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Galectin 3 and non-classical monocytes of blood as myocardial remodeling factors at ischemic cardiomyopathy

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ABSTRACT

Aims: To identify an imbalance of cardiac remodeling mediators and monocytes subpopulation in blood, distribution of myocardium macrophages in patients with ischemic cardiomyopathy (ICMP).
Methods: The study engaged 30 patients with ICMP, 26 patients with coronary heart disease (CHD) without ICMP, 15 healthy donors. Concentrations of TGFβ, MMP-9, MCP-1, galectin-3 were measured in plasma of blood from the coronary sinus and peripheral blood in CHD patients, as well as in peripheral blood in healthy donors, by enzyme immunoassay method. The ration of classical, intermediate, non-classical, transitional monocytes in peripheral blood of patients and healthy donors was assessed by flow cytometry (expression CD14, CD16); the content of CD68+ macrophages in myocardium – by immunohistochemistry method.
Results: In both samples of blood, the content of galectin-3 in patients with ICMP was higher than in CHD patients without ICMP and the level of TGFβ was comparable between the groups. At ICMP, the concentration of MMP-9 in sinus blood was higher than that in CHD patients without ICMP in whom an excess of MCP-1 in the general blood flow was determined. The density of distribution of CD68+ cells in the myocardium in patients with ICMP was higher in the perianeurysmal zone than in the right atrium appendage. ICMP was characterized by a deficiency of non-classical monocytes, and CHD without ICMP – by an excess of intermediate cells in peripheral blood.
Conclusion: Myocardium remodeling at ICMP is mediated by not so much TGFβ but intracardiac galectin-3, which determines the subpopulation composition of blood monocytes.

1. Introduction

Ischemic cardiomyopathy (ICMP) is the main cause of mortality from chronic heart failure (CHF), which demonstrates the priority importance of searching for treatment modalities of this disease [1]. At the same time, non-surgical treatment of ICMP is currently similar to that in coronary heart disease (CHD) without ICMP and surgical correction, in addition to bypass surgery, includes techniques for reconstructing of a left ventricular cavity, the expediency of which at ICMP is still being discussed [2,3].

In the pathogenesis of ICMP, the role of cardiomyocyte apoptosis, extracellular matrix (ECM) destruction with the participation of matrix metalloproteinases (MMPs), matrix formation in the presence of transforming growth factor-beta (TGFβ), microvascular dysfunction, synthesis of collagens with different properties, autoimmune and other mechanisms is being discussed [4–7]. Galectin-3 is considered as a marker of myocardium damage, fibrosis and unfavorable course of CHD [8–14]. At the same time, it is known that at different stages of the post-infarct period, the leading positions in the mechanisms of remodeling of the affected myocardium are occupied by macrophages attracted to the focus by chemoattractants, for example, monocyte chemotactic factor

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(MCP-1). In the process of inflammation in ischemic necrosis of the myocardium, M1-macrophages with significant phagocytic and pro-inflammatory functions play a primary role. Subsequently, their population in the affected tissue is replaced by alternatively activated M2-cells which have anti-inflammatory potential and are triggers of fibrosis, neoangiogenesis and tissue reparation [15,16]. It is possible that the accumulation and hyperactivation of M2-macrophages due to imbalance of the monocyte-macrophage system become the cause of inappropriate fibrosis of the myocardium after a heart attack at ICMP.

In response to the damaging effect, monocyte cells, which are capable of replacing all populations of resident cardiac macrophages, are actively recruited into the myocardium [17–19]. At the same time, in clinical practice, the assessment of the phenotype of macrophages is difficult due to their tissue localization. On the other hand, the precursors of macrophages, blood monocytes, are cells available for phenotyping. Taking into account the immunophenotypic and functional heterogeneity of monocytes and the existence of classical, intermediate, non-classical [15,20] and transitional [21–23] cells, changes in their subpopulation in the blood may represent the pathological process in the myocardium which makes it relevant to study monocytes subpopulations at ICMP.

Within the context, the aim of the study was to identify the imbalance of heart remodeling plasma mediators, the peculiarities of the subpopulation composition of blood monocytes, and the distribution of macrophages in the myocardium in patients with CHD suffering from ICMP. The outcomes of this study could make it possible to systematize and add new information about the immunopathogenesis of ICMP and to find potential blood markers of this disease.

2. Material and methods

2.1. Clinical characteristic of CHD patient groups

The study case-control engaged 56 CHD patients with angina of effort of II-IV functional classes and circulatory inefficiency of predominantly II-IV functional classes according to NYHA, who were in hospital of NRI of cardiology of Tomsk NRMC in the period from 10.01.2017 to 30.05.2020. Patients with CHD were divided into 2 groups: 30 individuals suffering from ICMP (left ventricular ejection fraction is $\leq$40%, acute myocardial infarction or revascularization, stenosis of the left main or proximal part of the left descending artery is $\geq$75% or stenosis of two or more epicardial vessels is $\geq$75%) [24] and 26 individuals without ICMP (left ventricular ejection fraction is >40%, acute myocardial infarction or revascularization, coronary stenosis of any site is $\geq$75%). The control group consisted of apparently healthy donors (13 men and 2 women, 86,67% and 13,33%, respectively, average age 57,63 ± 8,12 years) without any cardiovascular diseases. Taking into account the age of the examined donors, we assumed the presence of chronic pathology of other organ system outside the stage of exacerbation with a frequency similar to that in the groups of patients with CHD (Table 1). The sample size was calculated based on the effect size for the conducted Cohen's tests d = 1.0 and the study power of 80%.

CHD patients, with and without ICMP, were comparable in age, sex, body mass index, CHD duration, functional classes of angina of effort and circulation inefficiency, frequency of statin prescription, the value of systolic and diastolic blood pressure (it was achieved with drugs), the state of renal function (on the base of glomerular filtration rate). However, they were significantly differed in eventual diastolic and systolic indices, myocardium mass and left ventricular ejection fraction since a decrease in the last one was lower than 40% and other listed parameters were criteria for the diagnosis of ICMP and separation of patients into the groups (Table 1). The nature of co-morbidity in the patient cohorts was also comparable with the exception of the more frequent occurrence of type 2 diabetes mellitus in CHD patients without ICMP (Table 1). All patients with CHD underwent bypass surgery, patients with ICMP underwent bypass surgery in combination with reconstruction of the left ventricular cavity; all patients had a history of heart attack. At a pre-surgery stage, patients of both study groups received similar drug treatment according to the generally accepted principles of CHD therapy.

The criteria for excluding patients from the study were the following: the age over 70 years, autoimmune diseases, an allergic process in the stage of exacerbation, a tumor process, hypoplastic and megaloblastic anemias, virus hepatitis, syphilis, HIV-infection, pre-surgery therapy with iron-containing drugs, erythropoietin or immunosuppressive therapy, and the presence of acute infectious diseases less than 3 weeks before the surgery, as well as the patient's refusal of study.

The study conformed to the principles outlined in the Declaration of Helsinki and was carried out with the permission of the local ethics committee of Siberian State Medical University (protocols No.5046 (28.11.2016), No.7981 (16.12.2019)). All subjects gave written informed consent for the use of their data.

2.2. Study material

The study material was peripheral venous blood (peripheral blood) and blood from the coronary sinus (sinus blood), stabilized with heparin (25 IU/ml). On the day of surgery, 5 ml of peripheral blood was immediately sampled from the cubital vein in the morning on an empty stomach from both healthy donors and CHD patients of both study groups after an introduction to anesthesia. Peripheral blood was used for blood monocytes immunophenotyping, its plasma was used to assess the concentration of the studied mediators. 5 ml of sinus blood was taken intraoperatively from CHD patients only using transmyocardial puncture after surgical access to the heart but before connecting the heart-lung machine and carrying out the main stage of the surgery. The time between the sampling of peripheral and sinus blood in patients was 10–15 min. The plasma of sinus blood was used to assess the concentration of the studied mediators. After coronary sinus puncture completion, biopsy samples of the right atrial appendage were obtained; biopsies from the area adjacent to the aneurysm and the anterior wall of the left ventricular were obtained at the stage of aneurysmectomy. The total volume of material obtained for the study was no more than 2 mm^3 for each patient. Biopsy samples were used for analyzing the cellular composition of the inflammatory infiltrate and identifying the macrophages.

2.3. Blood monocytes immunophenotyping

The relative content of separate subpopulations of monocytes in venous blood from the cubital vein (peripheral blood) in healthy donors and CHD patients of both groups was determined by flow cytometry. Whole blood was lysed by adding lysing solution "FACS Lysingsolution" (BD Biosciens, USA), then the cells were washed three times by a 20-fold volume of Cell-WASH-solution BD buffer (Becton Dickinson, USA). FITC Mouse Anti-Human CD14 Clone M5E2 and CD16 PE Clone B73.1 monoclonal antibodies were used for monocytes immunophenotyping (according to the instructions of the manufacturer (BD Biosciens, USA)). The cells were being incubated with antibodies for 30 min at 4 °C in the dark. Afterward, the cells were washed twice in 2 ml of Cell-WASH-solution BD (Becton Dickinson, USA) and were being centrifuged for 5 min at 250 g. For the analysis, cells were resuspended in 300 μl of Stain-

Table 1
Indices of the clinical status of patients with coronary heart disease, with and without ICMP.

Index		CHD patients without ICMP	CHD patients with ICMP	P-value
Number of patients:		26	30	–
male		21 (80.8%)	27(90.0%)	0.547
female		5 (19.2%)	3(10.0%)	0.547
Age, years		64.0 [59.5; 67.5]	61.0 [56.0; 64.0]	0.147
CHD duration, years		5.00 [2.00; 9.25]	3.00 [1.00; 7.00]	0.171
Body mass index, kg/m^2		29.00 [26.00; 33.00]	28.00 [26.75; 31.25]	0.525
Functional class of angina of effort	II	5 (19.2%)	7 (23.3%)	0.963
	III	18 (69.2%)	20 (66.7%)	0.935
	IV	3 (11.5%)	3 (10.0%)	0.805
Functional class of circulation in efficiency (according to NYHA)	I	3 (11.5%)	2 (6.7%)	0.867
	II	10 (38.5%)	19 (63.3%)	0.112
	III	13 (50.0%)	9 (30.0)	0.210
LV ejection fraction, %		59.5 [51.00; 64.00]	30.00 [22.00; 36.00]	**<0.001**
Eventual systolic index of LV, ml/m^2		30.47 [25.54; 34.33]	14.58 [13.00; 15.83]	**<0.001**
Eventual diastolic index of LV, ml/m^2		18.07 [14.60; 27.05]	80.93 [72.16; 101.2]	**<0.001**
LV myocardium mass, g		187.5 [142.8; 215.0]	233.5 [222.3; 265.3]	**0.001**
Statin therapy		22 (84.6%)	25 (83.3%)	0.815
Hypertensive disease III degree		21 (80.8%)	21 (70.0%)	0.536
Systolic blood pressure, mm Hg		128.0 [119.0; 134.0]	125.5 [121.1; 134.5]	0.872
Diastolic blood pressure, mm Hg		79.0 [72.0; 86.0]	75.0 [72.0; 80.5]	0.662
Type 2 diabetes mellitus		9 (34.6%)	2 (6.7%)	**0.022**
Gastric and/or duodenal ulcer		6 (23.1%)	3 (10.0%)	0.335
Diseases of liver and biliary tract		4 (15.4%)	2 (6.7%)	0.536
Chronic kidney disease		6 (23.1%)	10 (33.3%)	0.582
Glomerular filtration rate, ml/min/m^2		70.96 [55,95; 89.14]	68.75 [53.92; 88.01]	0.836
Pulmonary diseases		3 (11.5%)	5 (16.7%)	0.870

Notes. Results are presented as Me [Pe25; Pe75] or n (%). The accepted level of statistical significance was P < 0,05. LV – left ventricular.

Buffer (Becton Dickinson, USA). The fluorescence intensity was measured by Accuri C6 flow cytometer (BD Biosciens, USA). The analysis of obtained data was carried out using software application BD Cell Quest for Mac OS® X (BD Biosciens, CIIIA). Monocytes were divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}$-$CD16^{+}$), non-classical ($CD14^{+}CD16^{++}$), and transitional ($CD14^{+}$-$CD16^{-}$) according to the expression of CD14 and CD16 molecules on the surface of cells, taking all cells positive for CD14 as 100%. The relative content of individual immunophenotypes of blood monocytes was expressed as a percentage (%).

2.4. Measurement of mediator concentrations in blood

Peripheral blood plasma of CHD patients of both study groups and healthy donors, as well as blood plasma from the coronary sinus in CHD patients of both groups, was aliquoted and was stored at −80 °C for no more than 12 months. TGFβ, MMP-9, MCP-1 and galectin-3 concentrations in blood plasma were determined by enzyme immunoassay. For this purpose, the following commercial kits for enzyme immunoassay were used according to the instructions of the manufacturers: "Human TGF beta 1 Platinum ELISA" (eBioscience, Austria), "Human MMP9 ELISA" (ThermoFisher Scientific, USA), "Human Galectin-3 ELISA" (Bender MedSystems, Austria), and "MCP-1-ELISA-Best" (Vector-Best, Russia). The results were expressed as pg/ml or ng/ml.

2.5. Assessment of the macrophage content in myocardial biopsies

Myocardial biopsy samples were being fixed in 10% neutral formalin solution for 24 h, then dehydrated in IsoPrep solution (BioVitrum, Russia). Biopsy samples were paraffinated in HISTOMIX® medium (BioVitrum, Russia). 4–5 μm sections were obtained using Thermo Scientific Microm STS Section-Transfer-System microtome (Thermo Scientific, USA) then they were being dewaxed in xylene and 96% alcohol solutions three times for 5 min and were being processed in Antigen Retrieval Buffer 4 Tris-EDTA Buffer (BioVitrum, Russia) buffer for antigen unmasking for 20 min at 97 °C. Biopsy samples were being washed in 0,001 M phosphate-buffered saline (PBS) with pH = 7,4 and were being treated with Peroxide Block (Cell Marque, USA) and Protein Block (Cell Marque, USA) reagents to block endogenous peroxidases and nonspecific binding respectively for 10 min, then they were washed by 0,01 M PBS with pH = 7,4.

After blocking, the sections were loaded with primary antibodies to the CD68 macrophage marker (Cell Marque, USA) and were being incubated for 30 min at room temperature. After washing with 0,001 M PBS with pH = 7,4, the sections were loaded with secondary antibodies labeled with biotin (Cell Marque, USA) and were being incubated for 10 min at room temperature, and the washing was repeated. Afterwards, the sections were loaded with HRP conjugate (Cell Marque, USA), washed and treated with DAB chromogen (Cell Marque, USA), and counterstained with hematoxylin-eosin. The stained preparations were dehydrated, clarified, immersed in BioMount medium (BioOptica, Italy), and microscoped with an Axioskop 40 microscope (CarlZeiss, Germany). Photomicrography of histological preparations was obtained using Canon G10 camera (Japan). The counting of macrophages in the myocardium was performed by the method of point counting of 5–7 random fields of view at a magnification of ×400 with a help of AxioVision graphic image processing program (CarlZeiss ImageJ, Germany), which corresponds to 1 mm^2 of tissue. The result was expressed by the number of $CD68^{+}$ cells detected per 1 mm^2.

2.6. Statistical data analysis

Statistical data analysis was performed using Statistica 10.0 program. When statistically describing the quantitative criteria, the median, 25th and 75th percentiles were calculated; when statistically describing the qualitative criteria, the sample rate was calculated. For the purpose of comparative analysis of sample data, Mann-Whitney (for independent samples) and Wilcoxon (for dependent samples) tests were used. To compare the frequencies of occurrence of the criteria in the group, we used the Chi-square

test with Yates correction for continuity. To assess the linear relationships between studied criteria, the Spearman rank correlation coefficient was calculated; to assess non-linear relationships between studied criteria, the correlation ratio was calculated. The results of statistical analysis were considered significant at $p < 0,05$.

3. Research results

3.1. Content of the heart remodeling mediators in peripheral and sinus blood in CHD patients, with and without ICMP

In peripheral blood, the content of galectin-3, MMP-9, and TGFβ in CHD patients corresponded to their concentrations in healthy donors. The concentration of MCP-1 in CHD patients without ICMP was higher than normal; in CHD patients with ICMP, it increased insignificantly (Table 2). At the same time, the content of MCP-1, MMP-9, and TGFβ in peripheral blood in both groups of CHD patients was comparable, while galectin-3 concentration at ICMP was 27,1% higher than in CHD patients without ICMP (Table 2).

The concentration of galectin-3 in sinus blood exceeded that in peripheral blood in both ICMP patients and CHD patients without ICMP (by 82,9% and 45,7%, respectively, Table 2), the level of TGFβ in sinus blood exceeded that in peripheral blood only in patients with ICMP (by 16,9%, Table 2). The content of MMP-9 and MCP-1 in sinus blood in patients of both groups varied at the level of peripheral blood values, but there was a tendency to a decrease in the concentration of MMP-9 in cardiac circulation in CHD patients without ICMP (Table 2). The analysis of intergroup differences in CHD patients showed that patients with ICMP had significantly higher concentrations of galectin-3 and MMP-9 in sinus blood than in CHD patients without ICMP (1,60 and 2,93 times, respectively, Table 2). The concentration of TGFβ and MCP-1 in sinus blood in CHD patients in both groups was comparable (Table 2).

3.2. Subpopulation composition of peripheral blood monocytes in CHD patients, with and without ICMP

The subpopulation composition of blood monocytes in CHD patients without ICMP was characterized by a deficiency of classical $CD14^{++}CD16^{-}$ and transitional $CD14^{+}CD16^{-}$ cells in a combination with an excess of intermediate $CD14^{++}CD16^{+}$ forms and the content of $CD14^{+}CD16^{++}$ non-classical monocytes, corresponding to the norm (Table 2, Fig. 1A, 1B). On the other hand, at ICMP, there was revealed a deficiency in non-classical $CD14^{+}CD16^{++}$ cells against the normal number of other forms of blood monocyte (Table 2, Fig. 1A, 1C).

With relatively similar median values, the number of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes in the blood of CHD patients with ICMP corresponded to the norm, and in CHD patients without ICMP it was different from the norm (Table 2, Fig. 1B, 1C). It is important to note that the content of non-classical $CD14^{+}CD16^{++}$ monocytes was the only one differential indicator of ICMP in CHD patients and it was lower in patients with ICMP than in patients without ICMP (Table 2, Fig. 1B, 1C).

3.3. Density of macrophages distribution in different myocardium areas in CHD patients with ICMP

$CD68^{+}$ cells, macrophages, were found in the myocardium of a patient with ICMP (Fig. 2A, 2B). The highest number of them (11,00 [6,00; 18,00] cells/mm^2) was observed in the area of aneurysm where it was higher than in the right atrial appendage (2,00 [1,00; 4,00] cells/mm^2, $p < 0,001$). The number of macrophages in the anterior wall of the left ventricular was 8,00 [6,00; 10,00] cells/mm^2 ($p = 0,106$ in comparison with the number of $CD68^{+}$ cells in the area of aneurysm and $p = 0,002$ in the right atrial appendage).

Table 2
The content of myocardial remodeling mediators and various subpopulations of blood monocytes in CHD patients, with and without ischemic cardiomyopathy, Me [Pe 25; Pe 75].

Blood parameters	Healthy donors	CHD patients without ICMP	CHD patients with ICMP
Blood from the cubital vein (peripheral blood)			
Classical monocytes, %	64.05 [59.30; 67.43]	46.35 [30.43; 56.42] **Pc = 0.017**	49.59 [42.33; 65.90] Pc = 0.089 P_2 = 0.274
Intermediate monocytes, %	17.47 [15.54; 18.27]	39.62 [27.42; 58.70] **Pc = 0.008**	39.53 [16.37; 49.56] Pc = 0.063 P_2 = 0.474
Non-classical monocytes, %	10.71 [9.52; 14.59]	8.11 [7.26; 13.21] Pc = 0.260	5.315 [3.920; 7.088] **Pc < 0.001** **P_2 = 0.004**
Transitional monocytes, %	6.84 [5.16; 7.00]	3.26 [2,64; 3.60] **Pc = 0.008**	2.73 [2.05; 5.83] Pc = 0.107 P_2 = 0.664
MMP-9, ng/ml	17.00 [10.85; 19.75]	12.00 [9,35; 13.40] Pc = 0.121	13.65 [7.05; 19.08] Pc = 0.457 P_2 = 0.307
Galectin-3, ng/ml	7.64 [6.28; 8.50]	6.45 [4.65; 7.41] Pc = 0.241	8.20 [7.28; 9.80] Pc = 0.230 **P_2 = 0.009**
TGFβ, pg/ml	68.00 [66.50; 70.50]	71.50 [61.00; 86.25] Pc = 0.537	65.00 [60.75; 75.00] Pc = 0.602 P_2 = 0.365
MCP-1, pg/ml	175.0 [145.0; 207.5]	225.0 [182.0; 280.0] **Pc = 0.027**	205.0 [170.0; 260.0] Pc = 0.104 P_2 = 0.660
Blood from the coronary sinus (sinus blood)			
MMP-9, ng/ml	–	4.38 [3.88; 10.25] P_3 = 0.108	12.80 [4.98; 20.60] **P_2 = 0.039** P_3 = 0.279
Galectin-3, ng/ml	–	9.40 [8.00; 11.80] **P_3 = 0.006**	15.00 [10.90; 46.20] **P_2 = 0.010** **P_3 = 0.002**
TGFβ, ng/ml	–	77.00 [55.00; 98.00] P_3 = 0.593	76.00 [66.50; 95.00] P_2 = 0.701 **P_3 = 0.028**
MCP-1, ng/ml	–	210.0 [148.8; 271.3] P_3 = 0.147	197.5 [147.5; 267.5] P_2 = 0.848 P_3 = 0.532

Notes. Results are presented as Me [Pe25; Pe75]. The level of statistical significance of differences in comparison: Pc - with control (healthy donors), P_2 - with patients with coronary heart disease, P_3 - with the concentration of the substance in the peripheral blood in patients of the corresponding study group (according to the paired Wilcoxon test).

3.4. Relationship between heart remodeling mediators and subpopulation content of blood monocytes in CHD patients, with and without ICMP

It was revealed that in patients with ICMP the concentration of galectin-3 in peripheral blood was negatively correlated with a proportion of intermediate $CD14^{++}CD16^{+}$ cells ($r_s = -0,59$, $p < 0,05$) and positively correlated with a proportion of classical

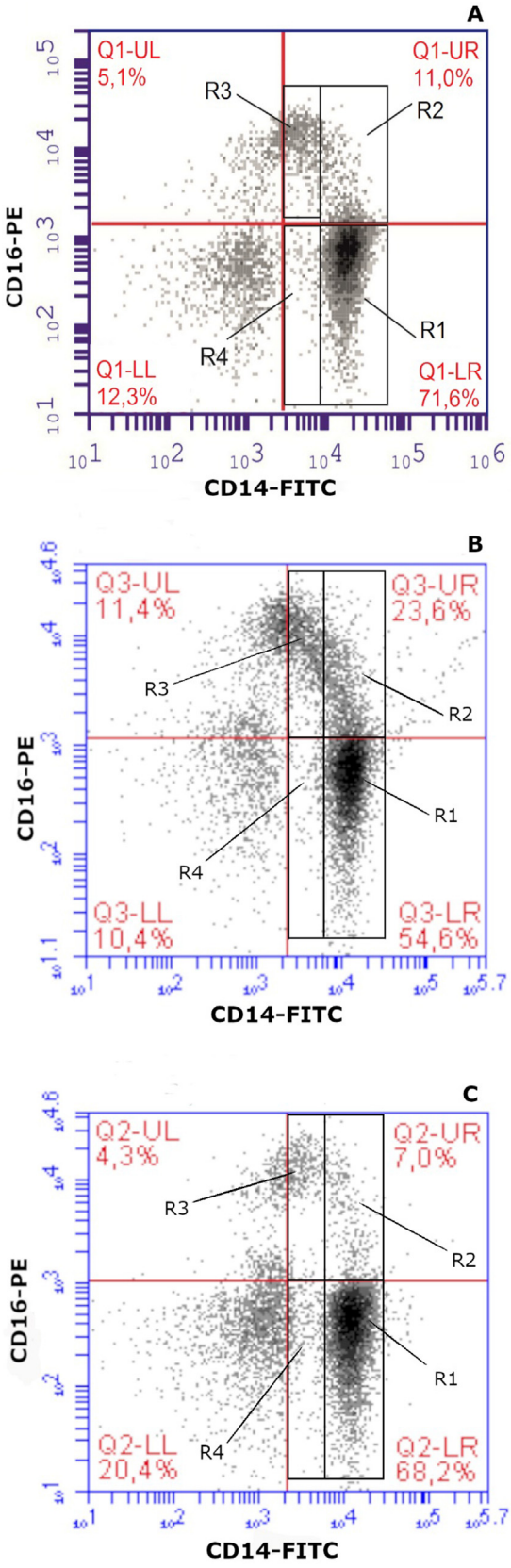

Fig. 1. The subpopulation composition of peripheral blood monocytes in (**A**) healthy donors and CHD patients: (**B**) without ICMP and (**C**) with ICMP. Distribution of monocytes into subpopulations in patient with ICMP: R1 – classical ($CD14^{++}$-$CD16^{-}$) monocytes, R2 – intermediate ($CD14^{++}CD16^{+}$) monocytes, R3 – non-classical ($CD14^{+}CD16^{++}$) monocytes, R4 – transitional ($CD14^{+}CD16^{-}$) monocytes.

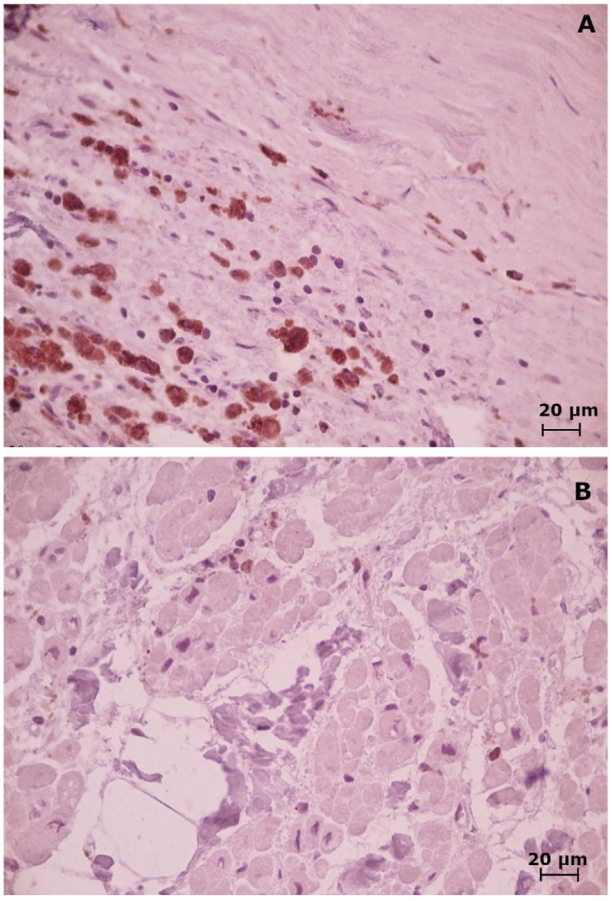

Fig. 2. The content of macrophages in biopsy samples of myocardium from the patients with ICMP. (**A**) The myocardium of the left ventricular aneurysm in a patient with ICMP. (**B**) The myocardium of the right atrium appendage in a patient with ICMP. Note. $CD68^{+}$ cells (brown stain), hematoxylin counterstain, ×400.

$CD14^{++}CD16^{-}$ monocytes (r_s = 0,67, p < 0,05). The number of the latter was directly proportional to the number of transitional $CD14^{+}CD16^{-}$ monocytes (r_s = 0,72, p < 0,05) and inversely proportional to the content of intermediate $CD14^{++}CD16^{+}$ forms (r_s = −0,94, p < 0,01), the proportion of which was positively correlated with the number of non-classical $CD14^{+}CD16^{++}$ cells (r_s = 0,61, p < 0,05). In CHD patients without ICMP, the parameters of the monocyte subpopulation composition had no relationships with the concentration of heart remodeling mediators in both blood samples. At the same time, a close negative correlation between the number of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes in peripheral blood was recorded (r_s = −0,96, p < 0,01).

It was revealed, that the concentration of TGFβ in sinus blood of patients without ICMP was positively correlated with the level of MCP-1 in the same biomaterials (r_s = 0,67, p < 0,05), and in patients with ICMP it was negatively correlated to the galectin-3 concentration in peripheral blood (r_s = −0,61, p < 0,05). A direct relationship between the content of MCP-1 in both blood samples in CHD patients without ICMP was revealed (r_s = 0,64, p < 0,05). For the rest of the mediators, a linear relationship between blood samples in any group of patients was not established. At the same time, a graphical representation of similar data for galectin-3 in pooled sample of the CHD patients, with and without ICMP, revealed non-linear relationship between the parameters of sinus and peripheral blood which was characterized with a high value of the correlation ratio (η=0,94, p < 0,01; Fig. 3).

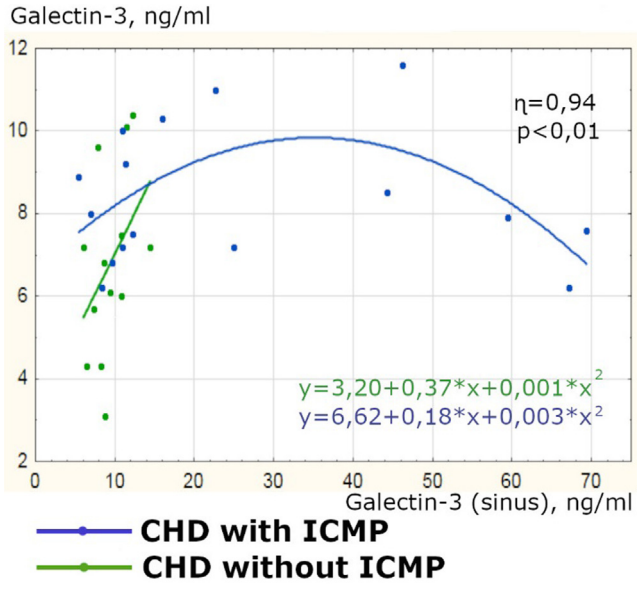

Fig. 3. The relationship between the concentration of heart remodeling mediators in peripheral and sinus blood in CHD patients, with and without ICMP. In pooled sample of CHD patients, with and without ICMP, a non-linear relationship between concentrations of galectin-3 in peripheral and sinus blood was demonstrated. Spearman's rank correlation coefficient (r) and correlation ratio (η) calculation were used.

4. Discussion

This study demonstrates the presence of specific features of the spectrum of plasma mediators, involved in the regulation of the heart remodeling processes, and subpopulation composition of blood monocytes at ICMP, in contrast to CHD without ICMP. The existence of a large number of pro-fibrotic factors, which include galectin-3, TGFβ и MMP-9 [8,9,12], justifies the need to search for the most significant of them for determining the therapeutic target at ICMP.

4.1. Factors of formation and destruction of the connecting tissue are synthesized in the heart of ICMP patients

The results of the research showed that, among the investigated pro-fibrotic factors, galectin-3 plays a leading role in the pathogenesis of ICMP, and TGFβ and MMP-9 have complex effects. Thus, the galectin-3 content in both blood samples in CHD patients with ICMP was higher than in CHD patients without ICMP; moreover, it was higher in sinus than in peripheral blood (Table 2). In patients with ICMP, the TGFβ concentration in sinus was higher than in peripheral blood, and MMP-9 content in sinus blood was higher than in CHD patients without ICMP (Table 2).

MMP-9 is a gelatinase B that is able to destruct several types of collagens, especially type IV collagen and components of the basal membrane of blood vessels [25]. In this regard, the high concentration of MMP-9 in sinus blood in patients with ICMP (compared to the CHD patients without ICMP, Table 2) represents the intensification of the ECM destruction processes in the heart at ICMP. In CHD patients without ICMP, the concentration of MMP-9 in cardiac circulation occurred lower than in peripheral blood, which can be explained by the entry of this enzyme into the bloodstream, not from the myocardium. At the same time, in the myocardium of CHD patients without ICMP, MMP-9 could be bounded by tissue inhibitors of matrix metalloproteinase (TIMP). In particular, TIMP-1 forms a complex with an active form of MMP-9 and does not allow this enzyme to exert its destructive effect [25]. Therefore, the higher level of MMP-9 in the cardiac circulation in patients with ICMP (than in CHD patients without ICMP) may be both a consequence of TIMP insufficiency and increased MMP-9 secretion in the myocardium. For example, E.I. Myasoedova et al. [26] demonstrated an increased MMP-1 content and a TIMP-1 deficiency in peripheral blood in patients with ICMP; T. Morishita et al. [27] described an increase in the MMP-9/TIMP-1 ratio in blood at CHD progression.

Along with the processes of ECM destruction in the heart in patients with ICMP, fibrosis formation is evidently enhanced. This is confirmed by the increased content of TGFβ in sinus blood in relation to the peripheral blood in patients with ICMP (which was not observed in CHD patients without ICMP) and a high concentration of galectin-3 in both blood samples at ICMP compared to CHD patients without ICMP (Table 2). TGFβ and galectin-3 induce regeneration and fibrosis but have a number of features [9,10,28].

TGFβ causes fibrosis by stimulating the differentiation of fibroblasts into myofibroblasts and enhancing their synthesis of ECM components [11,28]. The fact that the content of TGFβ in sinus blood in patients with ICMP is higher than in peripheral blood (Table 2) indicates its intramyocardial origin. Both TGFβ enhanced production (for example, by macrophages) and activation of its inactive form with the help of MMP-9 may be a mechanism of its accumulation in the heart [11,28]. However, the absence of differences in the concentration of TGFβ between the groups of CHD patients in both blood samples allows us to conclude that if this cytokine promotes myocardial fibrosis at ICMP, it is not to a greater extent than at CHD without ICMP.

Galectin-3, obviously, is a key inducer of fibrosis at ICMP. In patients with ICMP, its concentration in the sinus was higher than in peripheral blood. Also, its content in both blood samples was higher in ICMP patients than in CHD patients without ICMP (Table 2) with an equivalent state of renal function (on the base of glomerular filtration rate, Table 1). Therefore, the higher content of galectin-3 in both blood samples from patients with ICMP is due to its overproduction, rather than decreased excretion. It is common knowledge that galectin-3 is excreted in the urine (against the background of renal failure, its concentration in blood plasma increases) [29–32], but at the same time, it could also be formed in the heart in cardiomyopathy and heart failure [33]. Acting as a fibrosis trigger, galectin-3 is extensively produced by macrophages and stimulates fibroblasts' proliferation and differentiation into myofibroblasts. The secretion of ECM components in heart by the latter leads to an increase in types I and III collagens ratio, myocardium remodeling and its dysfunction [8,10]. Thus, in CHD patients without ICMP a moderate increase in the concentration of galectin-3 in the heart (by 45,7% relative to that value in peripheral blood, Table 2) was not accompanied by an increase in TGFβ concentration (Table 2). However, in patients with ICMP, an excess in galectin-3 in the heart (by 82,9% relative to its content in peripheral blood, Table 2) was combined with an increase in intramyocardial TGFβ concentration, which reflects the greater severity of the pathological process at ICMP.

An absence of intergroup differences in the content of TGFβ in CHD patients and the presence of those in the concentration of galectin-3 in both blood samples (Table 2) demonstrates a greater contribution of the latter to the pathogenesis of ICMP. An excess of TGFβ is dangerous since it causes collagen deposition, increased rigidity of the myocardium and diastolic dysfunction. However, TGFβ induces myocardial hypertrophy, which, under conditions of ICMP, could have a protective effect [28]. The activating effect of galectin-3 on myocardial hypertrophy has not been described. Moreover, it has been shown that a knockout of its gene in mice in experimental myocardial hypertrophy does not prevent hypertrophy [34]. Galectin-3 also has a damaging effect, since it mediates an increase in the reactive oxygen species production [13].

It is interesting, that, in patients with ICMP, an inverse correlation between the concentration of galectin-3 in peripheral blood and the content of TGFβ in sinus blood (see section 3.4, Fig. 4B), which was absent in CHD patients without ICMP, was revealed. Despite the ability of TGFβ and galectin-3 to activate each other secretion, the latter can «keep» TGFβ in the glycocalyx due to the building of a lattice from the galectin-3 molecules by their self-association [10,11]. Our data corresponds with the data of N. Wang et. al. [14], who also demonstrated the negative correlation between the concentration of TGFβ and galectin-3 in blood in patients with CHF [14].

The graphical presentation of the galectin-3 concentration in the pooled sample of CHD patients, with and without ICMP, revealed a parabolic relationship (Fig. 3) confirmed by valid and statistically significant value of correlation ratio (see section 3.4). The ascending part of the graph describes the desired direct relationship: the more galectin-3 is in the cardiac bloodstream, the more it is in peripheral blood. The descending part of the curve contradicts this relationship; however, this can be interpreted by dilution of the blood due to an increase in its volume upon activation of the renin-angiotensin-aldosterone system in CHD patients. Galectin-3 is a marker of CHD and its hyperproduction in the myocardium reflects a progressive loss of cardiac pumping function [8,10,11,13]; but its concentration in peripheral blood, on the contrary, decreases in some patients (Fig. 3). This important conclusion in combination with the normal content of galectin-3 in peripheral blood in CHD patients of both groups demonstrates the insufficient information content of the galectin-3 determination in the general circulation. Determination of the galectin-3 concentration in sinus blood could serve as an early marker of the progression of the pathological process.

4.2. In the myocardium of patients with ICMP, macrophages are distributed in an uneven manner, the accumulation of which is not associated with MCP-1

The histological analysis of biopsies obtained from ICMP patients right atrial appendage, the anterior wall of the left ventricle, and the area adjacent to the left ventricular aneurysm, revealed the presence of $CD68^+$ cells, namely macrophages, in the fragments of the myocardium of all the three localizations and, to the greatest extent, in the perianeurysmal area (see section 3.3, Fig. 2A, 2B). Therefore, at ICMP, macrophages in the heart play a predominantly pro-fibrotic role and this function is inherent with several subtypes of M2 cells (M2a, M2b, M2c) [15,16].

Also, macrophages in the heart of ICMP patients are also able to perform a protective function, eliminating ischemically damaged cardiomyocytes or cells that underwent apoptosis [5]. Therefore, it could be assumed that the accumulation of macrophages in the myocardium at ICMP is caused not only by apoptosis of cardiomyocytes, which was induced by cytotoxic lymphocytes but also by necrosis. Necrosis can be explained by the tendency to accumulation of macrophages in the area of the myocardium, not adjacent to the aneurysm, but most often subjected to ischemia (the anterior wall of the left ventricle). It was discovered that repeated episodes of short-term myocardial ischemia, which does not cause the formation of local necrosis, in several weeks, promote the migration of monocyte into the heart; firstly, they transformed into pro-inflammatory M1 cells (reaction to injury), and then into anti-inflammatory M2 cells [35].

Taking the above into consideration, $CD68^+$ cells found in the myocardium of ICMP patients are most likely to be M2 macrophages, which, as it is well-known, produce the main pro-fibrotic factors TGFβ, MMP-9, galectin-3 etc. [10,15,16]. The content of precisely these molecules was increased in the cardiac blood flow in

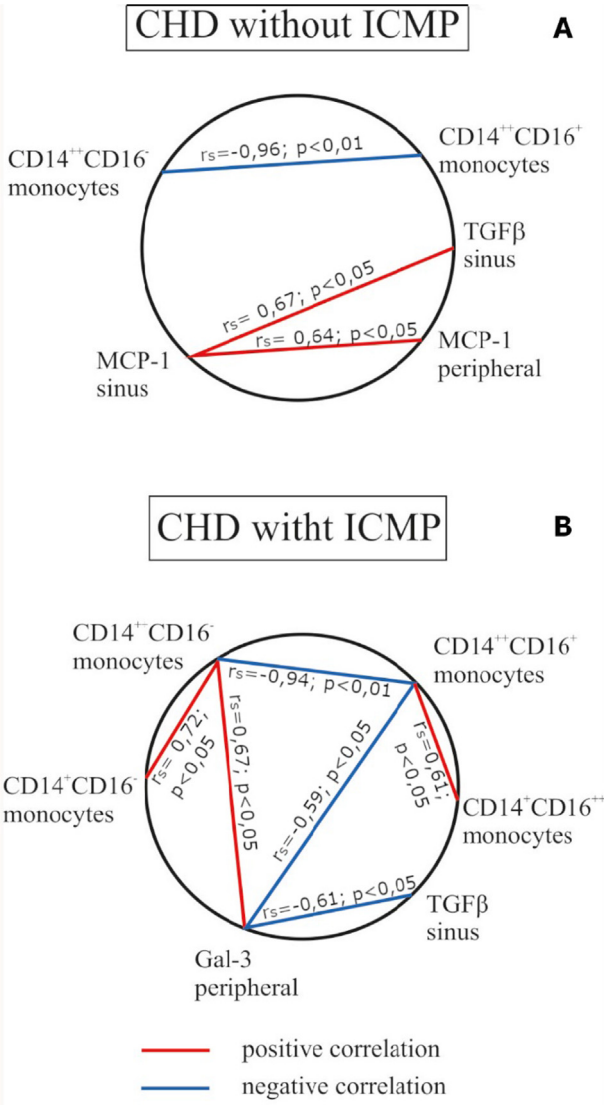

Fig. 4. An integral map of relationships between the concentration of myocardial remodeling mediators in peripheral and sinus blood and the subpopulation composition of peripheral blood monocytes in CHD patients: (**A**) without ICMP and (**B**) with ICMP. Note. r_s – Spearman's rank correlation coefficient, p – the level of statistical significance.

ICMP patients (Table 2). The accumulation of the M2-cells could occur under the influence of various substances.

The key monocyte chemoattractant is MCP-1 which is an important factor of the pathogenesis of CHD, since it recruits monocytes to atheroma, the focus of ischemia or necrosis [35,36]. In support of this, in our study, we found an increased peripheral blood level of MCP-1 in CHD patients without ICMP and only a tendency to increase in it in patients with ICMP (compared to the group of healthy donors) (Table 2). Probably, it is due to the more frequent occurrence of type 2 diabetes mellitus in CHD patients without ICMP (Table 1), in which the concentration of MCP-1 and the expression of its CCR2 receptors on monocytes increase [36]. When analyzing the structure of comorbidity in patients with CHD attention is drawn to the low incidence of type 2 diabetes mellitus at ICMP (Table 1). There is evidence that galectin-3 can bind to glycosylated proteins, promoting their utilization and exerting a protective effect on insulin resistance [10].

An excess of MCP-1 in peripheral blood at CHD without ICMP could have not only a pathological but an adaptive significance

since this cytokine is involved in neoangiogenesis [36]. In CHD patients without ICMP, a positive correlation between the concentration of MCP-1 in peripheral and sinus blood (see section 3.4, Fig. 4A) in the absence of significant differences in both blood samples (Table 2) reflects the ubiquity of the process of hyperproduction of MCP-1 in the body at atherosclerosis.

It should be recognized that physiological concentrations of MCP-1 in peripheral blood of patients with ICMP and the absence of differences in its content between samples of peripheral and sinus blood (Table 2) demonstrate an insignificant role of MCP-1 in macrophages accumulation in the myocardium at ICMP. Another activator of monocytes, the factor of the movement and retention of macrophages in tissues is galectin-3, the chemoattractant effect of which exceeds that for MCP-1. Since the level of galectin-3 in both blood samples of ICMP patients was higher than in CHD patients without ICMP, assuming that its concentration in the heart of CHD patients of both groups was higher than in peripheral blood (Table 2); then it is probably galectin-3, not MCP-1, which ensures macrophage accumulation in the myocardium and monocyte recruitment from the blood at ICMP.

4.3. ICMP is characterized by a subpopulation composition of blood monocytes, unusual for atherogenesis, with a deficiency of non-classical forms

The accumulation of intermediate monocytes in blood at CHD without ICMP revealed in this study (Table 2) is consistent with the literature data on their pro-inflammatory involvement in atherogenesis [15,20]. The immune function of interaction with T-lymphocytes is carried out by intermediate monocytes due to the higher expression of HLA-DR molecules [10,15]. It has been shown that an increase in the pool of intermediate monocytes correlates with the risk of cardiovascular diseases, compromised recovery after a heart attack, microvascular dysfunction and deterioration of clinical outcomes [19]. In addition, the number of non-classical monocytes was not associated with the risk of acute vascular events in cardiovascular diseases [20,37].

The number of classical and transitional blood monocytes in CHD patients without ICMP decreased (Table 2), which could be explained by the accelerated differentiation of these cells into intermediate monocytes under conditions of chronic inflammatory process in the vessel wall. The pool of classical monocytes could also be reduced due to their intensive migration into tissues in the setting of the excess of MCP-1 in CHD patients without ICMP (Table 2), since classical cells are characterized by the maximum expression of their CCR2 receptors [15,20]. It is supposed that the transformation of CD phenotype of monocyte subpopulations occurs sequentially: classical cells mature into intermediate ones and then into non-classical monocytes [19]. In our study, this property of classical cells is confirmed by the negative relationship between their number and the number of intermediate monocytes in patients of both study groups (see section 3.4, Fig. 4A, 4B).

Transitional monocytes are probably bone marrow precursors of classical and intermediate cells, since the number of these forms in medullary tissue is negatively correlated with a number of transitional monocytes [23], and the content of the latter is higher in the bone marrow than in blood [22].

It is noteworthy that in patients with ICMP, the deficiency of non-classical monocytes was the only one deviation from the norm of the subpopulation composition of blood monocytes. These cells provide a patrolling of the endothelium and are able to eliminate oxidized lipids, dead cells and pathogens from the surface of the vascular wall [15,19,38]. Consequently, in patients with ICMP, a deficiency of monocytes, which perform a protective function regarding the endothelium, is formed. These predispose to a lipid fixation in the wall of small coronary arteries, which usually is not affected by atherosclerosis and ischemia of the myocardium, no longer acquires a focal (as in CHD), but diffuse character, contributing to the microvascular dysfunction and the development of ICMP.

Despite atherogenesis, the number of intermediate blood monocytes in patients with ICMP remain normal (Table 2, Fig. 1C). This fact could be a consequence of either a reduced differentiation of intermediate cells from classical ones, or an accelerated migration of intermediate monocytes into tissues, or a combination of these processes. In patients with ICMP, the first mechanism is more likely to predominate in peripheral blood, and the second one is realized in the heart, where a high production of galectin-3 (Table 2), which has chemoattractant properties [8,10,11,13], was determined. The case for this hypothesis is the negative relationship between the number of intermediate monocytes and the level of galectin-3 in the blood (see section 3.4, Fig. 4B). It is also not implausible that this protein restrains macrophages in the heart, mediating the deficiency of non-classical monocytes in blood at ICMP (Table 2, Fig. 1C). It is believed, that non-classical monocytes are a recirculating population of cells of monocyte-macrophage system (since their content is greater in the lymph than in blood) [39]. The role of galectin-3 performing both as an attracting and retaining factors for monocytes could explain the established positive relationship between their types in blood: the fewer intermediate monocytes (due to increased migration into tissues), the fewer non-classical cells in the blood (due to the accumulation of macrophages in tissues) (see section 3.4).

4.4. Formation of pro-fibrotic factors in the heart mediates changes of subpopulation content of blood monocytes involving them into the vicious circle of ICMP progression

The results of this study significantly supplement the literature data on ICMP pathogenesis, demonstrating the priority role of galectin-3 in cardiac remodeling and modulation of the subpopulation composition of blood monocytes which are also involved in the development of this disease (Fig. 5). Thus, in CHD, exceedingly increased production of galectin-3 in the myocardium mediates its greater content in peripheral blood than at CHD without ICMP. Under the conditions of atherogenesis, in which intermediate monocytes actively differentiate from classical forms, an excess of galectin-3 maintains the number of both subpopulations in blood at the normal level, since it can inhibit this process and activate the migration of intermediate monocytes into the myocardium. At ICMP, these cells are attracted to the heart by intracardiac hyperproduction of galectin-3 rather than by the chemoattractant MCP-1 specific for these cells (Fig. 5).

In the myocardium of ICMP patients, monocytes are differentiated presumably into M2 macrophages, since TGFβ, galectin-3, MMP-9, which are characteristic of the secretory profile of M2 cells, are formed in the heart of these patients. A high concentration of MMP-9 in the myocardium in patients with ICMP contributes to the destruction of ECM and the loss of elastic properties of the connective tissue, formed as a result of post-infarction fibrosis. In addition, after its completion in patients with ICMP, fibrosis continues and is mediated by both TGFβ (but no more than in CHD patients without ICMP) and galectin-3, which plays a key role in ICMP being a powerful pro-fibrotic factor. The source of its accumulation in tissues is M2 macrophages and any damaged cells including ischemic cardiomyocytes. Moreover, an excess of galectin-3 in the myocardium, as a chemoattractant substation, could retain macrophages in the heart ensuring their accumulation (especially in the aneurysm area) and inhibiting their recirculation, which causes a deficiency of non-classical monocytes in the blood. Due to protective role of these cells for endothelium,

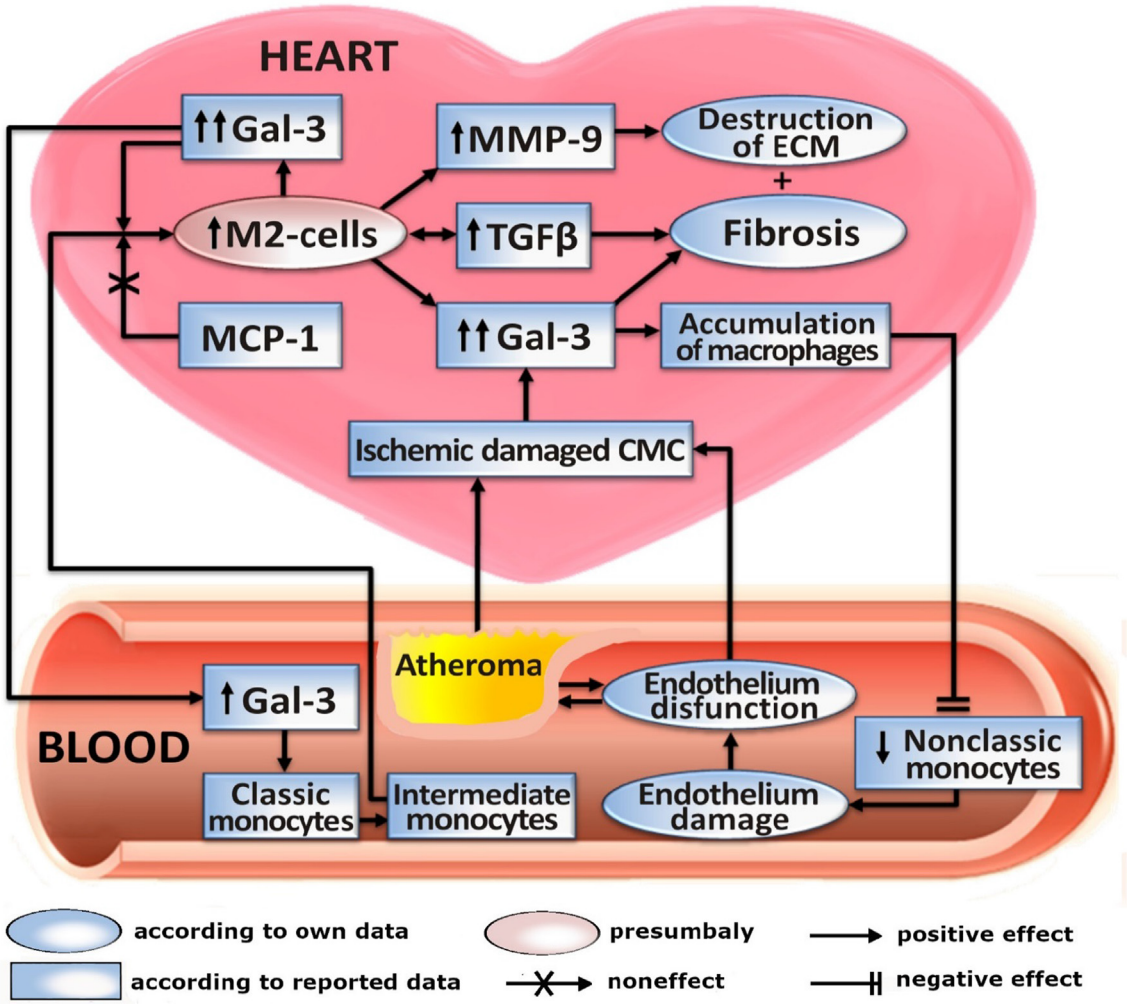

Fig. 5. The role of cardiac remodeling factors and subpopulation of blood monocytes in the pathogenesis of ischemic cardiomyopathy. Gal-3 – galectin 3, ECM – extracellular matrix, CMC – cardiomyocytes.

this contributes to its damage, dysfunction, and the spread of atherogenesis to smaller vessels. Implemented in the coronary arteries, this process aggravates ischemic damage of cardiomyocytes and following galectin-3 hyperproduction, which comes to a full vicious circle in the pathogenesis of ICMP (Fig. 5).

Thus, a number of significant pathogenic factors of ICMP development and progression, which create prospects for further clinical research, are shown. First, it was found, that in general group of CHD patients the level of galectin-3 in peripheral blood nonlinearly reflects its production in the myocardium. Therefore, its determination in sinus blood is more informative and may reflect the aggravation of the pathological process in the ICMP development before the concentration of galectin-3 exceeds the norm in the general blood flow. Secondly, ICMP is not based on excessive fibrosis or destruction of ECM, but a combination of these processes, as a result of which the formed connective tissue loses its elasticity and the heart chambers are dilated. This means, that ICMP treatment should be focused on both processes by suppressing the formation of both galectin-3 and MMP-9 in the myocardium. Thirdly, it was shown, that maximal density of distribution of macrophages at ICMP is observed in perianeurysmal area of the myocardium. Therefore, in a view of the ability of these cells to secrete galectin-3, its high intramyocardial production in patients with ICMP, it is advisable to combine cardiac revascularization with the removal of aneurysmal tissue. Next, ICMP development is characterized by a deficiency of non-classical monocytes in the blood. Consequently, this could be a marker of the disease and a target for its therapy, since the normalization of their number could provide protection of the endothelium and potentially reduce microvascular dysfunction.

5. Research limitations

The result of the study may be limited by the clinical status of CHD patients since the obtained data are valid for patients with hemodynamically significant multivascular disease of the main coronary arteries, which requires coronary artery bypass surgery. Thus, in patients at the initial stage of CHD with an existing or just emerging ICMP, the differences established in this study may not be detected yet, which requires further research. The small volume of samples should be noted as limitations, thus, the accumulation of data may reveal significant differences, which are currently traced at the level of tends. In this study, the absence of data on the distribution of macrophages in the myocardium in CHD patients without ICMP does not allow us to unambiguously estimate which of the mechanisms underlies the established hyperproduction of myocardial remodeling factors in the heart at ICMP: the accumulation of macrophages in the myocardium (which is more likely) or an increase in their secretory activity. It is not implausible that research limitations may relate to the

region of CHD patients' residency and nationality, since the results were obtained for people of European origin living mainly in the Siberian Federal District.

6. Conclusion

Myocardial fibrosis progression at ICMP is mediated to a lesser extent by TGFβ and to a greater extent by intramyocardial production of galectin-3, which determines the subpopulation composition of blood monocytes with a deficiency of non-classical cells, which is not characteristic for atherosclerosis. Myocardial fibrosis at ICMP is combined with the destruction of the ECM, mediated by the high concentration of MMP-9 in the heart and the accumulation of macrophages in the area of the aneurysm. MCP-1 does not play an important role in the pathogenesis of ICMP.

Further study of the properties of monocytes of various immunophenotypes, humoral mechanisms of differentiation of non-classical monocytes and the features of the M1/M2 macrophages balance in the myocardium at ICMP will allow us to study its mechanism in a more detailed way. This will serve as the basis for the development of the principles of early diagnosis of the pathological process leading to ICMP at CHD, its pathogenetically substantiated correction and methods for assessing its effectiveness according to the analysis of the mediator and cellular composition of the blood.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data

The data that support the findings of this study are available from the corresponding author, Chumakova S.P., upon reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcha.2021.100766.

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