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# MICRORNA EXPRESSION LEVELS IN LUNG RECIPIENTS: CORRELATIONS WITH CLINICAL AND LABORATORY DATA

*O.P. Shevchenko[1, 2], S.O. Sharapchenko[1], O.M. Tsirulnikova[1, 2], I.V. Pashkov[1], O.E. Gichkun[1, 2], D.A. Velikiy[1], E.F. Shigaev[1], D.O. Oleshkevich[1], M.T. Bekov[1]*

[1] Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russian Federation

[2] Sechenov University, Moscow, Russian Federation

**Objective:** to evaluate the expression levels of miRNA (miR-27, miR-101, miR-142, miR-339 and miR-424) and its relationship with clinical and laboratory parameters in lung transplant recipients. **Materials and methods.** The study included 57 lung recipients aged 10 to 74 years (35 ± 15), including six children (9%) – four boys 10, 12, 13 and 17 years and girls 13 and 14 years old – and 51 adult recipients, including 30 men (62.5%). The control group was made up of 14 healthy individuals that were not significantly different by gender and age. Expression levels of the microRNAs studied in blood plasma were determined via quantitative polymerase chain reaction (PCR). Correlations of miRNA expression levels with complete blood count and biochemical blood test indicators were analyzed. **Results.** Patients with end-stage chronic respiratory failure (potential lung recipients) were found to have significantly higher expression levels of miR-27, miR-101 and miR-339 in plasma than the healthy individuals ($p = 0.02$, $p = 0.03$ and $p = 0.01$, respectively). The expression level of miR-339 correlated with the age of potential lung recipients ($p = 0.04$). It was a negative correlation ($r = –0.46$). The expression levels of the other four miRNAs were age independent. The average expression level of miR-424 in lung recipients in the long-term period after lung transplant was higher than in waitlisted patients ($p = 0.03$). Analysis of the relationship between miRNA expression levels and external respiration function in the long-term post-transplant period showed that miR-142 expression level ($r = 0.61$; $p = 0.04$) positively correlates with the Tiffeneau-Pinelli index. This strong correlation, which exceeds 85%, indicates the presence of restrictive lung diseases. A year and more after transplantation, it was found that in the recipients, there were close positive correlations between miR-27, miR-142, miR-424 expression levels and blood leukocyte concentration, as well as between the miR-142 expression level and the sCD40L concentration during this period. **Conclusion.** A comparative study of the expression level of miRNAs (miR-27, miR-101, miR-142, miR-339 and miR-424) in the blood plasma of patients suffering from end-stage chronic lung diseases of various origin and in lung recipients enables us to conclude that further studies of the miRNA panels are needed in order to assess their effectiveness as potential molecular and genetic markers of post-transplant complications.

*Keywords: lung transplantation, biomarker, miRNA, miR-27, miR-101, miR-142, miR-339, miR-424, sCD40L, chronic respiratory failure.*

The recent significant increase in the survival rates of solid organ recipients have come with the challenge of providing long-term follow-up for recipients in order to detect early post-transplant complications, assess graft condition, and provide immunosuppression control. There are presently a considerable number of studies aimed at finding minimally invasive laboratory technologies for early preclinical diagnosis of complications in solid organ recipients. Changes in blood levels of some specific biomarker molecules involved in pathophysiological processes and acting as indicators of the risk of associated adverse events, are an objective reflection of the systematic nature of the processes occurring in the recipient's body [1].

Lung transplantation is a radical but effective remedy in severe respiratory failure. Transplanted lung biopsy followed by histological examination of the biopsy material allows to verify the graft condition. However, this comes with risks and limitations characteristic of invasive interventions. In recent years, miRNAs have been actively studied as potential biomarkers of post-transplant complications. MiRNAs are a family of small non-coding RNAs, about 22 nucleotides (18–25) in length, acting as regulatory elements of post-transcriptional genes. MicroRNAs inhibit protein synthesis by blocking translation by base pairing with complementary ribonucleic acid (RNA), thereby leading to degradation of a specific target [2]. It has been estimated that

**Corresponding author:** Sofya Sharapchenko. Address: 1, Shchukinskaya Str., Moscow, 123182, Russian Federation. Phone: (495) 190-35-62. E-mail: transplant2009@mail.ru

miRNAs may be able to regulate more than 30% of the protein-coding genes in the human genome. Moreover, miRNAs play a key role in regulating the functions of both healthy and damaged cells. They are closely related to various biological processes, including development and differentiation of hematopoietic cells, apoptosis and proliferation. It has been shown that expression of certain miRNAs is associated with a number of conditions, such as autoimmune diseases, malignant neoplasms, and graft rejection [3–5].

Small signaling molecules are studied in terms of their potential significance in the pathogenesis of post-transplant complications and use of targeted therapy as potential targets for solid graft rejection [2, 6, 7]. Recent studies have shown that data on changes in the expression levels of certain types of miRNAs in solid organ recipients can be useful for early diagnosis and monitoring of post-transplant complications, including rejection and fibrosis of heart, kidney, liver, and lung transplants [8–11].

The aim of this study was to evaluate the expression levels of miRNA (miR-27, miR-101, miR-142, miR-339 and miR-424) and its relationship with clinical and laboratory parameters in lung transplant recipients.

## MATERIALS AND METHODS

The study included 57 lung recipients aged 10 to 74 years (35 ± 15 average). From 2014 to 2019, they underwent lung transplant surgeries at the Shumakov National Medical Research Center of Transplantology and Artificial Organs. Among them were 6 children (9%) – 4 boys aged 10, 12, 13 and 17 years and 2 girls aged 13 and 14 years. There were also 51 adult recipients, aged 18 to 74 (37 ± 14) years, including 30 (62.5%) male patients. The diseases that caused respiratory failure and determined the indications for transplantation were cystic fibrosis (n = 22), chronic obstructive pulmonary disease (COPD) (n = 15), primary pulmonary hypertension (n = 9), pulmonary fibrosis (n = 6), lymphangioleiomyomatosis (n = 3) and bronchiectasis (n = 2). Maximum follow-up for lung transplant recipients was 1,808 days (median 294 [85; 545]). The control group consisted of 14 healthy individuals.

Scheduled examination of the patients was done in accordance with the clinical recommendations of the Russian Transplant Society. It included complete physical examination, general and biochemical blood tests, as well as virological and bacteriological studies. When studying external respiration function by spirometry, we measured the forced expiratory volume in one second ($FEV_1$) and forced vital capacity (FVC), and calculated the Tiffeneau-Pinelli index (FEV1/FVC × 100%). All patients received basiliximab induction. Immunosuppressive therapy included tacrolimus, mycophenolic acid and methylprednisolone preparations. Everolimus was administered if necessary [12].

Venous blood plasma served as the material for studying miRNA expression (1 to 3 samples from each patient, 1.22 on average). Peripheral blood samples were collected in disposable tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA), centrifuged for 10 minutes at 3,000 rpm. After that, the blood plasma was isolated from the cell pellet and immediately frozen at –20 °C. Total RNA was isolated from 100 μl of blood plasma using SerumPlasma kits (Qiagen, USA) with preliminary addition of $1.6 \times 10^8$ copies of synthetic microRNA cel-miR-39 (Qiagen) after plasma incubation with Qiazol phenolic mixture. Cel-miR-39 was used in real-time as an internal control of the efficiency of RNA isolation, complementary DNA (cDNA) synthesis, and quantitative polymerase chain reaction (PCR). MiRNA expression intensity was expressed in relative units that are equivalent to $2^{-\Delta Ct}$, where ΔCt are the working values of product cycle change relative to the internal control of cel-miR-39 expression. Statistical analysis of data obtained was performed using standard statistical processing methods – Microsoft Office Excel software and Statistica v.13.0 application package, StatSoftInc (USA). Data are represented by the values of the median and interquartile range for nonparametric variables. Expression values obtained were checked for distribution normality. Spearman rank-order correlation coefficient and Mann–Whitney U test were used to compare independent variables. Critical significance level was taken to be 5%, i.e., the null hypothesis was rejected at $p < 0.05$.

## RESULTS AND DISCUSSION

Comparative analysis showed that the expression level of three out of the five miRNAs (miR-27, miR-101 and miR-339) in candidates waitlisted for lung transplantation was significantly higher than in healthy individuals ($p = 0.02$, $p = 0.03$ and $p = 0.01$, respectively, Fig. 1).

Results obtained from measurement of miRNA expression levels are presented as median and interquartile range values, which is due to the distribution of values that is different from normal distribution. Data are given in relative units. There were no significant differences in the expression values of miR-142 and miR-424 in potential lung recipients and in healthy individuals ($p = 0.16$ and $p = 0.06$, respectively).

No significant differences were found in the expression levels of miR-27, miR-101, miR-142, miR-339 and miR-424 in men and women ($p = 0.37$, $p = 0.85$, $p = 0.98$, $p = 0.27$ and $p = 0.34$, respectively). The expression of four out of the five miRNAs was age independent; miR-339 expression level correlated with the age of potential lung recipients ($p = 0.04$), and the correlation was inverse ($r = –0.46$).

Fig. 2 shows comparative data on the change in the expression value of each of the five miRNAs in recipients in the early (n = 27) and long-term (n = 44) post-transplant period. The early post-transplant period in-

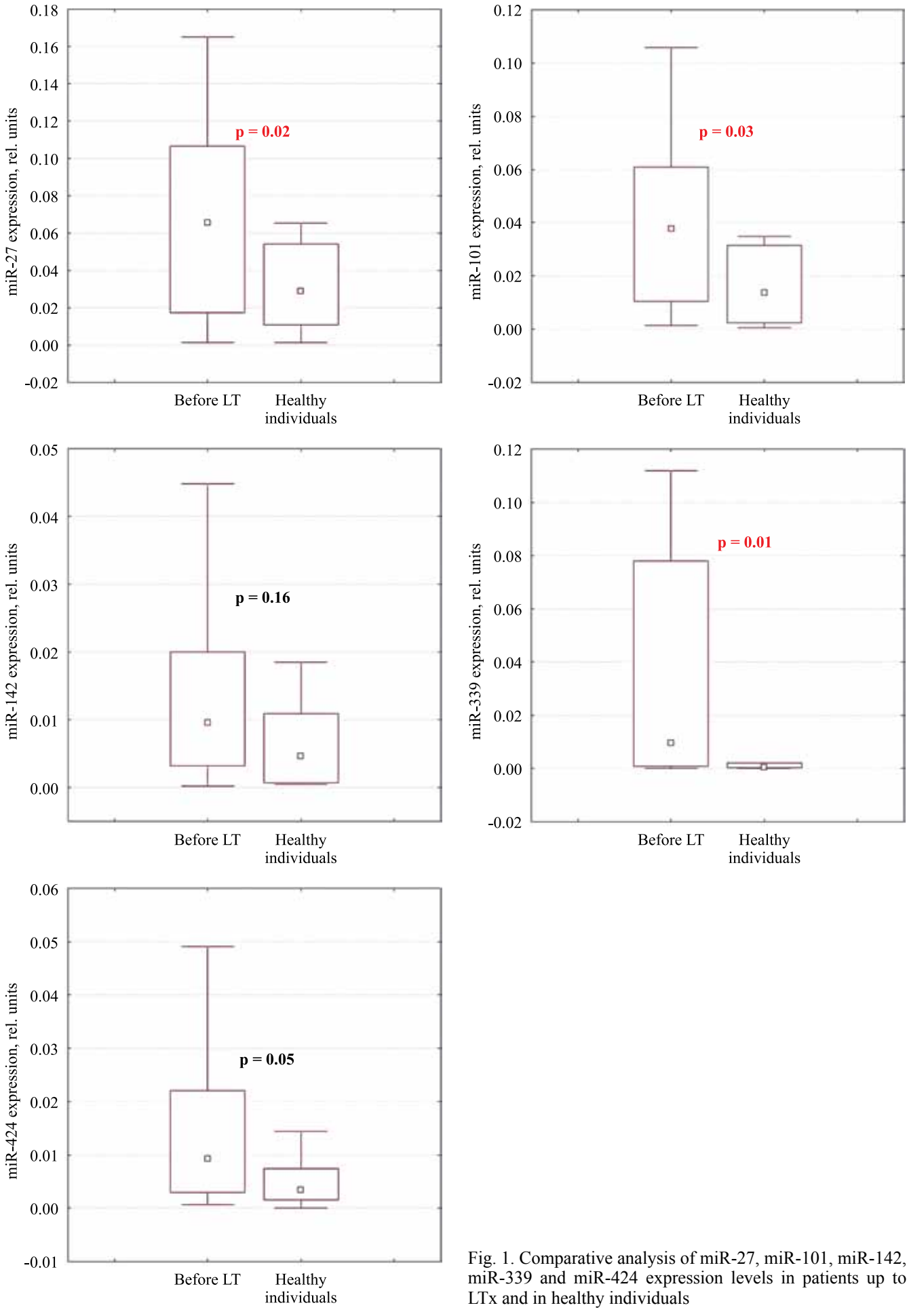



Fig. 1. Comparative analysis of miR-27, miR-101, miR-142, miR-339 and miR-424 expression levels in patients up to LTx and in healthy individuals

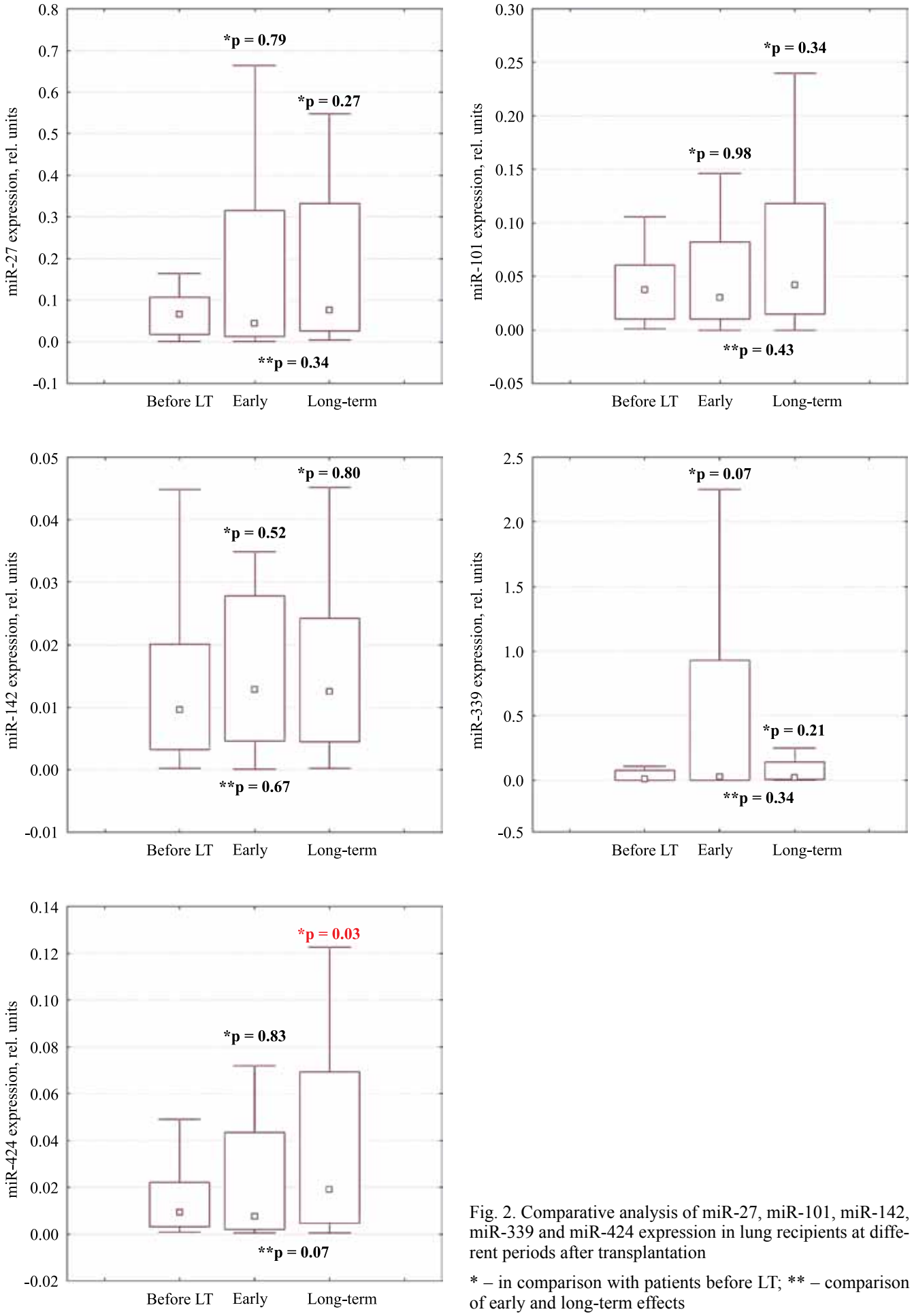



Fig. 2. Comparative analysis of miR-27, miR-101, miR-142, miR-339 and miR-424 expression in lung recipients at different periods after transplantation

* – in comparison with patients before LT; ** – comparison of early and long-term effects

Table

**Comparative analysis of microRNAs expression in lung recipients with and without postoperative complications**

| microRNA | Without complications | Complications | | | |
|---|---|---|---|---|---|
| | | infectious | *p | obstructive | *p |
| miR-27 | 0.07 [0.03; 0.25] | 0.06 [0.03; 0.11] | 0.64 | 0.07 [0.03; 0.28] | 0.98 |
| miR-101 | 0.04 [0.01; 0.08] | 0.05 [0.03; 0.13] | 0.81 | 0.04 [0.02; 0.14] | 0.58 |
| miR-142 | 0.01 [0.01; 0.02] | 0.01 [0.004; 0.01] | 0.44 | 0.01 [0.01; 0.02] | 0.94 |
| miR-339 | 0.02 [0.01; 0.07] | 0.02 [0.004; 0.06] | 0.83 | 0.03 [0.004; 0.15] | 0.98 |
| miR-424 | 0.02 [0.01; 0.05] | 0.07 [0.04; 0.07] | 0.50 | 0.03 [0.03; 0.07] | 0.86 |

*Note.* * – in comparison with the group without complications.

cluded recipients who were examined a median of 33 [23; 68] days after transplant surgery. The long-term post-transplant period included recipients examined 511 [388; 930] days after lung transplant surgery.

The average expression level of miR-424 in lung recipients in the long-term post-transplant period was significantly higher than in patients awaiting transplantation ($p = 0.03$). In all other cases, there were no significant differences in miRNA expression levels in recipients in the early and long-term post-transplant periods, as in patients awaiting lung transplant.

The Table presents a comparative analysis of the expression levels of individual miRNAs in recipients with complications associated with obstructive airway processes (n = 14), infectious complications (n = 6), as well as in recipients who were not diagnosed with these complications. The average expression level of each of the five miRNAs in the long-term post-transplant period represented by the median values [interquartile range], did not significantly differ in recipients with and without complications (see Table).

It cannot be ruled out that with increased number of observations, significant differences (in the expression of individual miRNAs) associated with post-transplant complications can be revealed.

At the same time, when studying the relationship between the expression level of each of the five miRNAs and the follow-up data obtained, it was found that the expression level of miR-27 inversely correlated with the patient's body mass index (BMI). The indicated dependence was revealed both in lung recipients in the long-term post-transplant period and in waitlisted patients with respiratory failure (Fig. 3).

The miR-27 expression level is higher in underweight patients; we found no correlation between the expression values of miR-101, miR-142, miR-339, miR-424 with the BMI.

When studying the external respiration function parameters in lung recipients, there was no significant correlation between the miR-27, miR-101, miR-142, miR-339, miR-424 expression levels and $FEV_1$ at a long-term post-transplant period ($p = 0.25$, $p = 0.64$, $p = 0.59$, $p = 0.14$ and $p = 0.48$, respectively). However, in the long-term post-transplant period, there was significant positive correlation between the expression level of miR-142 ($r = 0.61$; $p = 0.04$) and the Tiffeneau-Pinelli index.

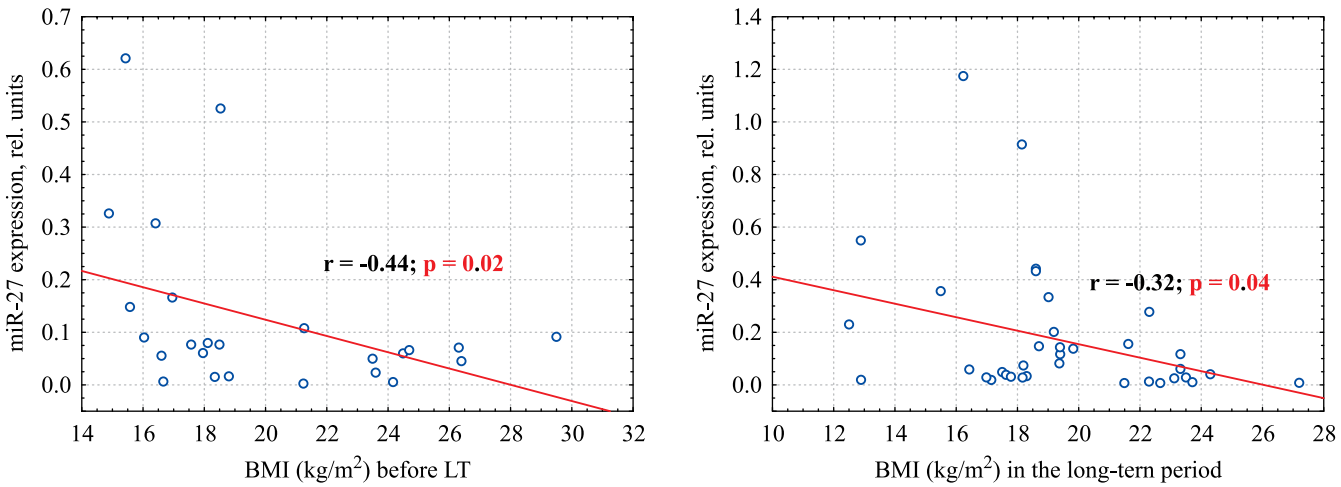



Fig. 3. Correlation of miR-27 expression with body mass index (BMI) in long-term lung recipients and in potential lung recipients

The value, which is more than 85%, suggests restrictive respiratory tract disorders (Fig. 4).

A positive correlation was found between white blood cell count and the expression levels of miR-27 (r = 0.63; p = 0.0002), miR-142 (r = 0.44; p = 0.04) and miR-424 (r = 0.56; p = 0.001) in recipients in the long-term post-transplant period (Fig. 5).

Analysis showed there were no significant correlations between microRNA expression levels and total plasma protein, albumin, creatinine, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) activity. In the long-term post-transplant period, there was moderate inverse correlation between ALT activity and miR-27 (r = –0.44; p = 0.01), and between AST and miR-27 (r = –0.41; p = 0.02) and miR-142 (r = –0.43; p = 0.04).

In lung recipients, the expression level of each of the five miRNAs in the long-term post-transplant period did not depend on blood levels of tacrolimus and everolimus. At the same time, concentration of the soluble CD40 ligand lymphocyte stimulating factor (sCD40L) correlated with the expression levels of miR-27 (r = 0.52; p = 0.02) and miR-142 (r = 0.53; p = 0.02) in the plasma of recipients in the long-term post-transplant period (Fig. 6).

No significant dependence of sCD40L concentration on the expression levels of other miRNAs has been established.

A number of recent studies have identified miRNAs as potential biomarkers of post-transplant complications

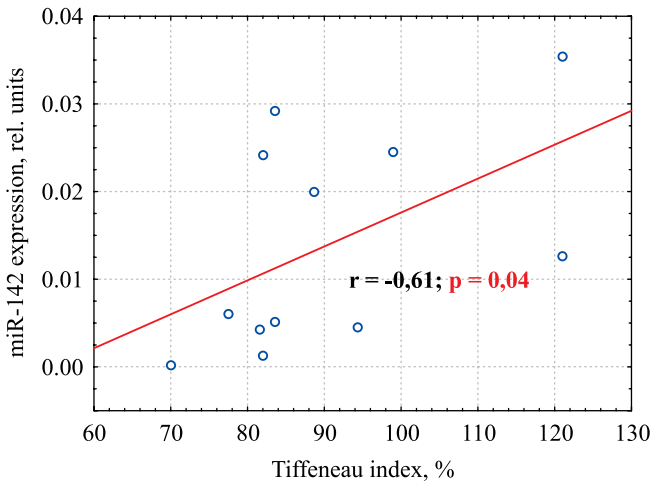
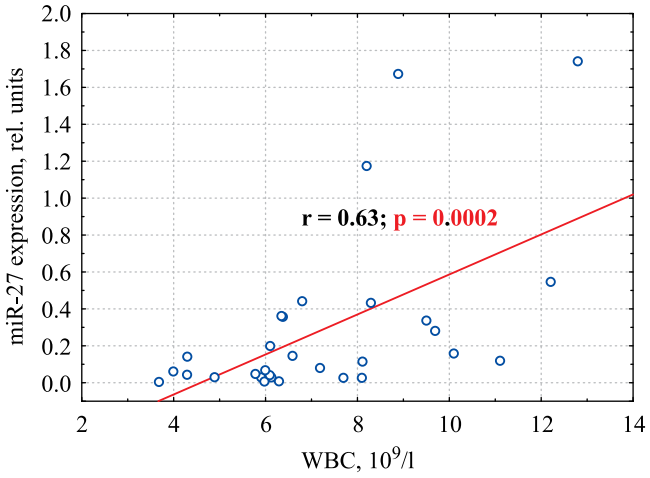
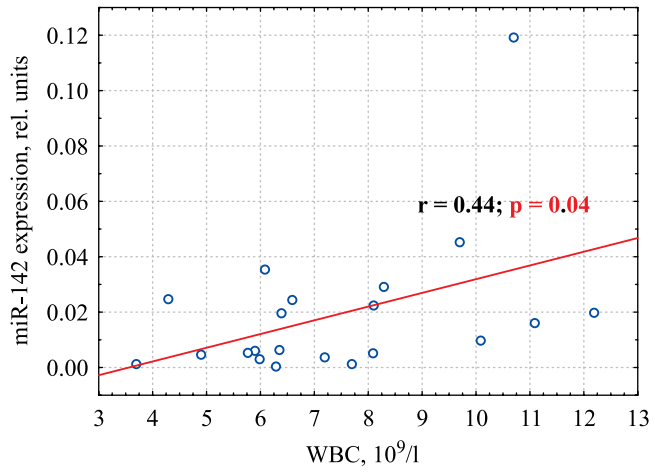



Fig. 4. Correlation of Tiffeneau index (%) and miR-142 expression in lung recipients in the long-term period



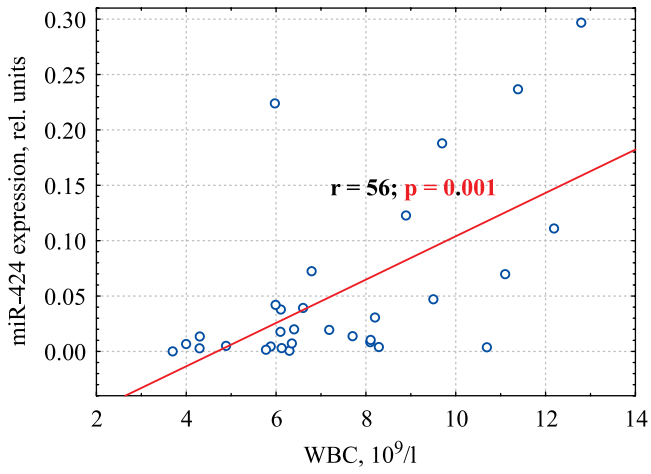




Fig. 5. Correlation of miR-27, miR-142 and miR-424 expression with leukocyte levels in lung recipients

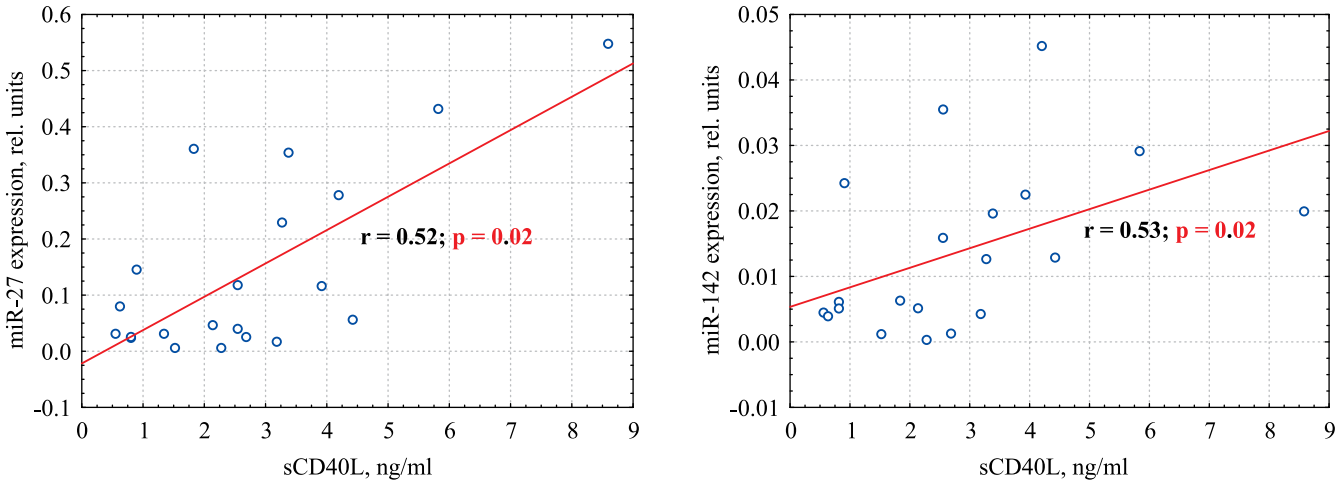



Fig. 6. Correlation of sCD40L and miR-27, miR-142 expression in lung recipients in the long-term period

[13]. Thus, significant differences in the expression levels of individual miRNAs (miR-10a, miR-31, miR-92a, miR-101, miR-142-3p, miR-155, etc.) were noted in heart recipients with and without acute cellular rejection [8]. Studies of the diagnostic potential for measuring miRNA expression in the plasma of kidney recipients revealed a number of molecules (miR-150, miR-192, miR-200b and miR-423-3p) associated with renal allograft rejection and allograft dysfunction [5]. Specific miRNAs were found (miR-122, miR-148a and miR-194), whose expression levels increase significantly in blood serum upon liver allograft rejection [9].

In experimental studies, the regulatory role and diagnostic significance of miRNAs in the development of post-lung transplant complications were established. It was shown that miR-124, through regulation of monocyte chemotactic protein-1 expression, affects proliferation and activation of pulmonary adventitial fibroblasts [14], which is of great importance in chronic graft dysfunction. Another study using an experimental rat lung transplantation model showed increased expression of miR-146a and miR-155 in obliterative bronchiolitis [15]. Similar data on possible diagnostic significance in lung graft rejection were obtained in the study of miR-376-5p, miR-338-3p, miR-16 and miR-195 [16]. It was shown that miRNA-199b regulates the severity of immune responses during lung graft rejection [17].

A significant increase in the expression of miR-21, miR-29a, miR-103, and miR-191 was observed in the blood serum of patients with end-stage respiratory disease at different times after lung transplantation [18].

Recent results from the study of microRNAs in solid organ recipients suggest that they could be characterized as potential biomarkers of post-transplant complications [19]. However, due to the small number of studies, there is a need to further study the participation of this group of molecules in biological processes in lung recipients.

The objective of this study was to establish a relationship between miRNA expression levels and changes in clinical and laboratory parameters in patients with severe respiratory failure and in lung transplant recipients, with subsequent determination of the possibility of using the microRNAs as potential biomarkers of post-transplant complications. MicroRNAs that regulate expression of genes potentially associated with post-transplant complications, primarily graft rejection, infection, and fibrosis, were selected for the study.

It was found that in potential lung recipients, expression levels of three of the five miRNAs (miR-27, miR-101 and miR-339) are higher than in healthy individuals. This may suggest that they are involved in the pathological process. We could not detect any significant changes in the expression level of each of the miRNAs, as compared to waitlisted patients and recipients in the early and long-term post-transplant period.

A comparative analysis of the expression level of the miRNAs in recipients with and without infectious-mediated, obstructive complications in the long-term postoperative period also did not reveal any major differences. It cannot be excluded that the absence of differences in the average expression levels between the indicated groups may be due to the small number of observations, especially if we take into account the wide range of variations in the miRNA expression value, as well as the wide variety of factors that can influence these parameters, as in the early and in the long-term post-transplant period.

A fairly large number of different types of miRNAs have been identified and characterized to date. Selected for the present study were five miRNAs that presumably play a role in the development of lung diseases, cardiovascular disease and/or are potentially significant for diagnosis of post-transplant complications in lung recipients [13]. The mechanisms of influence of individual miRNAs on transplant immunity and various aspects of

the relationship between a graft and the recipient's body have not been studied enough. Most of the available information is based on the results of experimental studies [13, 15, 20]. However, available data on the involvement of individual microRNAs in regulation of immune response to transplantation, in the processes of acute participation and chronic rejection, in their influence on the functions of fibroblasts, dendritic cells, T-lymphocytes are the basis for in-depth studies of the role of signaling molecules in the pathogenesis of post-transplant complications, with subsequent development of fundamentally new approaches to diagnosis and treatment [6, 14, 19].

In the present study, it was shown that the expression level of miR-27 in lung recipients is associated with a number of clinical and laboratory parameters – body mass index, hepatic transaminase activity, and white blood cell count. According to published data, miR-27 and its isoforms participate in regulation of metabolic processes and fibrosis by inhibiting the expression of transforming growth factor β (TGF-β) [12, 21].

The expression level of miR-142 also correlates with white blood cell counts and AST activity in lung recipients. MiR-142 is expressed on T lymphocytes, which play a major role in acute cellular rejection. The fact that miR-142 originates from immune cells, and not from transplant tissue, suggests the possibility of predicting rejection even before damage to the organ itself. Moreover, miR-142 has recently been shown to have a regulatory role in the pathogenesis of inflammatory lung diseases via suppression of macrophage activation [12, 22]. In this context, the dependence we found between miR-142 expression and Tiffeneau-Pinelli index, reflecting the restrictive pathology caused by alveolar disease of the bronchopulmonary system, may be of particular interest.

The presence of a direct relationship between expression levels of miR-27 and miR-142 and the blood concentration of soluble CD40 ligand, whose role in solid organ transplantation is mediated through co-stimulation of T-lymphocytes [23], may, on one hand, indicate one of the mechanisms regulating lung transplant functioning, and on the other hand, serve as the basis for development of diagnostic approaches.

Our study also established a connection between miR-424 expression and white blood cell count in lung recipients. MiR-424 is secreted by pulmonary arterial endothelial cells and plays an important role in the pathogenesis of pulmonary hypertension. It has recently been shown that miR-424 has a protective effect on alveolar epithelial cells in acute respiratory distress syndrome; this action is mediated through the nuclear factor kappa B (NF-kB) [24].

A comparative study (undertaken in this present work) of the level of miRNA expression (miR-27, miR-101, miR-142, miR-339 and miR-424) in the blood plasma of patients suffering from end-stage chronic lung diseases of various origins in lung recipients in early and long-term post-transplant period allows us to conclude that further study of the microRNA panel and evaluation of their effectiveness as potential molecular genetic markers in the observation of waitlisted patients and lung recipients are necessary.

The correlation found between miRNA expression levels and clinical, functional, and laboratory parameters may suggest that miRNAs play a role in regulation of graft-recipient relationships. It also shows that further investigation of the involvement of the five miRNAs in the immunological mechanisms of graft damage and their diagnostic effectiveness in post-transplant complications is needed.

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*The authors declare no conflict of interest.*

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