ORIGINAL INVESTIGATIONS

Metabolic aspects of clinical remission prediction from baseline blood gene expression in patients with rheumatoid arthritis treated with methotrexate

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Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, which is characterized by chronic erosive arthritis (synovitis) and systemic inflammation of the viscera. Methotrexate (MTX) is the drug of choice for RA treatment. However, it is currently impossible to predict the efficacy of MTX in a particular patient; the drug fails to produce the desired effect or causes adverse reactions in a considerable number of patients. The identification of patients who are responsive to MTX could significantly improve the results of therapy.

Objective: *to investigate the specific features of baseline (pretreatment) expression of genes responsible for major metabolic and energy production pathways in RA patients with different disease activity and to identify the genes, the baseline expression of which could serve as a predictor for remission attainment.*

Patients and methods. *Blood from 40 RA patients (mean age 47.5 years; mean disease duration 7.9 weeks) who had not previously received MTX and 26 healthy donors (mean age 45.1 years). All the patients had used MTX (15 mg/week) for 2 years. Clinical response was evaluated by DAS28 and the serum levels of anti-cyclic citrullinated peptide antibodies, C-reactive protein, and rheumatoid factor. Remission was diagnosed according to ACR/EULAR and DAS28 (DAS28 <2.6). Joint structural changes were radiographically evaluated. Gene expression was determined in peripheral blood cells by real-time reverse transcriptase-polymerase chain reaction.*

A control group consisted of 26 randomly recruited gender- and sex-matched patients without autoimmune diseases and a family history.

Results and discussion. *MTX treatment significantly decreased disease activity according to DAS28. At the end of the investigation, the majority of patients had moderate disease activity (3.2⩽ DAS28 ⩽5.1), 4 had high disease activity, while 12 attained remission (DAS28 <2.6).*

Gene expression analysis showed that RA patients who had achieved clinical remission after MTX therapy displayed higher baseline expression of the genes associated with glycolysis (Glut1, PKM), inflammation (TNF-α), autophagy (ULK1), apoptosis (caspase 3, p21), and hypoxia (HIF1α), compared with patients who had not attained remission and with healthy individuals. In addition, in patients who had achieved remission, the baseline expression of the CD1 gene was significantly higher than in healthy individuals, while in the remaining patients the expression of this gene was significantly lower than in the controls. While the disease activity remained high, the baseline expression of the p21, caspase 3, TGFβ1, and RUNX2 genes was significantly lower than in healthy individuals and other patients with RA.

Conclusion. *Remission achievement in RA patients who had not previously received MTX was associated with higher baseline (pretreatment) gene expression associated with glycolytic activity, inflammation, autophagy, apoptosis, and hypoxia compared with patients who failed to attain remission. Elevated baseline expression of the CD1 gene compared with that in healthy individuals may serve as a predictor of sensitivity to MT therapy.*

Keywords: *rheumatoid arthritis; DAS28; remission; gene expression; peripheral blood; inflammation; energy metabolism; cyclin D1; methotrexate.*
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Modern strategy for rheumatoid arthritis (RA) therapy consists in early diagnosis and active treatment to achieve the maximal suppression of the disease activity [1]. At the same time, the "window of opportunity", when patients respond best to therapy and achieve long-term remission, exists at an early stage of RA. Since at this stage, unlike the late stage of RA, patients are more sensitive to active therapy with basic anti-inflammatory drugs (disease-modifying anti-rheumatic drugs – DMARDs), it is possible to halt the progression of the disease. In addition, the destruction of joints develops faster at the onset of the disease [2]. Introduction to clinical practice of treatment strategies to achieve the goal of "T2T", which consists in periodic assessment of disease activity and timely correction of the therapy, is aimed at achieving remission or low RA activity, which helps prevent joint destruction and disability [3]. The goal of treating RA is to achieve remission, therefore, it is of particular importance to predict it before starting the therapy in order to determine the treatment strategy. However, at present, the optimal strategy of induction of remission in RA has not been studied enough [4].

In some studies it was noted that low basal activity of RA, minimal radiological changes, seronegativity for rheumatoid factor (RF) and anti-citrullinated protein autoantibodies (ACPA) in RA patients who had not previously received DMARDs, correlated with remission during treatment with methotrexate (MT) [5 , 6]. In addition, some biomarkers were found that characterize the pathophysiological process. In particular, a high serum concentration of chemokine CXCL13 – B-lymphocyte chemoattractant – turned out to be a marker of a severe course of RA and

destruction of joints [7]; at the same time it can serve as a predictor of remission in patients with early RA who have not received DMARDs [8, 9]. In another study, a higher concentration of naive T-lymphocytes was associated with subsequent remission during MT therapy [10]. When treating RA with tumor necrosis factor α inhibitors, it turned out that the basal levels of ADAMTS5 mRNA [11], serum concentrations of RANKL [12], CRP [13] and interleukin receptor (IL) 2 [14] were significantly lower in patients who achieved remission, compared with those who retained moderate and high disease activity. At the same time, the ineffectiveness of MT therapy in RA may be due to increased expression of FcγRIIIa / CD16 on CD14 ++ monocytes, which determines the increased sensitivity of these cells to stimulation by immune complexes [15]. However, the conducted studies are characterized by high heterogeneity in relation to the definition of remission, the time it was achieved and the duration of RA therapy. Therefore, there are currently no reliable predictors of remission.

Since the response to therapy depends on at least three parameters: drug concentration, stage, and pathophysiology of the disease, each of which is mediated by many other factors [16], in order to predict remission it is necessary to understand the nature of metabolic changes caused by both the disease and therapy. The system of generation and consumption of energy in the form of ATP is the most universal, it functions in all cells of the body and determines the efficiency of its vital activity. The method of ATP production depends on the functional needs of various subtypes of immune cells, mainly lymphocytes, since different T-cell subtypes with different functions activate different energy and biosynthetic metabolic programs [17]. In particular, resting non-proliferating cells: naive T-lymphocytes, T-regulatory cells (Tregs), and also memory cells usually generate ATP by oxidative phosphorylation in the mitochondrial electron transport chain – the most efficient way to produce energy [18]. At the same time, under aerobic conditions, there is oxidation of glucose (in glycolysis), amino acids, glutamine (in glutaminolysis) to pyruvate, which enters the tricarboxylic acid cycle (TCA), where high-energy reduced nucleotides are generated, which are further oxidized in the electron transfer chain. Due to the fact that glucose concentration is limited under oxidative phosphorylation conditions, it is associated with gluconeogenesis activity, which is responsible for the reduction of pyruvate to glucose in order to maintain a constant blood glucose concentration [19].

In contrast, activated T-lymphocytes and short-lived immunosenescent TEMRA-cells, despite the presence of oxygen, switch the metabolism to the use of glycolysis (aerobic glycolysis, Warburg effect) [20], which is necessary for the optimal production of cytokines by T-lymphocytes. A special state of T-lymphocyte anergy is also associated with the specificity of energy metabolism: with the arrest of the cell division cycle at the G1 / S phase, as well as with inability to activate glycolysis and reduce the use of glucose through oxidative phosphorylation, which prevents T-lymphocyte activation [21].

The main regulators responsible for switching energy flows in lymphocytes are such proteins as mammalian target of rapamycin (mTOR) that activates anabolic processes to stimulate cell growth and proliferation, including the initiation of the G1 / S cell division cycle by activating cyclin CD1; AMPK, which activates the production of energy through catabolic processes; and HIF1_, which is involved in the activation of anaerobic pathway for energy production, glycolysis [17].

Earlier, we showed that before the treatment the expression of all genes in the blood of RA patients was significantly increased compared with the controls. MT therapy resulted in a decrease in the expression of the mTOR, caspase 3 and tumor necrosis factor α (TNFα) genes to the same level as in healthy individuals. At the same time, gene expression of the indicator of autophagy (ULK1), inhibitor of cyclin-dependent kinases (p21), matrix metalloproteinase (MMP9), cathepsin K, transforming growth factor (TGFβ1) and Runt-dependent transcription factor (RUNX2) remained high. Moreover, a correlation was found between the basal expression of a number of genes in the blood and clinical and immunological parameters at the end of the therapy [22].

The aim of the study was to analyze the expression of genes responsible for the main pathways of metabolism and energy generation in the blood of RA patients with different disease activity who had not previously received MT, and to search for markers to predict the achievement of remission using basal gene expression during MT therapy.

Patients and methods. The study included 40 patients with RA who had not previously received MT, with the disease duration of not more than 2 years. Among them there were 5 men and 35 women aged 18 years and older (mean age 47.5 ± 15.5 years), who had not received DMARDs and systemic glucocorticoid therapy. Patients were treated at Nasonova Research Institute of Rheumatology in 2007–2008 under the program "RADICAL". The registration number of the clinical trial is 0120.0810610.

The study protocol was approved by the local ethics committee, and informed consent was obtained from all patients. The diagnosis was established according to the classification criteria of ACR (American College of Rheumatology), 1987. The criterion for excluding patients was the presence of contraindications for prescribing DMARDs in effective therapeutic doses.

All patients received MT at a dose of 10 mg / week. After 2 weeks of treatment, the dose was increased to 15 mg / week and the therapy continued for 2 years. In 11 of 40 patients with RA, methylprednisolone 8 mg / day was used in addition to MT. The patients were observed by the same rheumatologist every 6 months for 2 years.

The control group consisted of 26 randomly recruited blood donors without autoimmune diseases and burdened heredity, matched by sex and age with the group of patients.

Clinical, laboratory and instrumental methods. We determined the number of swollen joints (SJN) out of 44, the number of painful joints (PJN) out of 53, the duration of morning stiffness (in minutes). For a quantitative assessment of RA activity, the DAS28 index was used.

Immunological methods. The concentration of CRP and IgM RF in serum was determined by immunonephelometric method on an automated analyzer BN-100 (Dade Behring, Germany). The concentration of ACPA was determined by ELISA using a commercial kit from Axis-Shield Diagnostic Limited (United Kingdom) according to the instructions of the manufacturer.

Instrumental methods. All patients underwent radiography of the hands and distal feet in the direct projection. The progression of joint changes in RA was assessed by Sharp–van der Heijde modified scoring method. At the same time, the number of erosions and joint space narrowing was determined in 16 joints of

each hand and in 6 joints of each foot. The count of the number of erosions and joint space narrowing was recorded for each hand and each foot, calculating the average of the assessments of two researchers.

Molecular biological methods. Total RNA was isolated from whole blood using the commercial RIBO-sol-A kit (InterLabService, Moscow). The reverse transcriptase (RT) reaction was performed using the Reverta commercial kit (InterLabService, Moscow). For the real-time polymerase chain reaction (PCR), a 7300 Applied Biosystems instrument and gene expression kits (Applied Biosystems, USA) were used: mTOR (Hs0023522_m1), ULK1 (Hs00177504_m1), p21 (Hs00355782_m1), caspase 3 (Hs00263337_m1); TNF_ (Hs00174128_m1), MMP9 (Hs00234579_m1), cathepsin K (Hs00166156_m1), TGF_1 (Hs99999918_m1) and RUNX2 (Hs00231692_m1), cyclin, CCND1 (Hs00233365_m1), glucose transporter, Glut1, SLC2A1 (Hs00197884_m1), pyruvate kinase, PKM2 (Hs00987255_m1) , hypoxia-induced factor, HIF1_ (Hs00936368_m1), malate dehydrogenase, MDH2 (Hs00938918_m1), pyruvate carboxylase, PC (Hs00559398_m1), as described previously [23]. β-Actin was used as endogenous control. When setting real-time RT-PCR for each determination of the expression of each gene, cDNA of 16 control individuals and cDNA of patients with RA were placed onto the plate, therefore, expression in the controls was studied at each expression determination [23].

The study of clinical, immunological and molecular biological parameters was carried out before the beginning of MT therapy and after 24 months.

Statistical analysis. The data of quantitative experiments are presented as median [25th; 75th percentile]. Analyses were performed in duplicate. Statistical analysis was performed using the Statistic software version 6 (StatSoft version 6.0). For statistical processing of the results, Mann–Whitney and Wilcoxon tests were used. Differences were considered significant at $p \leqslant 0.05$.

Results

Characteristics of patients with RA. Previously, we presented a detailed description of this group of patients with RA [24]. Comparison of baseline clinical and immunological parameters in RA patients who achieved remission and in patients with persistent moderate to high disease activity did not reveal statistically significant differences (see the Table).

The initial characteristics of RA patients who achieved and did not achieve remission after MT, Me therapy [25th; 75th percentile]

Index	Patients with RA who did not achieve remission (n = 28)	Patients with RA who achieved remission (n = 12)	p
RF, ME/л	238.5 [9.5; 80]	110.2 [9.5; 81.2]	0.49
ACPA, U/л	55.3 [0.3; 100]	44.7[0.5; 98.5]	0.52
CPR, mg/ml	19.6 [8; 17.8]	19.4 [2.7; 37.5]	0.98
DAS28	5.52 [5.0; 5.9]	5.04 [4.4; 5.6]	0.24
Stiffness, min	134.4 [40; 180]	97.1 [30; 150]	0.34
SJN	10.1 [4; 13]	8.4 [6; 9]	0.55
PJN	10.8 [6; 13]	9.9 [6; 11.5]	0.76
Erosion score	0.59 [0; 1]	0.5 [0; 0.5]	0.79
Joint space narrowing	15 [7; 21]	13 [6; 20]	0.56

Association of gene expression with disease activity before and after therapy. To analyze the association of gene expression with disease activity, RA patients were divided into subgroups with high (DAS28> 5.1), moderate (3.2⩽DAS28⩽5.1) disease activity and remission (DAS28 <2.6) before and MT therapy (Fig. 1). It turned out that prior to the treatment, RA patients with high disease activity had higher expression of all the studied genes compared with the controls. In patients with moderate activity, the expression of most of the studied genes before therapy was also higher than in healthy individuals, whereas the expression of the p21, ULK1 and caspase 3 genes did not significantly differ from their expression in the controls.

After the therapy, the expression of most of the studied genes continued to be higher than in healthy individuals (see Fig. 1). Thus, the subgroup of patients in whom the disease activity remained high, had a significantly higher expression of all the studied genes compared with healthy individuals. In the subgroups of patients with moderate disease activity and patients who achieved remission, the expression of TNFα and mTOR decreased to normal. In addition, in the subgroup of patients with moderate RA activity, the expression of the p21, caspase 3, and RUNX2 genes after the therapy was comparable to that of the controls. In contrast, gene expression of ULK1, p21, caspase 3, MMP9, cathepsin K, TGFβ1 and RUNX2 in patients who achieved remission was higher than in healthy individuals.

Analysis of the initial (before therapy) expression of the examined genes in the subgroups of patients with RA classified according to the disease activity after MT therapy, showed that patients who achieved remission or moderate disease activity had a higher baseline (before therapy) expression of all the genes compared with healthy subjects (Fig. 2). At the same time, there was a tendency to a higher level of gene expression in patients who achieved remission, compared with those who achieved moderate RA activity.

On the contrary, when high disease activity was maintained after the therapy, the baseline (before therapy) expression of the mTOR, ULK1 and cathepsin K genes was comparable to that in the controls. The expression of MMP9 and TNFα exceeded the control values, and the initial expression of p21, caspase 3, TGFβ1 and RUNX2 was significantly lower than in healthy individuals. Therefore, low expression of these genes can serve as a predictor of insensitivity to MT.

Specific features of energy metabolism in patients with RA. Since the baseline (before therapy) expression of the examined genes in patients with moderate disease activity or those who reached remission after the therapy was higher than in healthy individuals, the analysis of the expression of these genes did not allow to predict before the therapy which of RA patients would

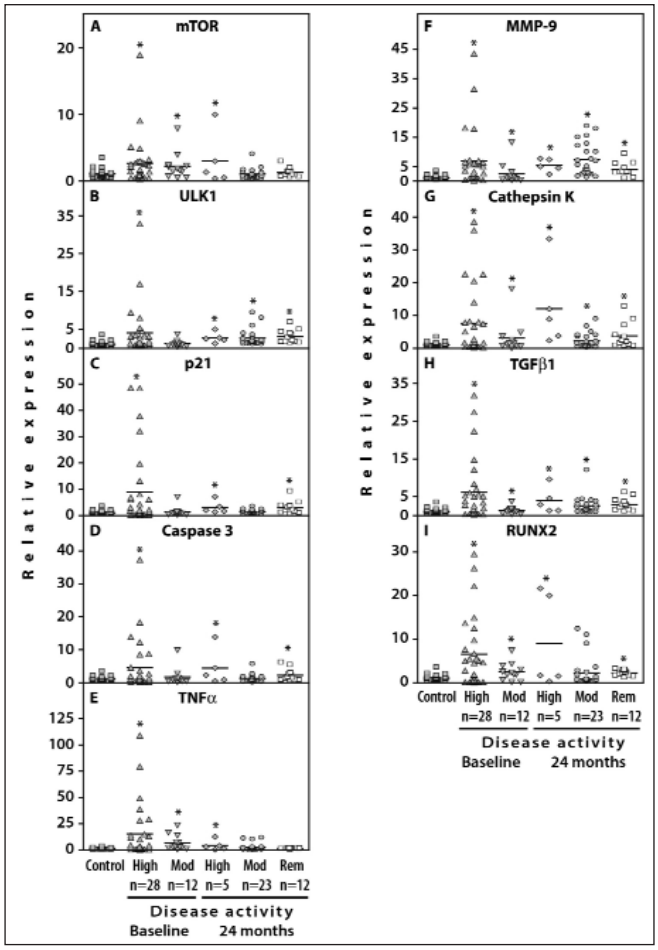

Fig. 1. *Relative expression of mTOR (A), ULK1 (B), p21 (C), caspase 3 (D), TNFα (E), MMP9 (F), cathepsin K (G), TGFβ1 (H) and RUNX2 (I) with respect to β-actin in the blood of patients with RA (n = 40) compared with healthy individuals (n = 26), measured before and after MT therapy in real-time PCR. Here and in Fig. 2: patients are divided into subgroups according to disease activity (DAS28): high (B) – DAS28> 5.1; moderate (Y) – 3.2⩽DAS28⩽5.1; remission (P) – DAS28 <2.6. Here and in Fig. 2: asterisks indicate statistically significant differences compared with the controls (K)*

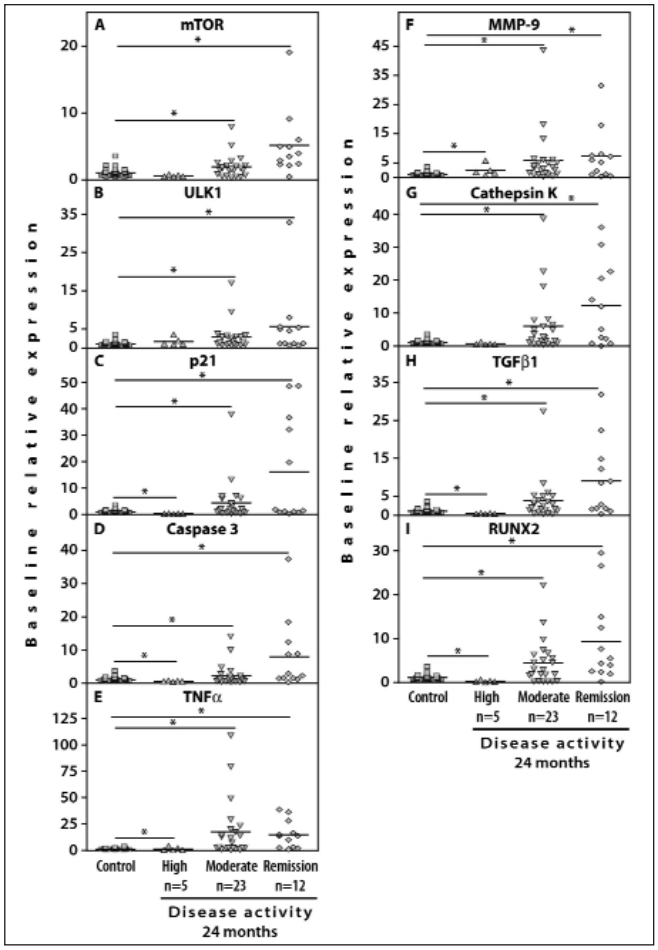

Fig. 2. *Baseline (before therapy) relative expression of genes mTOR (A), ULK1 (B), p21 (C), caspases 3 (D), TNFα (E), MMP9 (F), cathepsin K (G), TGFβ1 (H) and RUNX2 (I) in relation to β-actin in the blood of patients with RA (n = 40) compared with healthy individuals (n = 26), measured in real-time PCR*

achieve remission. Due to the fact that the development of RA may be associated with energy deficit caused by high energy expenditure to ensure an activated state of the immune system [19], we suggested that the achievement of remission in RA patients may be associated with a higher efficiency of their energy metabolism.

Indeed, an analysis of the gene expression of the glycolytic pathway of energy generation, Glut1 and PKM, showed that the patients who achieved remission had significantly higher levels of the initial expression of these genes compared with the rest of patients with RA (Fig. 3). In contrast, the initial expression of the TCA gene (MDH2) and the gene responsible for gluconeogenesis (PC) was comparable in both subgroups.

Analysis of the expression of glycolysis regulators HIF1α, mTOR, and cyclin CD1 was increased in the group of patients who achieved remission, compared with healthy individuals. At the same time, the differences with the controls were statistically significant for the mTOR and CD1 genes (see Fig. 3). Moreover, the expression of the CD1 gene was significantly lower in those patients who maintained a high or moderate disease activity after the therapy compared with healthy individuals. Therefore, the initial expression of the cyclin CD1 gene in the blood allows to identify patients with RA who will achieve remission, provided that they have not previously received MT.

Discussion. Since the use of drugs is often associated with significant adverse effects, they should be avoided if possible in those patients who cannot respond to this therapy. Therefore, an accurate and personalized strategy is required to predict the response to therapy and perspectives for achieving remission before the initiation of treatment [25].

There are different definitions of remission in RA. Although the experts of ACR and EULAR (European League Against Rheumatism) question the use of the DAS28 index for assessing remission and consider it more acceptable to use the SDAI and CDAI indices [26], recent studies have shown that, despite the

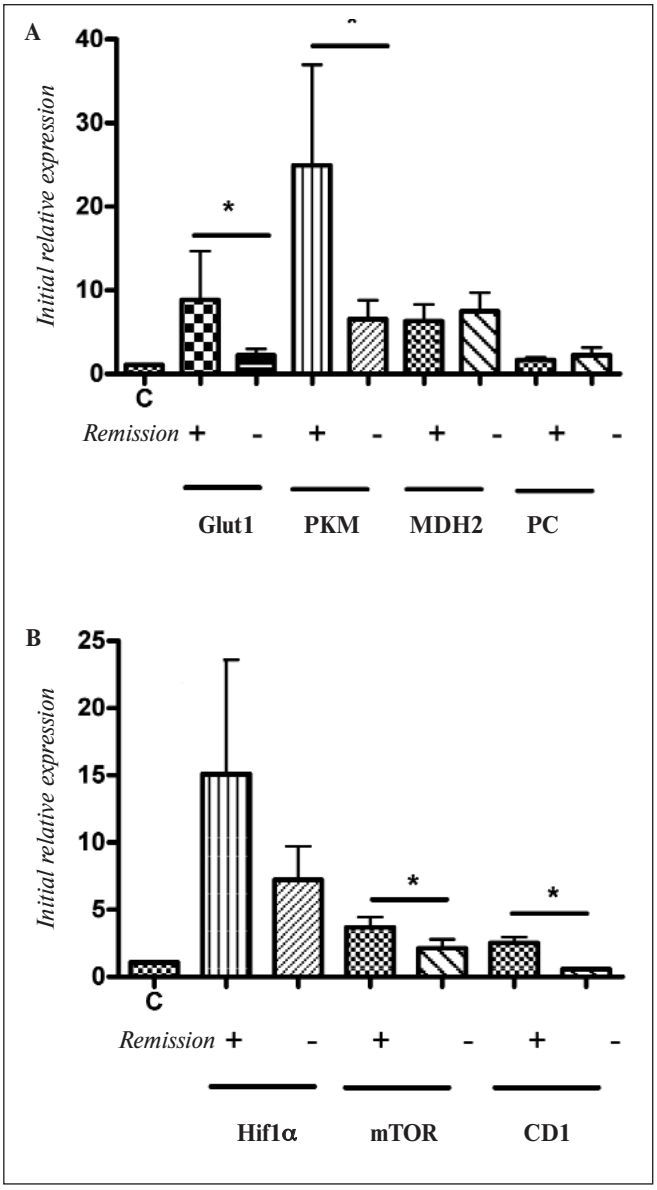

Fig. 3. *Baseline (before therapy) relative expression of the Glut1, PKM, MDH2, PC (A) and Hif1α, mTOR, CD1 (B) genes in relation to α-actin in the blood of RA patients who achieved remission (+) after MT therapy (n = 12), or who maintained (-) high or moderate disease activity (n = 28) compared with healthy individuals (control, K; n = 26) measured by real-time PCR. # – statistically significant differences between groups of patients*

remission confirmed by the CDAI index, "turned off" B – memory cells, when stimulated in cell culture, are capable of producing ACPA (predictor of adverse prognosis) with activity comparable to that of B-lymphocytes from patients with the active disease [27]. In this regard, in this study, the achievement of remission in RA patients who had not previously received MT was evaluated using the DAS28 index.

In those patients who achieved remission or moderate disease activity, a decrease in the expression of TNFα mediating the development of inflammation was accompanied by a decrease in disease activity according to DAS28. At the same time, the initial expression of TNFα in the patients who achieved remission was higher than in the patients who did not respond to treatment. Higher levels of baseline (before therapy) expression of pro-inflammatory cytokines were also previously observed in patients with RA, in whom anti-rheumatic therapy was effective [28].

Significantly lower basal expression of the p21, caspase 3, TGFβ1 and RUNX2 genes compared with that in healthy individuals (controls) while maintaining high disease activity after the therapy indicates a low efficacy of apoptosis and weakening of tissue regeneration processes characteristic of patients with RA [29, 30].

At the same time, the preservation of increased expression of a number of genes in those patients who achieved remission after MT therapy, compared with healthy individuals, confirms the results of the previous studies, which showed that the disease is not cured during remission and the possibility of relapse remains [31].

A significant excess of the expression level of the genes responsible for the glycolytic pathway for obtaining energy in RA patients who achieved remission, compared with other patients, suggests that they were able to activate aerobic glycolysis and proliferation characteristic of healthy T-lymphocytes [17]. This is evidenced by a higher expression of the Glut1, PKM, mTOR, CD1, and HIF1α genes, while the activity of the TCA-related (MDH2) and gluconeogenesis (PC) genes was comparable to the corresponding indicators in patients with persistent disease activity. Therefore, in patients resistant to therapy, there is a shortage of energy substrates, as in the case of anergy of lymphocytes [21].

Of particular interest is the basal expression of the CD1 gene. Although the classic function of cyclin D1 is regulation of the cell cycle [32], in which it provides progression from the G1 to the S phase by binding and activating Cdk4 / 6, cyclin D1 is also involved in many cellular metabolic processes. In particular, CD1 is able to activate and / or repress the expression of many genes [33]. Thus, an increase in CD1 expression leads to the suppression of gluconeogenesis [34] and mitochondrial function (oxidative phosphorylation) by blocking the association of the voltage-dependent anion channel protein (VDAC) with hexokinase 2, which reduces the access of ADP to the mitochondrial inner membrane [35]. In addition, it was previously shown that the activation of the CD1 gene stimulates glucose uptake and glycolysis in the presence of oxygen (the Warburg effect – aerobic glycolysis) [36].

In clinical practice, determination of baseline (before therapy) CD1 gene expression can be used to predict remission in RA patients who have not previously received MT, since it turned out to be significantly higher compared with the controls in those RA patients who achieved remission, and below normal in other patients and healthy individuals.

Conclusion. Thus, our study showed that a higher disease activity in RA patients who had not previously received MT was associated with a higher expression of all the examined genes, both before the onset of the disease and after the therapy. At the same time, in the patients who achieved remission, the baseline (before therapy) gene expression was the highest. This is accompanied by the activation of the glycolytic pathway of energy generation. The initial expression of the CD1 gene may serve as a predictor of remission in RA patients who have not previously received MT, since it was significantly higher than in the controls in those patients who achieved remission, unlike other patients in whom the initial expression of this gene was lower than in healthy individuals. Due to the small number of the examined sample, to confirm the results obtained, a similar study with a larger number of patients is required.

Transparency of the study
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Declaration of Financial and Other Relationships
All authors participated in the development of the concept and in writing the manuscript. The article was not previously published, and was not submitted for review and publication in another journal. The final version of the manuscript was approved by all authors. The authors did not receive a fee for the article.

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