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CORRELATION BETWEEN MICRORNA EXPRESSION LEVELS AND PLASMA CONCENTRATIONS OF BIOMARKERS OF POST-TRANSPLANT COMPLICATIONS IN HEART TRANSPLANT RECIPIENTS

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Objective: to analyze the correlation between the expression levels of microRNA-101, microRNA-142, microRNA-27, microRNA-339, and microRNA-424 and the plasma concentrations of biomarkers that are potentially significant for the diagnosis of post-transplant complications in heart recipients. **Materials and methods.** The study enrolled 72 heart recipients, among whom were 56 men (77.8%). The average age of recipients was 48.6 ± 10.9 (16 to 70) years. There were 38 patients with severe chronic heart failure, among whom were 29 men (76.3%). Patients' mean age was 48.8 ± 9.9 (26 to 70) years. The control group consisted of 12 healthy individuals who did not differ significantly by sex and age. microRNA expression levels in blood plasma were measured via quantitative polymerase chain reaction. Plasma concentrations of VEGF-A, PLGF, MCP-1, and sCD40L were determined using a multiplex method. ST2 and Galectin-3 concentrations were measured via enzyme-linked immunosorbent assay. **Results.** Patients with end-stage chronic heart failure were found to have significantly higher expression levels of microRNA-27, microRNA-339 and microRNA-424 in blood plasma compared with the healthy individuals. In potential heart recipients, the expression le vels of microRNA-339 and microRNA-424 correlated with serum galectin-3 concentrations, microRNA-101 expression levels correlated with PLGF-1 concentrations, while microRNA-27 expression levels correlated with plasma MCP-1 concentrations. In the early post-transplant period, the expression levels of microRNA-101, microRNA-339, and microRNA-424 in heart recipients were significantly lower than in patients with severe chronic heart failure. In the early post-transplant period (one year or more after transplantation), microRNA-101 and microRNA-27 expression levels were significantly higher than in heart recipients. A year or more after transplantation, the following correlations were found in heart recipients: microRNA-142 expression level correlated with serum levels of galectin-3 ($p = 0.05$), microRNA-27 and microRNA-424 expression levels correlated with ST2 concentrations ($p = 0.02$), microRNA-27 expression level correlated with PLGF-1 concentrations ($p = 0.02$), while microRNA-101 expression level correlated with serum levels of PAPP-A ($p = 0.05$). **Conclusion.** In heart recipients, the expression levels of microRNA-142, microRNA-27, microRNA-424, and microRNA-101 correlate with the concentration levels of biomarkers of fibrosis (Galectin-3), rejection (ST2), neoangiogenesis (PLGF), and tissue destruction (PAPP-A). A comprehensive analysis of pre- and post-translational markers may open up new perspectives in diagnosis, assessment of the risks of post-transplant complications, and in understanding the processes leading to their development.

Keywords: heart transplantation, rejection, microRNA, ST2, Galectin-3, placental growth factor (PLGF), pregnancy-associated plasma protein A (PAPP-A).

INTRODUCTION

Timely diagnosis of post-transplant complications is necessary to select and adjust the optimal dosages of immunosuppressive therapy and restore the function of the transplanted organ. To date, the standard for determining the degree and nature of acute rejection of a transplanted heart is endomyocardial biopsy (EMB), which is performed after transplantation, within the time frame stipulated by the protocol, as well as when signs of graft dysfunction are manifested [1]. Due to the fact that the innervation of the transplanted heart is disturbed, post-transplant complications in heart recipients are not accompanied by pain and may develop asymptomatically. To ensure long-term functioning of the transplanted organ, early diagnosis of complications after transplantation and the appointment of well-timed treatment are necessary [2].

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The participation of a number of biomarkers in the development of cardiovascular complications in patients with heart failure and in patients with heart transplants has been recently shown, as well as the possibility of exploiting the assessment of their concentration to predict and diagnose acute rejection, coronary artery disease, cardiac graft fibrosis [3–6].

A separate group of signaling molecules considered as promising candidates for the role of biomarkers of post-transplant complications in heart recipients is made up of microRNAs – small non-coding RNAs that regulate gene expression. It is believed that microRNAs play an important role in the regulation of the functions of both healthy and damaged cells. Changes in the expression of some miRNAs are associated closely with a number of pathological processes, such as autoimmune diseases, malignant neoplasms, and rejection of transplanted organs [7–9].

In this work, we analyzed the relationship between the expression level of miRNA-27, miRNA-101, miRNA-142, miRNA-339 and miRNA-424, determined in the blood plasma of cardiac recipients, with the concentration of biomarkers that are potentially significant for the diagnosis of post-transplant complications.

MATERIALS AND METHODS

The study included 72 patients who underwent heart transplantation (HT) in 2013–2016 at the Shumakov National Medical Research Center of Transplantology and Artificial Organs (Ministey of Health of Russia, Moscow, Russian Federation), among them men 56 (77.8%), the average age of recipients was 48.6 ± 10.9 (16 to 70), and 38 patients with severe chronic heart failure (III and IV functional class by NYHA), including 29 men (76.3%), the average age of patients was 48.8 ± 9.9 (26 to 70). Dilated cardiomyopathy (DCMP) was diagnosed in 20 (52.6%) patients with severe chronic heart failure, and coronary heart disease (IHD) in 18 (47.4%) patients. Follow-up of recipients after transplantation included: early period, the first month after HT (median 35 [15; 69] days); long-term period, a year or more after HT (median 404 [346.7; 783.5] days). The comparison group consisted of 12 healthy individuals, which did not differ significantly in gender and age.

All patients with indications for HT underwent a routine examination according to the National Clinical Guidelines "Heart transplantation and mechanical support of blood circulation" and the protocol for managing patients at the Shumakov National Medical Research Center. After transplantation, routine examinations of the recipient included: clinical assessment of the state, general and biochemical blood tests to determine the concentration of tacrolimus, 24-hour blood pressure monitoring (to correct antihypertensive therapy), echocardiographic examination, repeated myocardial biopsies, annual coronary angiographic examination. All recipients received three-component immunosuppressive therapy, including a combination of calcineurin inhibitors (tacrolimus) and cytostatics (mycophenolate mofetil or mycophenolic acid), as well as varying doses of oral prednisolone, depending on the time after surgery and the frequency of episodes of transplant rejection and adjuvant medication if required [1].

The material for the study of miRNA expression was venous blood plasma (1 to 3 samples from each patient, 1.44 on average). Patients' peripheral blood samples were collected in disposable tubes with the anticoagulant ethylenediamineacetic acid (EDTA), centrifuged for 10 minutes at 3000 rpm, after which the blood plasma was separated from the cell sediment and immediately frozen at –20 °C. Total RNA was isolated from 100 μL of blood plasma using Serum Plasma kits (Qiagen, USA) with preliminary addition of 1.6×10^8 copies of synthetic miRNA cel-miR-39 (Qiagen) after plasma incubation with Qiazol phenolic mixture. Cel-miR-39 was used as an internal control of the efficiency of RNA isolation, synthesis of complementary DNA (cDNA), and quantitative polymerase chain reaction (PCR) in real time. The intensity of miRNA expression was calculated using the $2^{-\Delta CT}$ method [10] and was expressed in relative units equivalent to $\log_2(2^{-\Delta Ct})$, where ΔCt are the working values of the change in the production cycle relative to the internal control of miRNA cel-miR-39 expression.

The concentration of VEGF-A, PlGF, MCP-1, and sCD40L in blood plasma was determined using a multiplex method that combines the principle of flow fluorimetry and enzyme immunoassay using polystyrene magnetic microspheres marked with red and infrared fluorophores, loaded with specific antibodies. The multiplex panel was generated based on the Simplex ProcartaPlex™ reagent kits. Determination of ST2 concentration in blood plasma was carried out by the method of enzyme-linked immunosorbent assay using the Critical Diagnostics Presage® ST2 Assay reagent kit (USA). The concentration of galectin-3 in blood plasma was measured by enzyme immunoassay using Human Galectin-3 Platinum ELISA reagent kits (Bender MedSystems GmbH, Vienna, Austria). Optical density at a wavelength of 450 nm was measured on a spectrophotometer.

Statistical data processing. Statistical analysis of the results was performed with the IBM SPSS Statistics 20 software package (IBM SPSS Inc., USA). The data obtained were statistically processed by nonparametric methods: when comparing dependent samples, the paired Wilcoxon test, and the Mann–Whitney U test to compare the independent variables. To assess the relationship between quantitative and qualitative ordered signs, the Spearman's rank correlation coefficient was calculated. The critical level of significance was taken equal to 5%, i. e., the null hypothesis was rejected at $p < 0.05$.

RESULTS AND DISCUSSION

Comparative analysis of the expression level of miRNA-101, miRNA-142, miRNA-27, miRNA-339 and miRNA-424 in healthy individuals and patients with terminal chronic heart failure is given in Table 1. Data are presented as a median of concentrations [interquartile range] with an indication of the significance of the differences, which is due to the distribution of values different from normal.

In patients with terminal chronic heart failure, the expression level of miRNA-27, miRNA-339, and miRNA-424 was significantly higher than in healthy individuals (Fig. 1).

The expression level of miRNA-101 and miRNA-142 in these groups did not differ significantly (p = 0.08 and p = 0.77, respectively).

In patients with severe chronic heart failure, the expression levels of miRNA-101, miRNA-142, miRNA-27, miRNA-339, and miRNA-424 did not differ between men and women (p = 0.93, p = 0.98, p = 0.63, p = 0.97 and p = 0.26, respectively). Indicators of miRNA expression did not depend on the patient's age (r = –0.304, p = 0.16; r = –0.236, p = 0.35; r = –0.361, p = 0.08; r = 0.146, p = 0.44 and r = –0.054, p = 0.82, respectively).

The expression level of miRNA-339 in patients with severe chronic heart failure caused by ischemic heart disease was significantly higher than in patients diagnosed with dilated cardiomyopathy (Fig. 2).

Table 1

Comparative analysis of miRNA expression in healthy individuals and patients with severe chronic heart failure

MiRNA ($\log_2(2^{-\Delta Ct})$)	Healthy individuals	CHF patients	Confidence, p
MiRNA-101	–5.66 [–6.85; –4.97]	–4.29 [–6.86; –3.21]	0.08
MiRNA-142	–7.39 [–10.26; –6.36]	–7.35 [–8.19; –6.25]	0.77
MiRNA-27	–5.79 [–6.70; 4.6]	–5.3 [–6.37; –1.22]	**0.02**
MiRNA-339	–11.14 [–11.75; –11.03]	–6.06 [–7.89; –4.86]	**0.0001**
MiRNA-424	–8.3 [–9.34; –7.41]	–5.46 [–7.54; –1.46]	**0.001**

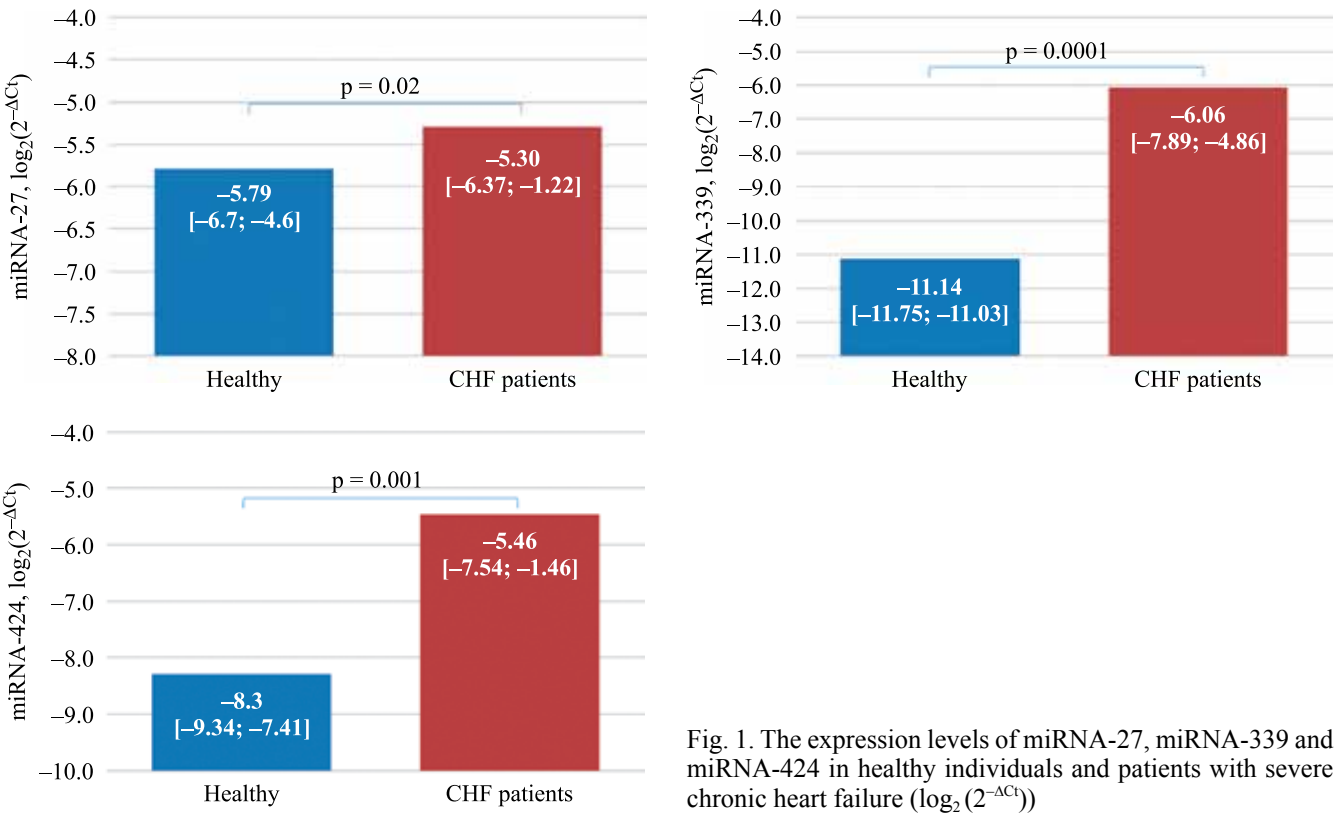

Fig. 1. The expression levels of miRNA-27, miRNA-339 and miRNA-424 in healthy individuals and patients with severe chronic heart failure ($\log_2(2^{-\Delta Ct})$)

There were no significant differences in the expression level of miRNA-101, miRNA-142, miRNA-27, and miRNA-424 depending on the initial diagnosis (DCMP or IHD) that caused the development of severe chronic heart failure (p = 0.6, p = 0.52, p = 0.87, p = 0.57 and p = 0.12, respectively).

The following biomarkers were included in the present study:

– vascular endothelial growth factor A (VEGF-A), produced by macrophages, fibroblasts, endothelial and other cells; involved in the activation, proliferation, migration and differentiation of endothelial cells of blood and lymphatic vessels [12];
– placental growth factor (PLGF), a biomarker of neoangiogenesis involved in the initiation of inflammatory processes in the vascular wall [13];
– macrophage chemoattractant protein (MCP-1) produced by vascular endothelial and smooth muscle cells, fibroblasts, lymphocytes and other cells; MCP-1 activates the migration of leukocytes into the vascular wall, is involved in the activation and degranulation of leukocytes, myelopoiesis, angiogenesis and fibrogenesis [14];
– soluble form of ligand CD40 (sCD40L), a component of the CD40 / CD40L lymphocyte costimulation system; participates in the processes of inflammation, thrombus formation, causes the activation and proliferation of smooth muscle cells [15];
– stimulating growth factor (ST2), the soluble form of which is the circulating form of the receptor for interleukin-33 and is a biomarker of acute rejection of a heart transplant; the secretion of interleukin-33 mainly occurs in response to mechanical stretching of fibroblasts and cardiomyocytes, leading to the activation of signaling pathways and preventing the development of myocardial hypertrophy [16];
– galectin-3, belonging to the family of β-galactoside-binding proteins that play an important role in the regulation of inflammation, immune response and fibrosis; at the site of injury, galectin-3 is able to be secreted into the extracellular space, which stimulates the process of fibrosis through the activation and reproduction of fibroblasts [17].

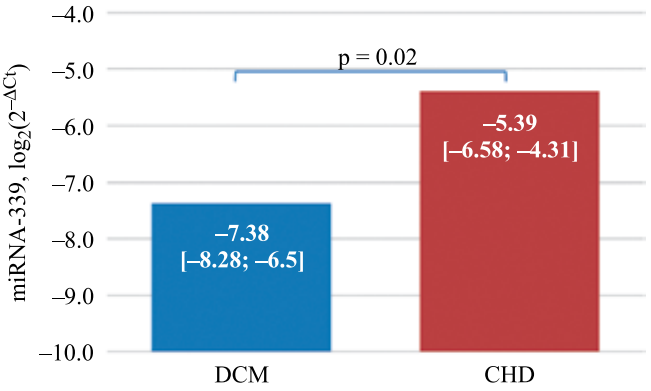

Fig. 2. The expression levels of miRNA-339 in patients with severe chronic heart failure depending on the initial diagnosis: DCM or CHD ($\log_2(2^{-\Delta Ct})$)

A comparative analysis of the correlation between the level of miRNA expression in the blood plasma of patients with end-stage heart failure and the content of protein (post-translational) biomarkers of post-transplant complications is given in Table 2.

In patients with terminal chronic heart failure, the expression level of miRNA-339 and miRNA-424 had an inverse correlation with the concentration of galectin-3 (Fig. 3).

The expression level of miRNA-101 had a direct correlation with the concentration of PLGF-1, and the level of expression of miRNA-27 was inversely correlated with the concentration of MCP-1 (r = 0.783, p = 0.01 and r = –0.717, p = 0.03, respectively).

No correlations were found between the expression level of miRNA-101, miRNA-142, miRNA-27, miRNA-339, and miRNA-424 and the concentration of VEGF-A, sCD40L, and PAPP-A in patients with end-stage chronic heart failure.

Table 2

Comparative analysis of the correlation between of the miRNA expression levels in the blood plasma of the patients with severe chronic heart failure and the concentration of biomarkers potentially significant for the diagnosis of post-transplant complications

MiRNA	Galectin-3	VEGF-A	PlGF-1	MCP-1	sCD40L	PAPP-A
MiRNA-101	r = 0.174, p = 0.55	r = 0.4, p = 0.29	**r = 0.783, p = 0.01**	r = –0.017, p = 0.97	r = 0.324, p = 0.28	r = 0.0182, p = 0.96
MiRNA-142	r = 0.321, p = 0.48	r = 0.5, p = 0.67	r = 0.5, p = 0.67	r = –0.5, p = 0.67	r = 0.314, p = 0.54	r = –0.6, p = 0.4
MiRNA-27	r = –0.139, p = 0.62	r = –0.183, p = 0.64	r = –0.417, p = 0.26	**r = –0.717, p = 0.03**	r = 0.0813, p = 0.78	r = –0.248, p = 0.49
MiRNA-339	**r = –0.519, p = 0.03**	r = –0.115, p = 0.75	r = 0.336, p = 0.31	r = 0.176, p = 0.63	r = –0.012, p = 0.97	r = 0.385, p = 0.19
MiRNA-424	**r = –0.714, p = 0.01**	r = 0.286, p = 0.49	r = 0.690, p = 0.06	r = 0.476, p = 0.23	r = 0.0091, p = 0.98	r = 0.238, p = 0.57

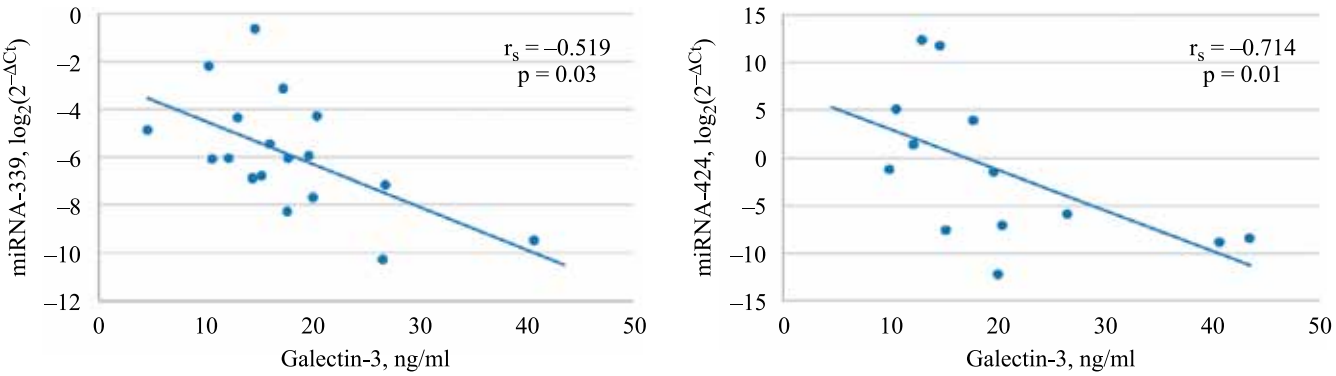

Fig. 3. Correlation between the expression level of miRNA-339, miRNA-424 and the concentration of Galectin-3 in patients with severe chronic heart failure ($\log_2(2^{-\Delta Ct})$)

Table 3

Comparative analysis of microRNAs expression in patients with chronic heart failure and heart transplant recipients at early and long-term after transplantation

MiRNA ($\log_2(2^{-\Delta Ct})$)	CHF patients	Heart recipients		Confidence, p	
		1 month after HT	1 year after HT		
MiRNA-101	–4.29 [–6.86; –3.21]	–8.28 [–9.74; –5.77]	–6.12 [–7.39; –4.61]	**0.005***	**0.03****
MiRNA-142	–7.35 [–8.19; –6.25]	–7.03 [–8.35; –6.01]	–6.52 [–7.39; –5.09]	0.89*	0.25**
MiRNA-27	–5.3 [–6.37; –1.22]	–5.76 [–6.24; –4.08]	–3.85 [–4.88; –1.73]	0.19*	**0.02****
MiRNA-339	–6.06 [–7.89; –4.86]	–9.91 [–11.29; –5.94]	–9.02 [–10.56; –5.80]	**0.02***	0.44**
MiRNA-424	–5.46 [–7.54; –1.46]	–7.21 [–8.25; –6.13]	–6.41 [–7.60; –5.50]	**0.01***	0.09**

Note. * – comparison between groups of patients with CHF and recipients after 1 month after HT; ** – comparison between groups of recipients after 1 month and 1 year after HT.

A comparative analysis of the expression level of miRNA-101, miRNA-142, miRNA-27, miRNA-339, and miRNA-424 in patients with chronic heart failure and heart recipients showed a tendency for a decrease in expression in the early stages after transplantation and a gradual increase in the long term (Table 3).

Fig. 4 shows the dynamics of the studied miRNAs in patients with severe chronic heart failure and recipients at different times after transplantation.

In heart recipients, 1 month after transplantation, the expression level of miRNA-101, miRNA-339, and miRNA-424 was significantly lower than in patients with terminal chronic heart failure.

The expression level of miRNA-101 and miRNA-27 in heart recipients 1 year or more after transplantation was significantly higher than after 1 month after HT.

There were no differences in the expression of the studied miRNAs in patients with severe chronic heart failure and long-term recipients after transplantation ($p \geq 0.05$).

In the early stages after transplantation, the expression level of miRNA-101, miRNA-339, and miRNA-424 is lower than in patients with end-stage chronic heart failure. A year or more after transplantation, the expression level of miRNA-101 and miRNA-27 in cardiac recipients increases as compared to those at early stages, which may reflect the development of fibrosis in the graft.

The presented data, obtained in the study of a larger number of heart recipients, confirm the results of our previous work [11] regarding the significance of differences in the level of miRNA-101 expression in patients with end-stage CHF and recipients in the early stages after HT, as well as the level of miRNA-101 and miRNA-27 in early and late periods after transplantation. At the same time, new results were shown regarding the reliability of differences in the expression levels of miRNA-339 and miRNA-424 in patients with end-stage CHF and recipients in the early stages after HT.

Analysis of the correlation between the level of miRNA expression in the blood plasma of recipients 1 year or more after transplantation with the content of biomarkers potentially significant for the diagnosis of post-transplant complications is given in Table 4.

In cardiac recipients 1 year or more after transplantation, the level of miRNA-142 expression had an inverse correlation with the concentration of galectin-3 (Fig. 5).

There is evidence that miRNA-142 is expressed by T-lymphocytes and is involved in the regulation of acute cellular rejection of the transplanted heart [18]. The correlation between the level of miRNA-142 expression and the concentration of galectin-3 suggests the involvement of immune mechanisms in the processes of myocardial fibrosis in cardiac recipients.

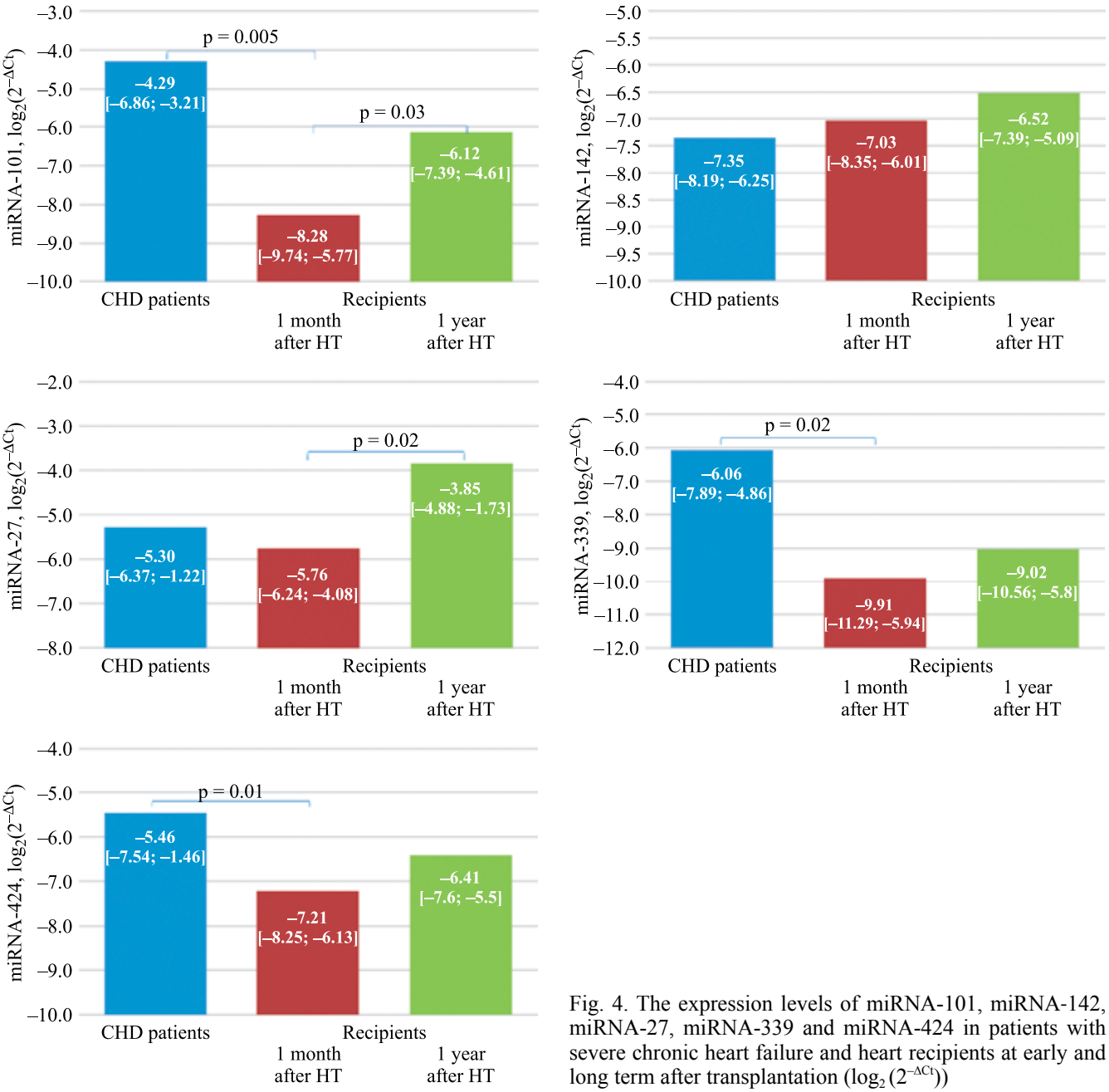

Fig. 4. The expression levels of miRNA-101, miRNA-142, miRNA-27, miRNA-339 and miRNA-424 in patients with severe chronic heart failure and heart recipients at early and long term after transplantation ($\log_2(2^{-\Delta Ct})$)

Table 4

Comparative analysis of the correlation between of the miRNA expression levels in the blood plasma of the recipients at long-term after transplantation and the concentration of biomarkers potentially significant for the diagnosis of post-transplant complications

MiRNA	Galectin-3	ST2	VEGF-A	PlGF-1	MCP-1	sCD40L	PAPP-A
MiRNA-101	r = 0.0673, p = 0.73	r = –0.209, p = 0.44	r = 0.119, p = 0.78	r = 0.491, p = 0.22	r = 0.143, p = 0.74	r = –0.429, p = 0.34	**r = –0.557, p = 0.05**
MiRNA-142	**r = –0.534, p = 0.05**	r = 0.1, p = 0.87	r = 0.5, p = 0.67	r = 0.9451, p = 0.21	r = 0.5, p = 0.67	н/д	r = –0.4, p = 0.6
MiRNA-27	r = 0.133, p = 0.51	**r = –0.585, p = 0.02**	r = 0.429, p = 0.34	**r = 0.847, p = 0.02**	r = 0.679, p = 0.09	r = –0.143, p = 0.79	r = –0.321, p = 0.34
MiRNA-339	r = 0.0433, p = 0.83	r = –0.041, p = 0.88	r = 0.0303, p = 0.93	r = 0.377, p = 0.28	r = –0.03, p = 0.93	r = –0.405, p = 0.32	r = –0.303, p = 0.29
MiRNA-424	r = 0.0148, p = 0.94	**r = –0.542, p = 0.02**	r = 0.286, p = 0.49	r = 0.539, p = 0.17	r = –0.381, p = 0.35	r = 0.2746, p = 0.55	r = –0.099, p = 0.76

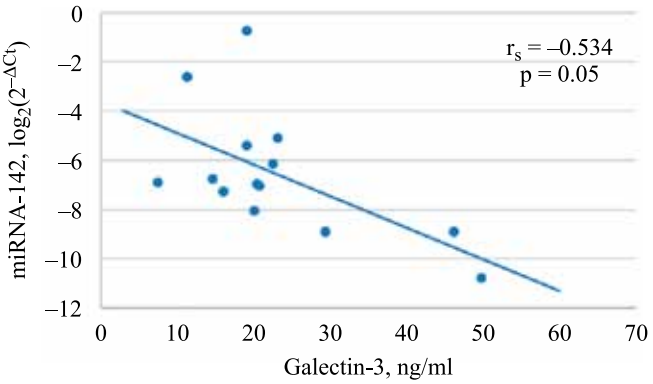

Fig. 5. Correlation between the expression level of miRNA-142 and the concentration of Galectin-3 in heart recipients at long-term after transplantation ($\log_2(2^{-\Delta Ct})$)

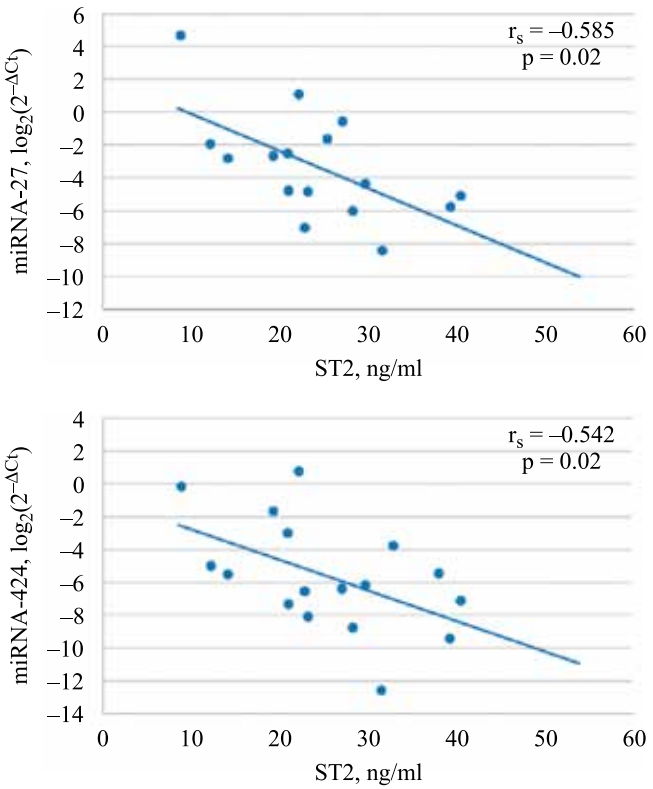

Fig. 6. Correlation between the expression level of miRNA-27, miRNA-424 and the concentration of ST2 in heart recipients at long-term after transplantation ($\log_2(2^{-\Delta Ct})$)

Expression levels of miRNA-27 and miRNA-424 in cardiac recipients 1 year or more after transplantation had an inverse correlation with the ST2 concentration (Fig. 6).

It has been shown that miRNA-424 plays an important role in the pathogenesis of pulmonary hypertension and subsequent right ventricular hypertrophy through inhibition of the function of the SMURF specific regulatory factor [19].

The expression level of miRNA-101 was inversely correlated with the concentration of PAPP-A, and the expression level of miRNA-27 was directly correlated with the concentration of PLGF-1 (r = –0.557, p = 0.05 and r = 0.847, p = 0.02, respectively). Changes in the expression profile of miRNA-101 and miRNA-27 are associated with the participation of these molecules in the regulation of myocardial fibrosis through interaction with the transcription factor RUNX1 and transforming growth factor β receptor 1 (TGFBR1) [20, 21].

No correlations were found between the expression level of miRNA-101, miRNA-142, miRNA-27, miRNA-339, and miRNA-424 and the concentration of VEGF-A, MCP-1, and sCD40L in heart recipients 1 year or more after transplantation.

In heart recipients a year or more after transplantation, there is a correlation between the level of expression of miRNA-142 and the concentration of galectin-3, the level of expression of miRNA-27, miRNA-424 and the concentration of ST2, the level of expression of miRNA-27 and the concentration of PLGF-1, the level of miRNA-101 expression and PAPP-A concentration. The relationship between the level of miRNA expression and the concentration of biomarkers potentially significant for the diagnosis of post-transplant complications in cardiac recipients not only confirms the available data on the involvement of these signaling molecules in the regulation of various pathological processes, including the graft, but also suggests their diagnostic potential for risk assessment. development of rejection and the possibility of minimizing immunosuppressive therapy. In this regard, the combined use of miRNAs with biomarkers of post-transplant complications for the formation of complex tests (multimarker panels) that make it possible to diagnose changes at various translational levels may become especially promising. Further comprehensive studies of pre- and post-translational markers can open up new perspectives both in diagnosis, assessment of the risks of post-transplant complications, and in understanding the processes leading to their development.

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The authors declare no conflict of interest.

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