ASSOCIATION OF FoxP3+ T REGULATORY LYMPHOCYTES WITH EPICARDIAL ADIPOSE TISSUE THICKNESS IN PATIENTS WITH CORONARY HEART DISEASE

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ABSTRACT

Background. *Increase of the epicardial adipose tissue (EAT) thickness is associated with development of inflammation and cardiovascular complications, however, there is no data on the relationship between EAT thickening and the number of immunosuppressive regulatory T lymphocytes.*

The aim. *To study the number of circulating T regulatory lymphocytes and nuclear translocation of the FoxP3 transcription factor in patients with stable coronary heart disease (CHD) depending on the epicardial adipose tissue thickness.*

Materials and methods. *We examined 30 patients with chronic stable CHD. The EAT thickness was measured by echocardiography. Patients were divided into groups depending on the presence and absence of EAT thickening above 5 mm (groups 1 and 2, respectively). Imaging flow cytometry was used to determine the number of T regulatory lymphocytes and the level of FoxP3 nuclear translocation. The concentration of cytokines and high sensitivity C-reactive protein (hsCRP) was determined using enzyme-linked immunosorbent assay in blood serum.*

Results. *In group 2, there was an increase in low-density lipoprotein cholesterol concentration (p = 0.043), ratio of low-density lipoprotein cholesterol to high-density lipoprotein cholesterol (p = 0.017) and the concentration of hsCRP (p = 0.044) and IL-1β (p = 0.005), a decrease in the number and relative count of T regulatory lymphocytes (p = 0.020 and p = 0.026, respectively), as well as the number of cells with FoxP3 nuclear translocation (p = 0.018) compared to group 1. According to multiple logistic regression, the concentration of hsCRP, IL-1β and T regulatory lymphocytes relative count in total were the predictors of EAT thickening (accuracy 80 %; sensitivity 75 %; specificity 84.6 %; AUC = 0.89).*

Conclusion. *Thickening of epicardial adipose tissue in patients with coronary heart disease is associated with a decrease in the number of T regulatory lymphocytes and FoxP3 nuclear translocation in them in presence of comparable anthropometric parameters of obesity and the severity of coronary atherosclerosis.*

Key words: *epicardial adipose tissue, T regulatory lymphocytes, FoxP3, interleukin 1β, lipids, imaging flow cytometry, atherosclerosis*

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ВЗАИМОСВЯЗЬ СОДЕРЖАНИЯ FoxP3$^+$ Т-РЕГУЛЯТОРНЫХ ЛИМФОЦИТОВ И ТОЛЩИНЫ ЭПИКАРДИАЛЬНОЙ ЖИРОВОЙ ТКАНИ У ПАЦИЕНТОВ С ИШЕМИЧЕСКОЙ БОЛЕЗНЬЮ СЕРДЦА

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РЕЗЮМЕ

Обоснование. *Увеличение толщины эпикардиальной жировой ткани (ЭЖТ) ассоциируется с развитием воспаления и сердечно-сосудистых осложнений, однако данные о взаимосвязи между утолщением ЭЖТ и количеством регуляторных Т-лимфоцитов отсутствуют.*

Целью исследования *являлось изучение содержания циркулирующих Т-регуляторных лимфоцитов и ядерной транслокации фактора FoxP3 у пациентов со стабильной ишемической болезнью сердца (ИБС) в зависимости от толщины эпикардиальной жировой ткани.*

Методы. *Обследовано 30 пациентов с хронической стабильной ИБС. Толщину ЭЖТ измеряли методом эхокардиографии. Пациенты были разделены на группы в зависимости от отсутствия и наличия утолщения ЭЖТ более 5 мм (группы 1 и 2 соответственно). Методом проточной цитометрии с визуализацией определяли содержание Т-регуляторных лимфоцитов и уровень ядерной транслокации FoxP3. Методом иммуноферментного анализа в сыворотке крови определяли содержание цитокинов и высокочувствительного С-реактивного белка (вчСРБ).*

Результаты. *В группе 2 выявлено увеличение содержания холестрола липопротеинов низкой плотности ($p = 0,043$), соотношения холестерола липопротеинов низкой плотности к холестеролу липопротеинов высокой плотности ($p = 0,017$) и концентрации вчСРБ ($p = 0,044$) и IL-1β ($p = 0,005$) и снижение относительного и абсолютного содержания Т-регуляторных лимфоцитов ($p = 0,020$ и $p = 0,026$ соответственно), а также количества клеток с ядерной транслокацией FoxP3 ($p = 0,018$) по сравнению с группой 1. По данным множественной логистической регрессии концентрации вчСРБ, IL-1β и доля Т-регуляторных лимфоцитов в совокупности являлись предикторами наличия утолщения ЭЖТ.*

Заключение. *Утолщение эпикардиальной жировой ткани у пациентов с ИБС ассоциируется со снижением содержания Т-регуляторных лимфоцитов в крови и ядерной транслокации фактора FoxP3 в них при сопоставимых антропометрических параметрах ожирения и выраженности коронарного атеросклероза.*

Ключевые слова: *эпикардиальная жировая ткань, Т-регуляторные лимфоциты, FoxP3, интерлейкин-1β, липиды, проточная цитометрия с визуализацией, атеросклероз*

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BACKGROUND

Epicardial adipose tissue (EAT) is a unique fat depot localised between the myocardium and the visceral layer of the epicardium. EAT is closely related to cardiac tissue both anatomically and functionally and contributes to the pathogenesis of a number of cardiovascular diseases such as coronary heart disease (CHD), atrial fibrillation and heart failure [1].

Increased EAT thickness determined by echocardiography is a reflection of ectopic fat deposition, which is associated with increased cardiovascular risk [2, 3].

It is evidenced that the content of immune cells in EAT is the highest compared to other fat depots and is about 20 % of the total number of cells [4]. EAT in patients with CHD is characterised by marked inflammation associated with infiltration by macrophages with predominantly proinflammatory M1 phenotype, mast cells and cytotoxic T-lymphocytes, and the severity of inflammation in EAT, as a rule, exceeds that not only in subcutaneous adipose tissue (SAT), but also in any other visceral fat depot, and the thickness of EAT directly correlates with the severity of inflammation in it [1].

Regulatory T lymphocytes (Treg) represent a distinct and important subpopulation of T lymphocytes that provides protection against the development of autoimmune reactions and excessive immune response. The $CD4^+CD25^{hi}FoxP3^+$ Treg subpopulation, which in norm is 5–10 % of all $CD4^+$ T-cells in peripheral blood and lymphoid organs, is the most characterized. Treg cell deficiency is associated with signs of subclinical inflammation in patients at high and very high cardiovascular risk and increased high-sensitivity C-reactive protein (hsCRP) [5]. Treg lymphocytes exert immunosuppressive function both by contact, through direct interreceptor interaction, and by secretion of anti-inflammatory cytokines. The key cytokines of Treg lymphocytes are interleukin (IL) 10 and transforming growth factor (TGF) β [6], while IL-1β suppresses their activity [7]. The state of lipid metabolism, including the activity of proprotein convertase subtilisin-kexin type 9 (PCSK9), has also been evidenced to be critical in maintaining Treg homeostasis [8, 9]. In the process of Treg lymphocyte activation, the expression of CD25 molecule may decrease, and cells with $CD4^+CD25^{lo}FoxP3^+$ phenotype appear. And the number of these cells increases with active autoimmune inflammation [10].

The suppressor activity of Treg is highly dependent on the stable expression and activity of the transcription factor FoxP3. When intracellular signalling is impaired, FoxP3 translocation to the nucleus is impaired, which is accompanied by Treg dysfunction [11]; however, post-translational regulation of FoxP3 activity has attracted the attention of researchers relatively recently, and many questions remain unexplored.

The existence of adipose tissue resident Treg lymphocytes has been evidenced to contribute to the maintenance of tissue insulin sensitivity by suppressing excessive inflammation. Visceral adipose tissue is most enriched with Treg lymphocytes, the content of which can reach 50 % of all $CD4^+$ T cells [12]. Fatty Tregs are characterised by a distinct repertoire of T cell receptors (TCR), produce more IL-10 and depend on PPAR-γ regulation, in contrast to Tregs of lymphoid origin [12].

Most of the information about the role of Treg in the regulation of visceral adipose tissue functional properties, however, has been derived from animal models, and studies in the human population are inconsistent. For example, when FoxP3 mRNA was assessed, it was revealed that visceral adipose tissue from obese patients contained less FoxP3 mRNA than visceral adipose tissue from normal weight individuals [13]. In contrast, in other studies, FoxP3 expression increased in obesity [14]. Considering that Treg in adipose tissue are most probably of thymic origin [15], it is of interest to investigate whether the changes occurring in EAT in patients with CHD are related to systemic immunoregulatory dysfunction and whether they are correlated with the content of circulating Treg lymphocytes.

The aim of the present study was to examine the content of $CD25^{hi}FoxP3^+CD4^+$ and $CD25^{lo}FoxP3^+CD4^+$ Treg lymphocytes and subcellular localisation of FoxP3 in them in patients with stable coronary heart disease depending on the thickness of epicardial adipose tissue.

METHODS

An observational single-center, single-stage comparative study was conducted. The study included 30 patients (22 men and 8 women) aged 44 to 78 years with chronic stable CHD.

All procedures were performed in accordance with the World Medical Association Declaration of Helsinki «Ethical Principles for Conducting Scientific Medical Research Involving Human Subjects» as amended in 2004 and «Rules of Good Clinical Practice in the Russian Federation» approved by Order of the Ministry of Health of the Russian Federation No. 200н dated April 01, 2016. The study was approved by the local ethical committee of the Research Institute of Cardiology, Tomsk National Research Medical Centre of the Russian Academy of Sciences (Minutes No. 210 dated February 18, 2021). All individuals included in the study signed informed consent to participate.

The study included only those patients in whom coronary angiography and echocardiography procedures with EAT thickness determination were previously performed as indicated. Selective coronary angiography was performed using the Artis One angiography suite (Siemens Shenzhen Magnetic Resonance Ltd., China) and the Digitron-3NAC computer system (Siemens Shenzhen Magnetic Resonance Ltd., China). Gensini Score index [16] was used to assess the severity of coronary atherosclerosis.

EAT thickness was measured along the free wall of the right ventricle at the end of diastole [2, 17] at a point perpendicular to the direction of the ultrasound beam

with respect to the free wall of the right ventricle using the aortic annulus as an anatomical landmark. EAT thickness was determined based on the mean of echocardiographic data obtained in three consecutive cardiac cycles. EAT thickness (tEAT) greater than 5 mm was considered EAT thickening [18]. All patients were divided into two groups: group 1, without EAT thickening (tEAT ≤ 5 mm); group 2, with EAT thickening (tEAT > 5 mm).

Exclusion criteria comprised: acute atherosclerotic complications in the preceding 6 months; any acute inflammatory disease; presence of current or recurrent infectious disease; chronic kidney disease above C3b class; presence of oncological, haematological or autoimmune diseases; and refusal to participate in the study.

All patients underwent anthropometric measurements to assess overall obesity (body mass index (BMI) was calculated) and abdominal obesity (waist circumference was measured).

All patients were on regular drug therapy approaching optimal.

The material for the study was venous blood sampled in the morning on an empty stomach. Blood was sampled in tubes with heparin and tubes with clotting activator. The tubes with clotting activator were centrifuged at an acceleration of 1500 g. After centrifugation, serum was sampled into plastic microtubes, frozen at -40 °C and stored until further examination.

Mononuclear leukocytes were obtained from blood with heparin by density gradient centrifugation (Histopaque 1077, Sigma Aldrich, USA). To assess the content of $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ Treg lymphocytes, cells were stained with monoclonal antibodies to surface antigens (anti-CD4 conjugated with fluorochrome FITC; anti-CD25 conjugated with fluorochrome PE) (Becton Dickinson, USA), cells were fixed and permeabilized using a set of buffers for intracellular and intracellular staining (Becton Dickinson, USA). Cells were then stained with monoclonal antibodies to FoxP3 conjugated to fluorochrome AF647 (Becton Dickinson, USA), fixed and 7-aminoactinomycin D (7-AAD) was added to stain the nucleus. Cells were harvested on an Amnis FlowSight instrument (Luminex, USA) with 488 nm and 642 nm lasers in the INSPIRE program (Amnis Corporation, USA). For subcellular localization analysis of FoxP3, IDEAS 2.0 software (Amnis Corporation, USA) including the Nuclear Localization Wizard for cell image analysis was used. Results were expressed as % positive cells from the desired population. The absolute number of cells in the peripheral blood was calculated according to the data of the general blood analysis.

Concentrations of hsCRP, IL-10, IL-1 β (all kits – Vector-Best, Russia), TGF-β (Invitorgen kit, ThermoFisher Scientific, Austria), PCSK9 (R&D Systems kit, Bio-techne, USA) were assessed in serum by solid phase enzyme immunoassay.

Blood lipid spectrum (total cholesterol (TCL), triglycerides (TG), CL of high-density lipoprotein (CL-HDL), CL of low-density lipoprotein (CL-LDL) was studied (kits of CJSC «Diakon-DS», Russia). Blood glucose and glycated haemoglobin contents were estimated on Konelab 60i (ThermoFisher Scientific, USA) and Cobas 6000 C501 (Rosche, USA) automated analysers, respectively. The CL-LDL/CL-HDL ratio was calculated.

Statistical processing of the obtained data was performed using Statistica 10.0 software package (StatSoft Inc., USA). Results were presented as median and interquartile range (Me (Q1; Q3)). The Shapiro – Wilk criterion was used to test the hypothesis of normality of distribution of quantitative indicators. Since the distribution of quantitative indices differed from normal, the statistical significance of differences in quantitative indices in independent groups of patients was assessed using the Mann – Whitney U-criterion. The frequencies of occurrence in independent groups of patients were compared by Pearson's χ^2 test or Fisher's exact test. Spearman's rank correlation coefficient(r_s) was used to assess the relationship between the traits. Differences were considered statistically significant when the achieved level of $p < 0.05$. To reveal statistically significant factors affecting the increase in epicardial adipose tissue thickness, a multiple logistic regression model was constructed; to assess the quality of the model, a ROC-curve (Receiver Operator Characteristic) was constructed and the area under curve (AUC), accuracy, sensitivity and specificity of the model were calculated.

RESULTS

The groups of patients with EAT thickness ≤ 5 mm and > 5 mm were comparable to each other in terms of sex, age, presence and duration of type 2 diabetes mellitus (DM) and arterial hypertension (AH), smoking status, anthropometric measures of obesity and severity of atherosclerosis (Table 1). The frequency of intakes of statins and the type of drug taken (atorvastatin / rozuvastatin) did not differ between patient subgroups (Table 1), but the dosages taken were higher in the group of patients with thicker EAT: 20 (20; 40) vs. 40 (40; 60) mg/day for atorvastatin ($p = 0.019$) and 20 (10; 20) vs. 30 (20; 40) mg/day for rosuvastatin ($p = 0.030$)).

When metabolic parameters were assessed, it turned out that patients with EAT thickness > 5 mm were characterised by higher CL-LDL concentrations and CL-LDL/CL-HDL ratio values (despite more intensive therapy with statins), and tended to have lower CL-HDL and higher PCSK9 concentrations. The glycemic indices in both groups were comparable (Table 2).

In patients with EAT > 5 mm, we revealed a decrease in the relative and absolute content of $CD25^{hi}FoxP3^+$ Treg lymphocytes, as well as a decrease in the absolute content of $CD25^{hi}FoxP3^+$ Treg lymphocytes with intracellular FoxP3 localization (Fig. 1). The content of $CD25^{lo}FoxP3^+$ Treg lymphocytes did not differ in the studied groups (Fig. 1).

The content of pro-inflammatory hsCRP and IL-1β was higher in patients with EAT thickness > 5 mm with comparable concentrations of TGF-β and IL-10, key Treg lymphocyte cytokines (Table 3).

In the general group of CHD patients, the content of $CD25^{hi}FoxP3^{+}$ Treg lymphocytes was negatively correlated with triglyceride concentration (r_s = -0.345; p = 0.034) and triglyceride/CL-HDL ratio (r_s = -0.388; p = 0.016), and the absolute content of $CD25^{hi}FoxP3^{+}$ Treg lymphocytes and $CD25^{hi}FoxP3^{+}$ Treg lymphocytes with intracellular localization of FoxP3 were negatively correlated with the concentration of IL-1β (r_s = -0.374; p = 0.038 and r_s = -0.389; p = 0.031, respectively). Only in the group of patients with EAT thickness ≤ 5 mm, we found an inverse relationship between PCSK9 content and absolute $CD25^{lo-}FoxP3^{+}$ Treg lymphocytes content (r_s = -0.608; p = 0.036). In patients with greater EAT thickness, this relationship was absent.

TABLE 1

CHARACTERIZATION OF PATIENTS ACCORDING TO THE THICKNESS OF EPICARDIAL ADIPOSE TISSUE

Indices	Patients with tEAT < 5 mm (*n* = 14)	Patients with tEAT > 5 mm (*n* = 16)	*p*-value
Sex: male/female	10/4	12/4	0.999
Age, years	67 (59; 69)	61 (58; 67)	0.224
AH history, *n* (%)	14 (100)	15 (93.8)	0.998
AH duration, years	19.5 (15.0; 30.0)	15.0 (12.0; 20.0)	0.217
type 2 DM history, *n* (%)	7 (50)	4 (25)	0.477
DM duration, years	0.5 (0; 5)	0 (0; 2)	0.652
BMI, kg/m^2	29.2 (25.7; 31.9)	27.0 (24.3; 32.1)	0.854
Waist circumference, cm	100 (96; 105)	100.6 (93.0; 111.5)	0.697
tEAT, mm	4.8 (3.9; 5.0)	9.1 (7.8; 11.4)	< 0.001
Gensini Score, points	30.0 (17.5; 77.0)	53.0 (38.8; 68.5)	0.313
Tobacco smoking, *n* (%)	3 (21.4)	8 (50.0)	0.142
Statin intake, *n* (%)	14 (100)	15 (93.8)	0.998
Atorvastatin, *n* (%)	9 (64.3)	8 (50.0)	0.484
Rosuvastatin, *n* (%)	5 (35.7)	7 (43.7)	0.722

TABLE 2

METABOLIC PARAMETERS OF PATIENTS DEPENDING ON THE THICKNESS OF EPICARDIAL ADIPOSE TISSUE

Indices	Patients with tEAT < 5 mm (*n* = 14)	Patients with tEAT > 5 mm (*n* = 16)	*p*-value
Fasting glucose, mM	6.3 (5.3; 6.8)	5.8 (5.2; 7.6)	0.886
Glycated hemoglobin, %	6.1 (5.8; 8.3)	6.2 (6.0; 6.6)	0.701
Total cholesterol, mM	3.3 (2.9; 3.8)	4.2 (3.0; 5.4)	0.154
Triglycerides, mM	1.2 (0.8; 1.9)	1.6 (1.1; 2.1)	0.240
CL-LDL, mM	1.6 (1.1; 2.1)	2.4 (1.5; 2.8)	0.043
CL-HDL, mM	1.1 (1.0; 1.3)	1.0 (0.8; 1.1)	0.077
CL-LDL/CL-HDL	1.4 (0.9; 1.9)	2.0 (1.6; 3.0)	0.017
PCSK9, ng/mL	211.1 (180.7; 244.5)	273.9 (199.3; 294.8)	0.087

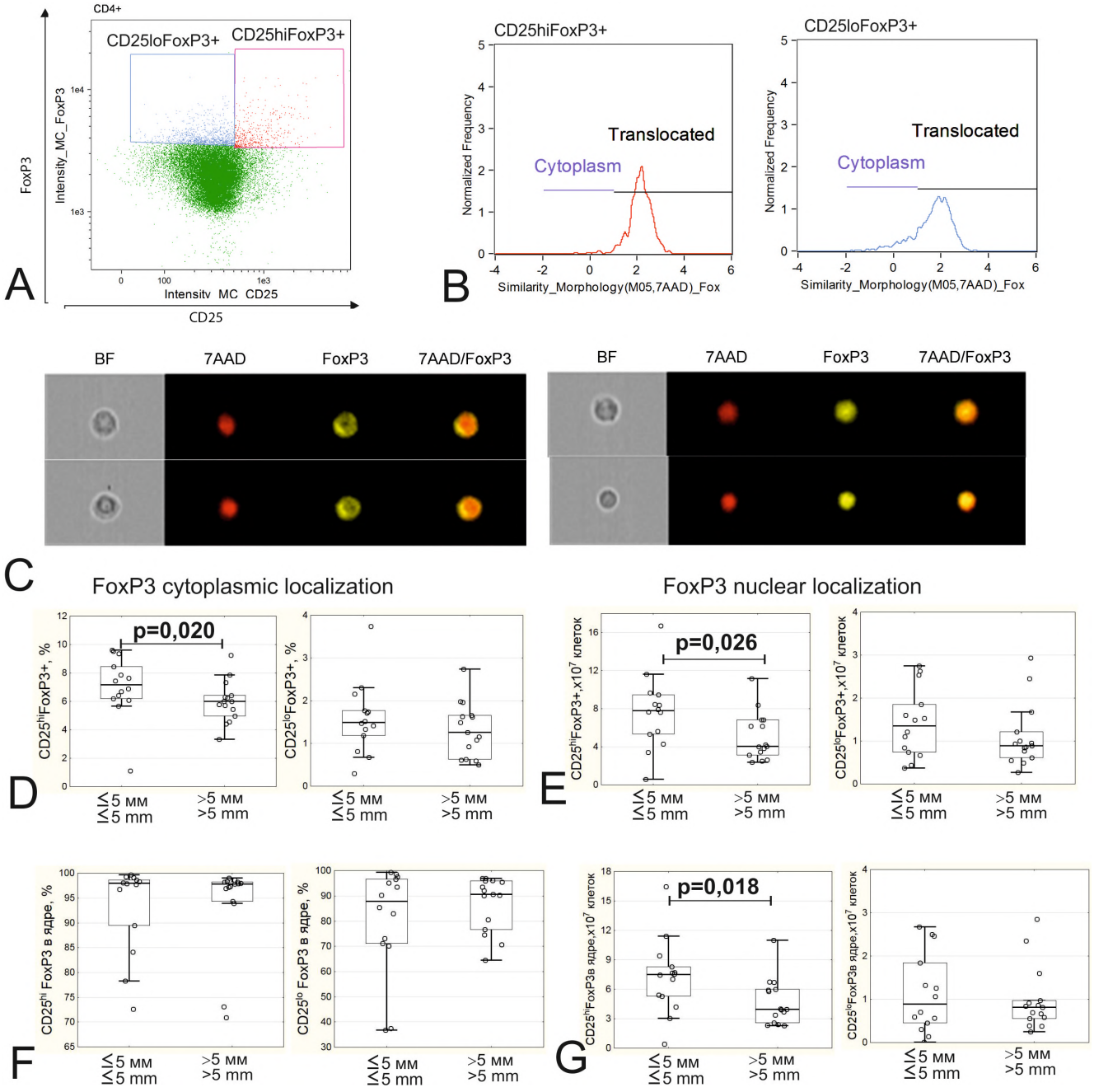

FIG. 1.
The content of FoxP3+ T-regulatory lymphocytes and translocation of FoxP3 into the nucleus: ***A*** *– an example of a dot diagram reflecting the content of CD25hiFoxP3+ and CD25loFoxP3+ cells among CD4+ lymphocytes;* ***B*** *– histograms reflecting the degree of translocation of FoxP3 into the nucleus;* ***C*** *– examples of cellular images obtained by flow cytometry with visualization with cytoplasmic and nuclear localization of FoxP3 (the nucleus is colored red, FoxP3 is colored yellow);* ***D*** *– the relative content of CD25hiFoxP3+ and CD25loFoxP3+ cells at different tEAT;* ***E*** *– the absolute content of CD25hiFoxP3+ and CD25loFoxP3+ cells at different tEAT;* ***F*** *– the proportion of cells with nuclear localization of FoxP3 among CD25hiFoxP3+ and CD25loFoxP3+ cells at different tEAT;* ***G*** *– the absolute content of CD25hiFoxP3+- and CD25loFoxP3+ cells with nuclear localization of FoxP3 at different tEAT*

TABLE 3

CONTENT OF KEY CYTOKINES AND HIGH-SENSITIVITY C-REACTIVE PROTEIN IN PATIENTS ACCORDING TO THE THICKNESS OF EPICARDIAL ADIPOSE TISSUE

Indices	Patients with tEAT < 5 mm (*n* = 13)	Patients with tEAT > 5.4 mm (*n* = 13)	*p-value*
hsCRP, mg/L	2.1 (1.0; 4.5)	4.9 (3.1; 17.5)	0.044
TGF-β, ng/mL	36.2 (29.1; 38.2)	36.6 (32.4; 42.4)	0.948
IL-10, pg/mL	1.9 (1.7; 3.2)	2.3 (1.8; 2.6)	0.545
IL-1β, pg/mL	0.7 (0.5; 0.9)	1.1 (0.9; 1.4)	0.005

TABLE 4

ESTIMATES OF LOGISTIC REGRESSION MODEL COEFFICIENTS AND THEIR LEVELS OF STATISTICAL SIGNIFICANCE

Indices	Ratio estimation	*p value*
Absolute term	0.228	0.929
hsCRP, mg/L	–0.101	0.283
IL-1β, pg/mL	–2.378	0.101
$CD25^{hi}FoxP3^{+}$ Treg, %	0.461	0.143

According to correlation analysis, EAT thickness in all patients with CHD was positively correlated with BMI (r_s = 0.336; p = 0.037), waist circumference (r_s = 0.379; p = 0.017), serum concentrations of hsCRP (r_s = 0.400; p = 0.019) and IL-1ß (r_s = 0.444; p = 0.008) and negatively – with a relative $CD25^{hi}FoxP3^{+}$ Treg lymphocytes content (r_s = -0.353; p = 0.032).

We constructed a multiple logistic regression model according to which the significant predictors of EAT thickening in patients were hsCRP concentration, IL-1β concentration, and relative content of $CD25^{hi}FoxP3^{+}$ T-regulatory lymphocytes (Table 4).

Although the individual contribution of each of the predictors in the model individually was not statistically significant, the level of statistical significance of the model as a whole was high (p = 0.00403). The predictive performance of the model: accuracy 80.0 %, sensitivity 75.0 %, specificity 84.6 %, AUC = 0.891. The ROC curve of the model is shown in Figure 2. The threshold probability of different degrees of EAT thickening was 0.63.

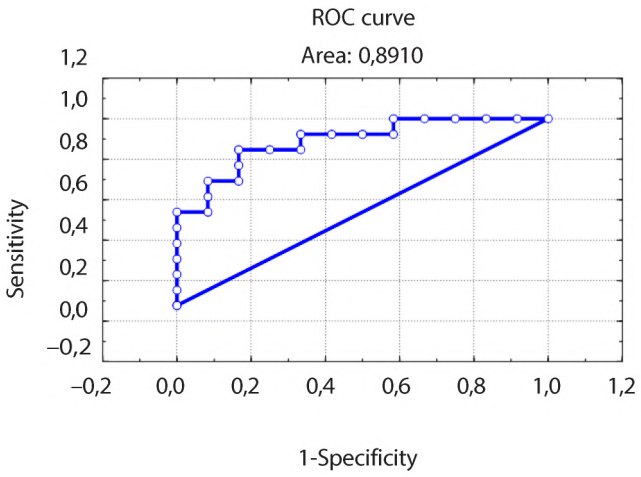

FIG. 2.
ROC curve of the multiple logistic regression model for classifying patients into groups with and without thickening of epicardial adipose tissue thickness greater than 5 mm

DISCUSSION

Although increased EAT thickness is associated with increased local production and accumulation of proinflammatory cytokines [19], the relationship between the properties of this fat depot and $FoxP3^{+}$ T lymphocytes, important regulators of the intensity of the inflammatory response, has long remained unexplored. In this study, we have recently revealed that greater EAT thickness in CHD patients with comparable anthropometric parameters of general

obesity (BMI, waist circumference) and severity of coronary atherosclerosis (Gensini Score index) is associated with a decrease in relative and absolute Treg content in peripheral blood, with a decrease in the absolute number of cells with intranuclear localisation of FoxP3.

The relationship between the development of inflammation and EAT thickening may be ambivalent. EAT thickening has been revealed in many inflammatory diseases including rheumatoid arthritis, psoriasis, multiple sclerosis, and human immunodeficiency virus infection [19]. Meanwhile, the conditions of inflammation provoke an increase in adipogenesis, although initially this process is protective [20].

No evidence for a relationship between FoxP3$^+$ Treg and EAT accumulation is available. However, it is known that Treg lymphocytes are able to interact with adipocytes and other immune cells in subcutaneous and visceral adipose tissue [21]. It has been evidenced that during adaptive transfer, circulating Treg cells in animals have tropism to adipose tissue, where they promote mRNA expression of genes regulating thermogenesis and the transformation of subcutaneous and epididymal white adipose tissue into beige tissue, which is beneficial in maintaining a favorable metabolic profile [21]. This is of particular importance since in healthy individuals EAT adipocytes have a phenotype close to brown adipose tissue, and with accumulation of epicardial fat they change their properties and acquire more and more features of white adipose tissue [19].

Preservation of immunoregulatory function in obesity depends on cell localisation. Specifically, the development of obesity was associated with a decrease in the relative and absolute numbers of Treg lymphocytes in visceral adipose tissue, but Treg in subcutaneous adipose tissue and spleen remained intact [12, 14]. Although we have also demonstrated an inverse relationship between the content of FoxP3$^+$ Treg lymphocytes in peripheral blood and EAT thickness, further study of the cellular composition of the stromal-vascular fraction of the EAT proper is necessary, as the number of Treg cells in it and the subcellular localisation of FoxP3 may differ from the circulatory pool. Therefore, a comparative analysis of Treg content in the circulation and epicardial fat depot with further evaluation of the relationship of these parameters with tEAT is of interest for further studies.

A number of mechanisms can be speculated that may have caused the decrease in Treg lymphocyte content in patients with thicker EAT. Patients with EAT thickness > 5 mm were characterised by increased CL-LDL and CL-LDL/CL-HDL ratio. Considering that statin therapy was more intensive in this group of patients, it is most likely associated with the baseline more unfavourable lipid profile in these patients. A recent study evidenced that increased CL-LDL was associated with pro-inflammatory changes in EAT in patients with chronic CHD [22]. It is known that elevated LDL concentrations are directly capable of causing impairment of the relative content, function, stability and migratory activity of Treg cells, thus contributing to the progression of inflammation in patients with atherosclerosis [23]. Meanwhile, the doses of statins in all patients included in our study were relatively low as a result of the development of side effects or reaching the maximum tolerated dose of the drug. Pleiotropic effects of statins are known, including in relation to Treg lymphocytes, unrelated to their hypolipidaemic action. Thus, it was evidenced that intensification of therapy with atorvastatin (increasing the dosage from 20 to 80 mg/day) led to an increase in the content of Treg lymphocytes in patients with stable CHD, while increasing the dosage of hydrophilic rosuvastatin did not cause such an effect [24]. However, it should be emphasised that culturing Treg cells with high doses of atorvastatin *in vitro* resulted in a decrease in their immunosuppressive properties and reduced expression of key molecules, including the transcription factor FoxP3 [25]. It is urgent to conduct prospective studies aimed at closer examination of hypolipidaemic and pleiotropic effects of statins against content and functional activity of Treg lymphocytes in comparison with the study of EAT in patients with CHD. In addition, patients' adherence to statin therapy was not studied in our study, but could potentially influence the obtained results.

In patients with thicker EAT, we observed a trend towards increased *PCSK9* concentrations. *PCSK9*, whose main pool is produced by hepatocytes, regulates lipid metabolism by promoting lysosomal degradation of the low-density lipoprotein receptor [26]. *PCSK9* has also been evidenced to affect the immune system: suppression of *PCSK9* gene expression resulted in increased production of IL-10, TGF-β and Treg lymphocytes [27]. Notwithstanding the important role of *PCSK9* in regulating Treg lymphocyte homeostasis and lipid metabolism, the change in its concentration in patients depending on EAT thickness was only at the trend level, and there were no correlations with the content of FoxP3$^+$-cell subpopulations at greater EAT thickness. Further studies are required to elucidate the place of *PCSK9* in the development of epicardial obesity and immunoregulatory imbalance in high cardiovascular risk patients.

With EAT thickening, we did not reveal changes in the content of key Treg lymphocyte growth factors, TGF-β and IL-10. However, Treg cell deficiency was associated with an increase in the pro-inflammatory cytokine IL-1β and the systemic inflammatory marker hsCRP, which together with the relative content of CD25^{hi}FoxP3$^+$ Treg lymphocytes determined the increase in EAT thickness. A study performed earlier by another group of scientists also evidenced a relationship between circulatory concentration of hsCRP and EAT thickness in patients with metabolic syndrome [28]. According to the results of the CANTOS study, inhibition of IL-1β by the monoclonal antibody drug canakinumab in patients with hsCRP concentration > 2 mg/l who had undergone myocardial infarction was accompanied by a reduction in the number of adverse cardiovascular events [29]. Considering the relationship between EAT

thickness and pro-inflammatory biomarkers revealed in this pilot study, it is important to consider the properties of the epicardial fat depot when further studying therapeutic approaches aimed at controlling inflammation in patients with atherosclerosis.

The study has several limitations, the main ones being small sample size, lack of prospective follow-up of patients, and lack of data in patients without CHD. To date, in addition, there is currently no uniform approach to assess EAT thickness in patients. Although many groups of researchers assess EAT thickness at the end of systole [30], assessment of EAT thickness at the end of diastole, such as that performed in our study, is also warranted for better correlation with the results obtained by CT and MRI [2, 17]. The borderline EAT thickness value of 5 mm used in our study is not standardised to determine the presence of epicardial obesity, but was proposed by A.G. Bertaso et al. (2013) as a result of analysis of data from the largest EAT studies by echocardiography performed in diastole [18]. The inverse correlation between EAT thickness and Treg lymphocyte content revealed in our work, however, is a reason to conduct larger prospective studies aimed at translating the findings into the clinic, since Treg lymphocytes are characterised by a high therapeutic potential [12].

CONCLUSION

The results of the pilot study reveal a decrease of $CD4^{+}CD25^{hi}FoxP3^{+}$ Treg lymphocytes content in peripheral blood and nuclear translocation of transcription factor FoxP3 in them with increasing thickness of epicardial adipose tissue in patients with CHD. The revealed changes are not associated with anthropometric parameters of obesity and severity of coronary atherosclerosis and are associated with signs of systemic inflammation and more unfavourable serum lipid profile.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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