ORIGINAL INVESTIGATIONS

# Association between a low baseline level of gene expression of energy metabolism in the blood and the development of clinical remission in response to tofacitinib therapy in patients with rheumatoid arthritis

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***Background.*** *Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, characterized by erosive arthritis (synovitis) and systemic inflammation. Janus kinase (JAK) inhibitors (JAKi) are small molecules that block major signal pathways of many cytokines a growth factors, associated with RA. Identification of patients sensitive to JAKi before treatment could significantly improve therapy outcomes. Currently it is not possible to predict JAKi efficacy in every patient, while some patients are non-responsive to the drug, other develop adverse effects. JAKi effect in RA patients has been recently associated with alterations in mitochondrial function and ATP production. Therefore, we hypothesized that baseline metabolic status of RA patients prior to drug administration can predict the therapeutic outcome.*

***Objective:*** *to investigate the predictive value of baseline expression of genes involved in energy generation in the blood of RA patients, for treatment response to JAKi.*

***Patients and methods.*** *We examined peripheral blood of 28 RA patients aged 52.2±15.6 years, average disease duration 3.5 years (range 0.6–19), treated with Tofacitinib (TOFA, 5–10 mg twice a day) during three months and 26 healthy age-matched control subjects. Clinical response was assessed by disease activity score (DAS28-ESR), immunological status by measurements of serum levels of anti-citrullinated protein antibodies (ACPA), rheumatoid factor (RF), and C-reactive protein (CRP). Gene expression was assessed in peripheral blood cells by real-time reverse-transcription polymerase chain reaction (RT-PCR). At baseline all patients had Steinbrocker radiographic stage II–III. Most patients (85.7%) were ACPA and RF positive. Thirteen patients had medium, others – high RA activity.*

***Results and discussion.*** *JAKi treatment significantly decreased the inflammatory disease activity according to DAS28. At the end of the study 17 patients demonstrated moderate disease activity (3.2<DAS28<5.1), 4 patients retained high disease activity while 7, attained remission (DAS28 <2.6). Disease remission, achieved on TOFA treatment, was accompanied by significant decrease in CRP and the number of swollen and tender joints. ESR values were not changed significantly. Gene expression analysis revealed that RA patients, which attained clinical remission after TOFA treatment, demonstrated significantly lower baseline expression of genes associated with glycolysis (pyruvate kinase, PKM2) and oxidative phosphorylation (succinate dehydrogenase, SDHB) compared to other examined RA patients, but higher expression of the abovementioned genes compared to control subjects. Moreover, RA patients who attained clinical remission demonstrated a trend to increase of these gene expressions within follow-up period, while in the rest of patients these gene expression was tending to downregulate.*

***Conclusion.*** *Clinical remission in RA patients treated with JAKi is associated with significantly lower baseline expression of genes associated with energy generation pathways (PKM2 and SDHB) compared to other examined subjects.*

***Keywords:*** *rheumatoid arthritis; gene expression; JAK-inhibitors; energy generation pathways; blood.*

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Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, characterized by chronic erosive arthritis and systemic inflammatory lesions of internal organs. The prevalence of RA among the adult population is 0.5P2%. The disease affects all age groups, including children and the elderly, and the peak incidence occurs in the working age (30P55 years). RA has a wide heterogeneity of clinical manifestations due to various combinations of disease phenotypes characterized by varying degrees of joint damage (from minor cartilage damage to bone erosions), as well as course and outcomes [1].

Current strategies for the treatment of RA are aimed at blocking proinflammatory cytokines, cellular receptors, or intracellular pathways leading to the expression of proinflammatory molecules, thus achieving low activity and alleviating disease symptoms [2]. However, the loss of efficacy of the drug and / or the development of undesirable phenomena arising over time limit its long-term use [3], therefore, it becomes necessary not only to change therapy, but also to develop new methods of treatment. Although many patients with RA respond adequately to treatment with methotrexate (MT), 40% of patients are resistant to first-line therapy and require the appointment of genetically engineered biological (GIBD) or targeted synthetic drugs [4]. In turn, 40% of patients are resistant to both the first and the second GIBD, therefore, 20% of them will need to change this therapy

[5]. Patients resistant to several drugs with different structures and mechanisms of action, in particular those who are unable to achieve low disease activity after therapy with three or more basic anti-inflammatory drugs (DMARDs), of which one must be a GIBD, are considered refractory and account for 6–21% of all patients with RA [6].

New cytokine antagonists blocking interleukin (IL) 6 and IL17, granulocyte macrophage colony-stimulating factor, signaling pathways including NF-kB and mitogen-activated phosphokinase p38 (p38 MAPK), as well as stem transplantation, or mesenchymal stromal cells [7]. However, there is currently no significant progress in this area.

Due to the high cost of GIBD, it is important to assess the potential for patient response to therapy. It is proposed to use analysis of genetic mutations (SNP), sets of genes for response to interferon, serological parameters or stratification of patients by the nature of damage to synovial tissue as predictors of response to treatment, but the results of such an assessment are ambiguous [6]. At present, ideas about the mechanisms of development of the RA are changing. There is evidence that systemic inflammation is caused by metabolic changes due to disturbances in energy metabolism in cells of various tissues [8].

JAK / STAT signaling pathways play the role of a key mediator of proinflammatory pathways in autoimmune diseases and are considered the main driving force of disorders associated with RA [9], since they are activated by a number of cytokines (interferons, IL2 and IL6) that control the viability, proliferation and differentiation of various types cells [10]. Recently, in vivo studies have shown that synovial oxygen partial pressure is associated with the activation of pSTAT-3, which mediates the migration of synovial fibroblasts and their invasiveness [11]. In addition, during inflammation in response to hypoxia, STAT-3 in some cell types activates the hypoxia-inducible factor (HIF1α) [12], its reciprocal interactions with pSTAT-3 and the main glycolysis enzyme, pyruvate kinase (PKM2) [13]. These data indicate complex interactions between key signaling pathways within the inflamed synovial microenvironment and suggest that the JAK / STAT signaling pathway may be involved in the regulation of metabolic pathways associated with energy production in these cells.

Janus kinase inhibitors (iJAKs) are synthetic targeted drugs that have shown more significant results in RA therapy compared to MT in primary patients, as well as with an inadequate response to GIBD [14, 15]. Recent studies have shown that iJAK reduce ROS production and regulate the expression of the most important mitochondrial genes in synovial tissue explants from RA patients [16], significantly increasing oxidative phosphorylation and adenosine triphosphate production. This leads to a decrease in the activity of glycolysis, assessed by the level of glycolysis enzymes, hexokinase 2, glycogen synthase kinase 3?, lactate dehydrogenase A, and HIF1α [16].

Since it is difficult to obtain joint tissue in the early stages of the disease, and collection of blood samples is technically less difficult and painful, peripheral blood mononuclear cells (PBMC) from patients with RA were used in this study. Based on previously obtained data that T-lymphocytes of RA patients produce insufficient amounts of ATP [17], it was suggested that both sensitivity and resistance to JAK therapy may be associated with the characteristics of the energy metabolism of immune cells. In particular, under the influence of iJAK, immune cells can activate metabolic pathways for energy production, which provides a response to treatment, whereas in patients resistant to therapy, immune cells are not able to sufficiently affect these pathways.

**The aim of the** study was to analyze the expression of genes responsible for the main pathways of energy metabolism, as well as genes associated with the production of energy in the blood of JAK-naive RA patients with different disease activity; search for markers for predicting remission by gene expression before and JAK therapy.

**Patients and methods.** The study included 28 patients with RA (6 men and 22 women, mean age 52.2±15.6 years) who met the ACCR / EULAR classification criteria (American College of Rheumatology / European League Against Rheumatism ) 2010 or ACR 1987, who have not previously received a JAK. All patients were treated at the «Research Institute of Rheumatology n.a. V.A. Nasonova» from 2015 to 2016.

The study was approved by the local ethics committee. All patients signed voluntary informed consent to participate in the study.

Most patients were refractory to previous therapy: MT at a dose of 20–25 mg/week (92.6%), including in combination with methylprednisolone 8 mg/day (14.3%), and various GIBDs (32.1%) ). All patients received iJAK tofacitinib (TOFA) at a dose of 5–10 mg 2 times a day. The therapy lasted for 3 months. The majority of patients – 23 (82%) – in addition to TOFA received MT 20–25 mg/week, 4 (14%) – methylprednisolone 8 mg/day, 1 (0.03%) patient – hydroxychloroquine 200 mg/day, leflunomide 20 mg/day and adalimumab 40 mg subcutaneously every 2 weeks.

Study inclusion criteria : reliable diagnosis of RA (according to ACR / EULAR 2010 or ACR 1987 criteria); medium and high disease activity (DAS28> 3.2); age from 20 to 80 years old; signed informed consent to participate in the study; lack of effect or intolerance and / or ineffectiveness of previous therapy; adequate contraception for patients of childbearing age.

*Exclusion criteria:* pregnancy and lactation; severe active infections (AIDS, tuberculosis, viral hepatitis, etc.); severe dysfunction of internal organs (renal, hepatic, heart failure, high uncontrolled arterial hypertension, decompensated diabetes mellitus, etc.); hematological disorders (Hb <85 g/l, tr. $<100 \cdot 10^9$/l, l /$<3 \cdot 10^9$/ , n. – absolute value <2 thousand, lymph. – absolute value <0.5 thousand .), alanine aminotransferase, aspartate aminotransferase >1.5 upper limit of normal, triglycerides >10 mmol/l; demyelinating diseases of the nervous system; any malignant neoplasms or precancerous conditions, or a history of precancerous conditions in the past 5 years; alcohol and drug addiction; the impossibility of monitoring the patient for 1 year; a history of allergic reactions to protein drugs; immunization with live and attenuated vaccines 4 weeks before enrollment in the study.

The control group for the analysis of gene expression consisted of 26 healthy blood donors without autoimmune diseases and burdened heredity for rheumatic diseases, comparable in gender and age with the group of patients with RA.

*Clinical, laboratory and instrumental examination methods*. All patients underwent direct projection of the hands and distal parts of the feet. PA activity was determined using the DAS28 index.

The concentration of CRP and IgM RF in blood serum was assessed by the immunonephelometric method on an automatic analyzer BN-100 (Dade Behring, Germany), antibodies to cyclic citrullinated peptide (ACCP) – by an enzyme immunoassay using a commercial kit from Axis-Shield Diagnostic Ltd. (UK) according to the manufacturer's instructions.

Total RNA was isolated from whole blood samples from healthy individuals and patients with RA, which was converted into complementary DNA by means of a reverse transcriptase reaction, as described previously [18]. Since the relative expression of the studied genes was determined, the deviation of the expression of each gene in each RA patient was assessed in comparison with the average expression of the same gene in the control.

Using quantitative polymerase chain reaction (PCR) in real time in peripheral blood samples, the expression levels of key genes associated with the main pathways of energy production were investigated: in glycolysis – by expression of PKM2 and during oxidative phosphorylation – by expression of the Krebs cycle enzyme gene succinate *dehydronenase (SDHB )* associated with the generation of ATP; energy conversion: the uncoupler of oxidative phosphorylation *(UCP2)* and superoxide dismutase *(SOD1)* – a protein that destroys free radicals generated during the movement of electrons along the electron transport chain. We used ready-made primers and probes for the TaqMan method (Applied Biosystems Inc., USA):

– glycolysis: PKM2 (Hs00987255_m1);

– oxidative phosphorylation (OXPHOS): SDHB (Hs01042482_m1);

– UCP2 (Hs01075227_m1);

– SOD1 (Hs00533490_m1);

– α -actin as a housekeeping gene.

The quantitative assessment of mRNA levels was carried out on a QS5 instrument (Applied Biosystems Inc., USA) according to the previously described method [18]. In a real-time PCR system, the relative expression of each gene is calculated compared to a control of 1.

The study of clinical, immunological and molecular biological parameters was carried out before the start of therapy with TOFA and after 3 months.

*Statistical analysis.* Quantitative experimental data are presented as median and interquartile range (Me [25th; 75th percentile]). The analyzes were performed in duplicate. Statistical analysis was performed using the Statistica software package (version 12.0 of StatSoft Inc., USA). For statistical processing of the results, the Mann P Whitney and Wilcoxon tests were used. Differences were considered significant at pI0.05.

**Results.** *Characteristics of RA patients.* The average duration of RA was 24 (from 4 to 156) months. The majority of patients – 21 (75%) – had II radiological stage of the disease according to Steinbrocker, 7 (25%) – III stage. Three patients were segonegative for ACCP, and four were seronegative for RF, the rest of the patients (25 and 24, respectively) were seropositive for both indicators. Initially, 15 patients had high disease activity (DAS28> 5.1), the remaining 13 had moderate (3.2 <DAS28 <5.1).

During therapy, there was a significant decrease in the activity of the disease according to the DAS28 index (p <0.001), the number of painful (NPJ) and swollen (NSJ) joints (p <0.001). At the end of the study, 4 (14%) patients remained high RA activity, 7 (25%) achieved remission (DAS28 <2.6) and 17 (61%) – moderate disease activity.

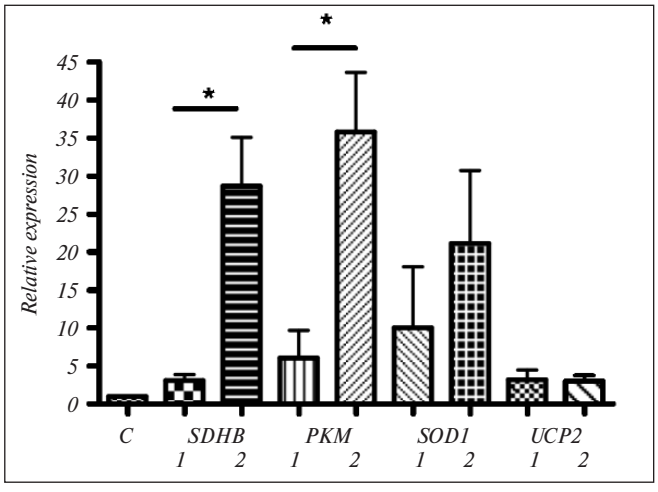
*Clinical indicators of RA patients during therapy with TOFA.* Depending on the results of the TOFA treatment, the RA patients were divided into three groups. Group 1 included 7 patients who achieved disease remission (DAS28 <2.6); group 2 – 12 patients who responded to therapy but did not reach the target DAS28 values, in whom the disease activity significantly decreased to moderate (3.2 <DAS28 <5.1); group 3 – 9 patients who did not respond to treatment (ЖKDAS28 <0.6).

**Table 1. Characteristics of RA patients depending on the results of therapy and JAK**

| Parameter | 1st group (n = 7; DAS28 <2.6) | | p | 2nd group (n = 12; ΔDAS> 1.2; DAS28> 2.6) | | p | 3rd group (n = 9; without significant dynamics DAS28) | | R |
|---|---|---|---|---|---|---|---|---|---|
| | before treatment | after 3 months | | before treatment | after 3 months | | before treatment | after 3 months | |
| Age, years | 53 [36; 51] | | | 61.5 [56; 68] | | | 40 [29; 55] | | |
| Duration of RA, months | 33 [16; 143.5] | | | 24 [10.5; 54] | | | 24 [11; 51] | | |
| CRP, mg / ml | 15 [4.4; 43] | 5.4 [0.25; 10] | 0.06 | 27.6 [14.6; 40.3] | 1.3 [0.9; 18.6] | 0.08 | 17.5 [6.9; 48.4] | 8.2 [2.3; 30.9] | 0.31 |
| ESR, mm | 26 [22; 62] | 16 [10; 20] | 0.06 | 32 [18; 42,5] | 20 [13; 55] | 0.62 | 38 [22; 49] | 60 [46; 76] | 0.06 |
| DAS28 | 5.04 [4; 5.5] | 1.96 [1.68; 2.21] | 0.01 * | 5.77 [5.0; 6.4] | 3.4 [2.7; 4.5] | <0.001 * | 5.4 [4.6; 6.1] | 5.1 [4.4; 5.6] | 0.37 |
| ΔDAS28 | | 3.08 | | | 1.92 | | | 0.25 | |
| NSJ | 7 [3; 9] | 0 | | 8.5 [6.5; 10] | 1.5 [0.5; 4.5] | 0.001* | 9 [3; 13] | 5 [2; 6] | 0.03 * |
| NPJ | 5 [3; 9] | 0 | | 10,5 [6; 12.5] | 1 [1; 2] | 0.001* | 6 [5.5; 14] | 4 [1; 12] | 0.07 |

**Note.** * – statistically significant differences. Here and in table. 2: data are presented as Me [25th; 75th percentile]; the Wilcoxon t-test was used to calculate the statistical significance.

Patients in groups 1 and 2 were significantly older (p=0.05) than patients in group 3 (Table 1). In addition, these patients had




*Initial gene expression in patients who achieved (1) and did not achieve (2) remission of RA during TOFA therapy. C – expression of the studied genes in healthy individuals (control). * – statistically significant differences (Mann–Whitney U-test)*

**Table 2. Changes in the expression of genes for glycolysis and the Krebs cycle in RA patients who have achieved remission and are refractory to therapy and JAK**

| Gene | 1st group (n = 7; DAS28 <2.6) | | R | 3rd group (n = 9; no significant dynamics DAS28) | | R |
|---|---|---|---|---|---|---|
| | before treatment | after 3 months | | before treatment | after 3 months | |
| *SDHB* | 2.53 [2.0; 3.48] | 9.24 [3.33; 267.5] | 0.07 | 38.5 [9.87; 55] | 6.0 [2.67; 25.93] | 0.19 |
| *PKM2* | 2.24 [1.63; 5.15] | 18.9 [3.68; 259.2] | 0.15 | 48.66 [39; 8.46] | 12.22 [3.1; 27] | 0.07 |
| *SOD1* | 1.55 [0.69; 7.3] | 4.05 [1.92; 220] | 0.15 | 36.1 [0.42; 44.77] | 8.21 [1.04; 9.99] | 0.08 |
| *UCP2* | 1.84 [0.77; 4.51] | 26.45 [4.1; 253] | 0.06 | 3 [2.5; 3.94] | 0.5 [0.3; 0.7] | 0.15 |

more pronounced ($p<0.05$) a decrease in disease activity according to DAS28, NSJ and NPJ compared with patients who did not respond to treatment, in whom a statistically significant decrease in NSJ and a tendency towards a decrease in NPJ were revealed ($p=0.07$). However, according to the level of CRP (pi0.06) and ESR (pi0.06), statistically significant differences between the groups were not found during treatment, although there was a tendency ($p=0.06$) to decrease these indicators in the 1st group. and to an increase in ESR ($p=0.06$) in the 3rd group (see table. 1).

Association of gene expression with disease activity before and after therapy and JAK. In RA patients, no statistically significant changes in the levels of all studied genes were revealed before and after 3 months of TOFA therapy, but these indicators were significantly higher than in the control group.

At the same time, before the initiation of TOFA therapy, the expression of the SDHB and PKM2 genes in patients who achieved remission was significantly lower ($p <0.05$) than in patients of groups 2 and 3 (see figure). For the rest of the studied genes, the revealed trend persisted, but was statistically significant.

In addition, in patients of group 1 against the background of treatment with TOFA, the expression of all studied genes tended to increase, while in group 3 – to decrease (Table 2), but the differences were statistically insignificant.

**Discussion.** Currently, thanks to the introduction of innovative treatment methods, significant success has been noted in suppressing symptoms and achieving remission of RA. At the same time, the cure of the disease, which implies the complete absence of manifestation of clinical and subclinical manifestations, relapses, progression and, as a consequence, the complete cessation of therapy, remains a difficult task [19]. To solve it, it is necessary to understand the underlying mechanisms that are the sources of continuous inflammation in RA. It is proposed to investigate disorders in the adaptive immune system, synovial cells, as well as factors not directly related to joints, for example, the function of mucous membranes and neuroendocrine parameters, as hidden reasons for the preservation of the potential for RA activation in patients who have achieved remission [19]. In addition, one of the reasons may be metabolic disorders caused by the production and redistribution of energy in the cells of patients with RA, which was shown in the present study: the improvement in the condition of patients with the use of iJAK was associated with a change in the functioning of the ways of obtaining energy in the form of ATP. In particular, the study of the expression of genes responsible for the production of energy in glycolysis and the Krebs cycle in the blood cells of RA patients before the start of TOFA therapy revealed a relationship between the state of the energy potential of blood cells and the effectiveness of treatment. Thus, as a result of therapy and JAK, patients (group 1) achieved remission with a significantly lower initial expression of the *PKM2* and *SDHB* genes, which regulate the production of ATP in glycolysis and the Krebs cycle, respectively. The results obtained are consistent with the data that the JAK-STAT signaling pathway is associated with metabolic pathways of energy production and use, since the activation of AMPK, the main regulator of ATP content in the cell, is able to block JAK-STAT-dependent pro-inflammatory signaling pathways [20]. These data can be used to predict the response to therapy in order to prevent adverse events due to drug ineffectiveness in patients who are unable to respond to treatment [21].

In addition, our results support the previously presented evidence that inhibition of the JAK-STAT pathway alters mitochondrial cell function [16]. However, in contrast to in vitro culture of synovial explants [16], blood cells from RA patients responded differently to therapy. In particular, in patients who achieved remission, the expression of genes responsible for the production of ATP in glycolysis and the Krebs cycle slightly increased during therapy with iJAK. This may indicate that the normalization of metabolism is associated with an increase in energy production in the form of ATP, since it was previously reported that T-lymphocytes of RA patients produce insufficient amounts of ATP [17]. Probably, maintaining the ability to increase ATP production to ensure the normal functioning of T-lymphocytes can lead to the normalization of the patient's condition. It is possible that in patients who did not respond to therapy (group 3), in whom the expression of these genes tended to decrease, metabolism is at the limit of activity and its further increase is impossible.

In addition, in all the RA patients we examined, there was a decrease in the heart rate and heart rate, including those who did not respond to treatment, in whom the inflammatory activity changed very insignificantly, which is explained by the previously noted association between the action of and JAK and a decrease in pain indicators caused by both inflammation and a number of others. reasons [22].

**Conclusion.** Thus, our study showed that the response to iJAK therapy is associated with the state of energy metabolism of blood cells in RA patients. At the same time, the expression of the *PKM2* and *SDHB* genes can probably predict the patient's response to therapy with iJAK, but this requires confirmation of the results obtained in a large cohort of RA patients. Further studies of disorders of energy metabolism in blood cells in RA in response to the action of drugs will reveal the internal «drivers» of inflammatory processes in this disease, which will facilitate the search for ways for its complete cure.

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**Conflict of Interest Statement**

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