the heart, above the free wall of the right ventricle, and in the atrioventricular groove. The mean values of the measurements were calculated. Body mass index and waist circumference of the examined patients were analyzed. All patients with CAD received antiplatelet drugs, beta-blockers, angiotensin-converting enzyme inhibitors/sartans, and statins, according to the national and international guidelines [22, 23].

The collected tissue samples were frozen in liquid nitrogen and stored at –80°C until the time of analysis. Blood samples were centrifuged at 3000 rpm 20 minutes to separate serum. Serum samples were then collected into separate sterile test tubes and put in a freezer to be stored at –80°C until testing.

The study was approved by the Ethics Committee of First Pavlov State Medical University of St. Petersburg. All patients signed the informed consent form to participate in the study.

Total RNA was isolated from the EAT/SAT biopsies using a RNeasy Mini Kit (Qiagen, USA). Complementary deoxyribonucleic acid (cDNA) was obtained by reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). The purity of an RNA preparation was assessed on a SmartSpecPlus spectrophotometer (Biorad, USA) in terms of absorption at 260 and 280 nm (purity ratio). The absence of RNA degradation was verified using electrophoresis in 1% agarose gel by the intensity ratio of the band corresponding to 28S and 18S rRNA (2:1 if there is no degradation).

The relative levels of *ADIPOQ* mRNA in EAT/SAT was determined by a real-time polymerase chain reaction (PCR) using TaqMan fluorescent probes and a CFX96

device (Biorad, USA). PrimerExpress primers and probes labeled with different fluorophores (DNK-Sintez, Russia) were used. The primer and probe sequences are given in Table 1.

Amplification was carried out in 30 μL of a mixture containing 2.5 mM of MgCl2, 0.25 mM of each dNTP, 0.2 μM of each primer, 0.4 μM of the *ADIPOQ* gene probe, 0.1 μM each reference gene primer and probe, 50 ng cDNA, 2.5′ TaqM polymerase, and 3 μL relevant 10X PCR buffer (AlcorBio, Russia) in the following temperature-timing mode: 1 cycle at 95 °C, pre-denaturation for 15 minutes; melting at 95 °C for 10 seconds, annealing at 58°C for 20 seconds, synthesis at 72°C for 10 seconds; 40 cycles. All samples were measured at least in triplicate to ensure accuracy and precision. Each plate contained a reference sample, i.e., a pooled adipose tissue cDNA obtained from the reference group, which was used for the entire cycle of experiments, and negative control (no template) in triplicate, respectively. The relative level of *ADIPOQ* gene mRNA was calculated using the ΔΔCt relative measurement method modified for several reference genes and expressed in relative units [24].

The level of H-adn in EAT/SAT was determined by the western blotting technique. Adipose tissue samples were lysed in a solution (50 mM Tris, pH 8.0. 150 mM NaCl, 1% Triton X-100. 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (Roche, Switzerland). The total protein was quantified by the Bradford method using a BioRadxMark microplate spectrophotometer (USA). Electrophoresis was performed under the conditions necessary for the preservation of H-adn without adding β-mercaptoethanol 4x Laemmli Sample Buffer (Biorad, USA) and boiling the samples to avoid denaturation and degradation of the complexes, in a VE-2 vertical electrophoresis chamber (Helicon, Russia). Rabbit polyclonal adiponectin primary antibodies (1:100; Almabion, Russia), beta-actin antibodies (1:1000; NB600–503, Novus Biologicals, USA) and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:3000; ab6721, Abcam, UK) were used. Secondary antibodies bound to the corresponding protein on the membrane fragments were identified using an Amersham ECL TM Plus System kit (Amersham, UK). Western blotting data were analyzed using Image J (version 1.38a for Windows, http://rsb.info.nih.gov/ij/). The content of H-adn was normalized to beta-actin content. H-adn bands were visualized above 250 kDa (Figure 1).

Levels of total serum adiponectin were measured using the Human Adiponectin ELISA kits (BioVendor, Czech Republic) according to the manufacturer's instructions. Absorbance was measured on a BioRad microplate spectrophotometer. The standard curve was constructed using the standards from the kit.

Data were processed in SPSS 23.0. The conformity of data to normal distribution was verified using the Kolmogorov-Smirnov test. If data conformed to the normal distribution (most clinical and biochemical characteristics), the Student's T-test was applied. In case of non-normal distribution, the Mann – Whitney U-test was used. The results are presented as the mean ± standard deviation or the median (min-max). The correlation between quantitative characteristics was analyzed using the Spearman test. The critical significance level was 0.05.

Results

The characteristics of the patients examined are provided in Table 2. The percentage of patients with multivessel coronary disease did not differ between normal-weight patients and those with abdominal obesity. The absence of significant differences in the serum levels of total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol in

Table 1. Structure of oligonucleotide primers and probes

Gene	Forward (F) and reverse (R) primers and TaqMan® probes
ADIPOQ (target gene)	F: 5'- CCTGGTGAGAAGGGTGAGA - 3' R: 5'- GGTTTCACCGATGTCTCCCT - 3' 5' - (FAM) AGGAGATCCAGGTCTTATTGGT (BHQ1) - 3'
RPLPO (reference gene)	F: 5'- GATCAGGGACATGTTGCTGG - 3' R: 5'- GACTTCACATGGGGCAATGG - 3' 5' - (ROX) CAATAAGGTGGCAGCTGCTGC (BHQ2) - 3'
ACTB (reference gene)	F: 5'- CGTGCTGCTGACCGAGG - 3' R: 5'- ACAGCCTGGATAGCAACGTAC - 3' 5' - (HEX) CCAACCGCGAGAGGATGACCCAGAT (BHQ1) - 3'

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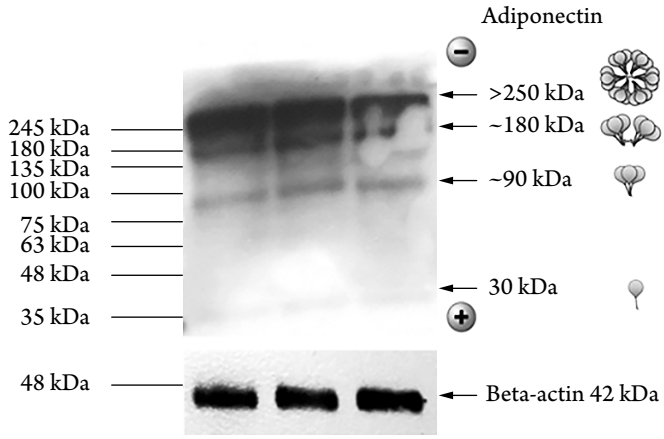
Figure 1. Example of a western blot with adiponectin forms: multimer, hexamer, trimer, monomer



Electrophoresis: 10% polyacrylamide gel, molecular weight marker (ab116028, Abcam, UK)

patients with CAD and the reference group appears to be associated with the fact that patients with CAD used lipid-lowering drugs.

The levels of serum adiponectin, *ADIPOQ* gene expression, and EAT / SAT H-adn in the study groups are shown in Table 3. The levels of total serum adiponectin in patients with CAD were lower than in the reference group ($p<0.001$). A negative correlation was found between the levels of total serum adiponectin and body mass index in all patients ($r = –0.419$, $p<0.01$).

The levels of *ADIPOQ* gene expression in EAT were not different from the expression levels in SAT ($p = 0.6$), while the levels of H-adn were higher in EAT versus SAT ($p<0.001$). The levels of *ADIPOQ* mRNA and relative levels of H-adn in SAT in patients with CAD were lower than in the reference group ($p = 0.020$ and $p = 0.003$, respectively) (Table 3). No statistically significant differences in *ADIPOQ* gene expression or the H-adn levels in EAT between patients with CAD and the reference group were found.

There were no differences in the levels of serum total adiponectin or levels of mRNA and H-adn in EAT / SAT in the patient subgroups formed using the degree of coronary artery lesions and overweightness or obesity. We suggested that the levels of mRNA and H-adn in EAT might depend on the degree of epicardial obesity. Since there is no direct relationship between the thickness of EAT and the measurement used in this study, and no reference values for the thickness of EAT were established, we performed a comparative analysis of the levels of mRNA and H-adn in EAT based on the quartile division according to EAT thickness (1.9–4.4 mm = 1st quartile, 4.5–8.0 mm = 2nd and 3rd quartiles, 8.1–11.3 mm = 4th quartile). The lower levels of H-adn in EAT were found in the subgroup of patients with EAT thickness>8 mm versus patients with EAT thickness ≤8 mm (Figure 2).

Discussion

This study was performed to explore *ADIPOQ* gene expression and content of a biologically active multimer form of adiponectin in EAT / SAT in CAD. The relative levels of H-adn in paired EAT / SAT samples in patients with CAD were evaluated for the first time.

Table 2. Clinical and biochemical characteristics of patients with coronary artery disease and the experimental group

Parameter	CAD group (n = 74)	Experimental group (n = 16)	p
Age, years	61.8±8.5	60.5±13.0	ns
Sex			
Male	55	8	–
Female	19	8	–
BMI, kg/m2	28.5±4.2	25.5±4.0	ns
WC, cm	98.0±9.2	86.4±14.1	ns
EAT thickness, mm	6.5±2.5	5.3±1.9	ns
Abdominal obesity, N (%)	35 (47)	7 (43)	
Number of coronary arteries affected			
1–2	27	0	–
3 or more	47	0	–
TC, mmol/L	4.8±1.4	4.6±1.0	ns
HDL, mmol/L	1.2 (0.7–2.4)	1.1 (0.5–1.8)	ns
LDL, mmol/L	2.6±1.4	2.8±0.5	ns
TG, mmol/L	1.5 (0.4–5.1)	1.1 (0.4–3.8)	0.048

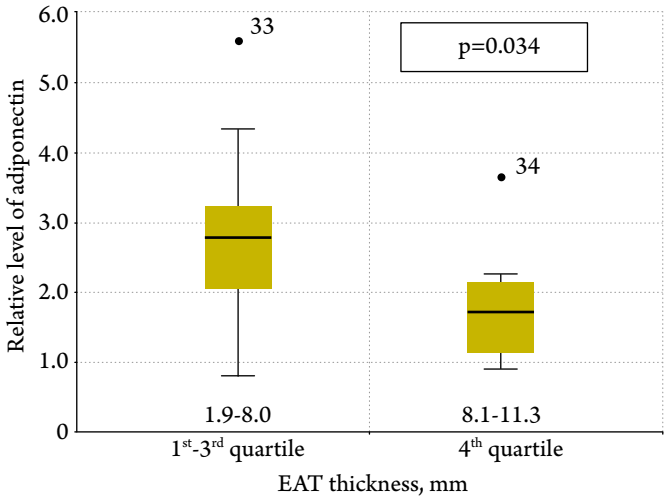
BMI, body mass index; WC, waist circumference, EAT, epicardial adipose tissue; TC, total cholesterol; HDL, high-density lipoproteins; LDL, low-density lipoproteins; TG, triglycerides; p, level of statistical significance; ns, no statistically significant differences found.

Table 3. Levels of serum adiponectin, relative levels of *ADIPOQ* gene expression, and relative levels of high molecular weight adiponectin in the study groups

Parameter	CAD patients, median (min-max)	Experimental group, median (min-max)	p*
Concentration of serum adiponectin (ng/mL)	22.10 (6.00–54.00)	29.60 (7.00–79.00)	0.001
EAT			
ADIPOQ mRNA	0.31 (0.01–5.61)	0.16 (0.02–3.18)	ns
H-adn	2.35 (0.80–5.61)	1.93 (0.75–4.34)	ns
SAT			
ADIPOQ mRNA	0.27 (0.01–7.34)	0.74 (0.27–8.25)	0.020
H-adn	0.77 (0.31–2.41)	1.10 (0.70–2.40)	0.003

EAT, epicardial adipose tissue; SAT, subcutaneous adipose tissue; H-adn, relative level of high-molecular-weight adiponectin; p, level of statistical significance; ns, no statistically significant differences found, *ADIPOQ* mRNA, relative level of expression of the messenger RNA of ADIPOQ gene. *, the Mann–Whitney U-test.

Figure 2. Relative level of high-molecular-weight adiponectin in epicardial adipose tissue in the patients studied depending on the thickness of epicardial adipose tissue



Pischon et al. found in their prospective study that low levels of adiponectin in peripheral blood plasma is associated with the development of CAD and a higher risk of myocardial infarction in male patients [16]. In our study, it was found that the levels of total serum adiponectin were higher in the reference group, i.e., those who had no clinically significant coronary artery atherosclerosis, than in patients with CAD. On the other hand, since H-adn is considered to be a biologically active form of adiponectin [14], it can be responsible for the anti-atherogenic effect of adiponectin. The low levels of serum H-adn were shown to be associated with cardiovascular risk factors such as smoking, obesity, and low levels of high-density lipoprotein cholesterol [25, 26]. The reduced incidence of subclinical atherosclerosis was, on the contrary, observed when the H-adn levels were elevated [27].

We found that the relative levels of H-adn in SAT and the levels of *ADIPOQ* gene mRNA were lower in patients with CAD versus the reference group. However, no correlation was detected between the levels of *ADIPOQ* gene mRNA and the levels of H-adn in SAT. Jonas et al. previously demonstrated their findings of lower levels of *ADIPOQ* gene expression in SAT (mRNA, protein) in obese patients versus a reference group [28]. Similar data on the reduced expression of the *ADIPOQ* gene in SAT in overweight and obese women versus the expression of this gene in normal-weight women was obtained earlier by us and other researchers [29, 30]. The reduced expression of the *ADIPOQ* gene in SAT was also detected earlier in patients with diabetes [31]. Our study found no differences in *ADIPOQ* gene expression depending on the degree of obesity. The reduced expression and low levels of adiponectin in SAT may be associated with the presence of CAD regardless of obesity.

We did not obtain statistically significant differences in the expression of the *ADIPOQ* gene in EAT in patients with CAD and the reference group, i.e., subjects without signs of coronary artery atherosclerosis. The data on *ADIPOQ* gene expression in EAT in the presence of cardiovascular pathology are contradictory. It was shown earlier that the levels of *ADIPOQ* gene mRNA in EAT in patients with CAD are reduced, this parameter being negatively correlated with an increase in the number of arteries affected by atherosclerosis [32]. Moreover, it was published that patients with CAD and diabetes mellitus had lower levels of *ADIPOQ* mRNA in EAT than reference group patients [17, 18, 31]. In our study, no differences in the levels of total serum adiponectin and *ADIPOQ* gene expression (mRNA, H-adn) in SAT and EAT were detected depending on the degree of coronary artery lesions. Similar findings previously reported that the levels of *ADIPOQ* mRNA in EAT

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did not differ depending on degrees of coronary artery lesions in groups of patients with CAD [17].

We, and other authors, already showed that *ADIPOQ* gene expression is higher in SAT than in visceral adipose tissue [30. 33]. EAT is a type of visceral adipose tissue adjacent to the myocardium and coronary arteries [5]. According to our study, the levels of *ADIPOQ* gene mRNA in EAT were comparable to that in SAT, which is consistent with the findings of Du et al. who also did not report statistically significant differences in the *ADIPOQ* gene expression between EAT and SAT in the mixed groups [17]. In our study, higher levels of H-adn were detected in EAT than in SAT. EAT adipokines EHT can be locally involved in the regulation of inflammatory and atherogenic processes in the walls of coronary arteries, thus, adiponectin EHT has local anti-inflammatory and vasoprotective effects [32, 34, 35].

On the other hand, the levels of H-adn in EAT were not statistically significantly different in patients with CAD versus the reference group. The absence of differences between the study groups may be due to the fact that the EAT thickness in the reference group subjects was comparable to that in patients with CAD. While the levels of *ADIPOQ* gene mRNA in EAT did not correlate with the thickness of EAT, we found reduced levels of H-adn in EAT with a thickness>8 mm. There are data indicating that the development of CAD and the thickness of EAT are correlated [36, 37]. According to a meta-analysis, the EAT thickness measured by echocardiography is higher in patients with CAD versus subjects without CAD [38]. According to several studies, EAT thickness >7 mm was associated with subclinical atherosclerosis [39] and CAD in female patients [40]. It was also demonstrated that patients with EAT thickness >6 mm are at a higher risk of developing CAD in older age regardless of sex [41]. Lu et al. showed in their study, including 500 patients, that there is an association between the 4th quartile (EAT volume >216.15 cm^3) and adverse cardiovascular outcomes, e.g. myocardial infarction and death [42]. According to Sadea et al., the thickness of EAT may be a predictor of a reduced coronary blood flow reserve in female patients [43]. Thus, it is reasonable to evaluate several potential risk factors of CAD in the subgroups of patients divided by the EAT thickness. The lower levels of H-adn in EAT detected in the subgroup of patients with EAT thickness>8 mm versus the subgroup with a thickness of ≤8 mm could be due to an abnormal process of the multimerization of adiponectin when EAT is growing. For example, the multimerization of adiponectin in adipocytes may become difficult due to endoplasmic reticulum stress caused by obesity [44]. The reduced levels of adiponectin may also be observed during the increased expression of pro-inflammatory cytokines when EAT is growing [32].

Our study had two main limitations: a small number of subjects in the reference group and the comparable thickness of the EAT in the study groups due to the difficulties in collecting EAT samples from individuals without CAD who needed no coronary artery bypass surgery.

Conclusion

The levels of adiponectin expression (mRNA, H-adn) in SAT and EAT were compared in patients with CAD and a reference group. The findings showed comparable relative expression of the *ADIPOQ* gene in EAT/SAT and suggested that the levels of H-adn in EAT may be associated with the thickness of epicardial fat.

The levels of serum total adiponectin, *ADIPOQ* gene mRNA, and H-adn in SAT are lower in patients with CAD than in subjects without signs of clinically significant coronary artery atherosclerosis.

Our findings suggest *ADIPOQ* gene expression in SAT affects the development of CAD.

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No conflict of interest is reported.

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